Mechanisms of hyperexcitability and efficacy of antiepileptic drugs in hippocampal-entorhinal networks in the Reduced Intensity Status Epilepticus (RISE) model of chronic epilepsy

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A third of epilepsy patients are resistant to anti-epileptic drug (AED) treatment leading to reduced quality of life, increased treatment costs and complexities surrounding polytherapy. The overall aim of this project was to explore dynamic network changes in the excitability and efficacy of AEDs in: acute models of epileptiform activity, chronic models of epileptogenesis and in resected human tissue, *in vitro*.

Initial studies investigated the differences in the neuronal network excitability induced by 0$[Mg]^{2+}$ in rat brain slices prepared using either a standard NaCl-based aCSF or a sucrose-based aCSF. Standard prepared slices were more excitable in comparison to sucrose-based aCSF prepared slices. Immunohistochemical investigations for parvalbumin demonstrated a reduction of interneurons in slices prepared in the standard way. There was little difference in response to combination AEDs, but this could be due to increased latency to first seizure in sucrose prepared slices. LTP was suggested to play a role in the resistance to AEDs. These results suggest sucrose prepared slices better preserve the neuronal network *in vitro*, and serve as a better acute model for assessing AEDs and mechanisms of resistance.

Sucrose perfused slices were prepared from rodents that had undergone a refined chronic Li-pilocarpine-based model of epileptogenesis (RISE) to investigate the effects of six AED combinations on network excitability (24 hrs and 1, 5 and 12 weeks post status). Ictal-like discharges (IDs) were seen in significantly greater numbers in slices from RISE animals compared to age-matched controls. Additionally, RISE slices showed a consistently shorter latency to first seizure across all time points. Investigations exploring the efficacy of different AED combinations during epileptogenesis showed that the tiagabine and carbamazepine combination was most effective in reducing measures of ictal activity whilst the combination of lamotrigine and gabapentin was least effective. The resistance of different drug combinations was also variable depending on the stage of epileptogenesis. These findings suggest that vulnerable networks show underlying hyperexcitability even at stages when chronic behavioural seizures are not yet developed, and that the RISE model may provide insights into the variable efficacy of AEDs.

In comparison to chronically epileptic rodent tissue, epileptic human tissue from the temporal lobe was not as excitable, and often required stronger ID inducing manipulations. Once IDs were initiated *in vitro*, inter-event intervals between seizures were longer in comparison rodent epileptic tissue. Discrepancies in excitability could be attributed to the likelihood that damage within human tissue is likely to be subtle, hence require more stimulation to induce ictal-like activity (Gabriel et al., 2004).

There was a developmental trend for excitability, in response to low concentrations of the NMDA antagonist MK801 (100-300 nM), to decrease in controls and remain elevated in epileptic animals. The NOS inhibitor, 7-nitraindazole, failed to stop the induction of IDs by low concentrations of MK801. Additionally, low concentrations of MK801 had no significant effects on the frequency and amplitude of field IPSPs in control and SE latent period slices. Further investigations are required to elucidate the mechanisms of how altered excitatory drive of inhibition may promote network excitability in epilepsy.

Overall, my findings suggest network changes in excitability occur at stages when chronic behavioural seizures are not yet developed, and that the RISE model may provide insights into the variable efficacy of AEDs and underlying mechanisms of epileptogenesis.

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I have had an unforgettable experience the past three years, contributing to understanding how the human brain works and the mechanisms underlying drug resistant epilepsy. I have developed and learnt so much about myself and could not have done it without the support of my mentors, colleagues, friends and family. I feel extremely privileged to have had the opportunity to complete this PhD and overcome all the challenges I faced, and I am extremely grateful, thank you.

Table of Contents

List of Figures

List of Tables

Abbreviations

4-AP - 4-aminopyridine

aCSF - Artificial Cerebrospinal Fluid

AED - Antiepileptic Drug

AHP - Afterhyperpolarisation

AMPAr - 2-amino-3-(5-methyl-3-oxo-1, 2- oxazol-4-yl) **propionic** acid receptor

ATP - Adenosine Triphosphate

BBB - Blood Brain Barrier

BZD - Benzodiazepine

Ca^{2+} - Calcium Ion

$CaCl_2$ - Calcium Chloride

CamKII - Calmodulin Dependent Protein Kinase II

cAMP -Cyclic Adenosine 3', 5' -Monophosphate

CBZ - Carbamazepine

CCh - Carbachol

cGMP - Cyclic Guanosine Monophosphate

Cl^- - Chloride Ion

CNQX - 6-Cyano-7-nitroquinoxaline-2, 3-dione

CYP - Cytochrome

DG - Dentate Gyrus

DRE - Drug Resistant Epilepsy

e.g. - Exempli Gratia

EC - Entorhinal Cortex

ECoG - Electrocorticography

EEG - Electroencephalogram

EHs - Epoxide Hydrolases

Enos - Endothelial Nitric Oxide Synthase

EPSCs - Excitatory Postsynaptic Currents

ESM - Ethosuximide

FBM - Felbamate

FR - Fast Ripples

GABA - Gamma (γ)-Aminobutyric Acid

$GABA_A$ - Gamma (γ)-Aminobutyric Acid - A receptor

$GABA_B$ - Gamma (γ)-Aminobutyric Acid - B receptor

GABAT- GABA Transaminase

GAD - Glutamate Decarboxylase

GPT - Gabapentin

$HCO3^-$ - Bicarbonate Ion

HFO - High Frequency Oscillation

HVA - High Voltage Activated

IDs - ictal discharges

i.e. - Id est

IEI - Inter event Interval

IFMT - isoleucine, phenylalanine, methionine and threonine (amino acid stretch)

ILAE - International League Against Epilepsy

i/m - Intramuscular

ING - Interneuron Network Gamma

iNOS - Inducible Nitric Oxide Synthase

IP3 - Inositol 1,4,5-trisphosphate

IPSCs - Inhibitory Postsynaptic Currents

IPSPs - Inhibitory Postsynaptic Potentials

K^+ - Potassium Ion

KA - Kainic Acid

KAR - Kainate Receptor

KCl - Potassium Chloride

Kg - Kilogram

LEC - Lateral Entorhinal Cortex

LEV - Levetiracetam

LFP - Local Field Potential

Li - Lithium

LiCl - Lithium Chloride

LFS - Latency to First Seizure

L-NAME - L-N^G-Nitroarginine Methyl Ester

LRD - Late Recurring Discharges

LTD - Long Term Depression

LTG - Lamotrigine

LTP - Long Term Potentiation

LVA - Low Voltage Activated

Mg^{2+} - Magnesium Ion

mGluR - Metabotropic Glutamate Receptor

$MgSO_4$- Magnesium Sulphate

MEC - Medial Entorhinal Cortex

mEPSPs - Miniature Excitatory Postsynaptic Potentials

MES - Maximal Electroshock Shock

mM - Millimolar

µM - Micromolar

Ms - Millisecond

Na^+ - Sodium Ion

NaCl - Sodium Chloride

NaH_2PO_4 - Sodium Phosphate

$NaHCO_3$ - Sodium Hydrogen Carbonate

nM - Nanomolar

NMDA - N-Methyl-D-aspartic acid

nNOS - Neuronal Nitric Oxide Synthase

NO - Nitric Oxide

PB - Phenobarbital

PC - Piriform Cortex

PING - Pyramidal Interneuron Network Gamma

PG - Persistent Gamma

PGB - Pregabalin

PHT - Phenytoin

PKA - Protein Kinase A

PKC - Protein Kinase C

PMCA - Plasma membrane Ca^{2+} ATPase

PRC - Phase Response Curve

PSBB - Post-seizure Behavioural Battery

PTZ - Pentylenetetrazole

PV - Parvalbumin

RISE - Reduced Intensity Status Epilepticus

SC - Stellate cell

s/c - Subcutaneous

SE - Status Epilepticus

SEM - Standard Error of the Mean

sEPSCs - Spontaneous Excitatory Postsynaptic Currents

SERCAs - Sarcoplasmic Reticulum Ca^{2+} ATPase

sIPSC - Spontaneous Inhibitory Postsynaptic Current

SLE - Seizure Like Event

SRS - Spontaneous Recurrent Seizures

SSRIs - Selective Serotonin Reuptake Inhibitors

SVA2A - Synaptic Vesicle Protein 2A

SWO - Slow Wave Oscillations

TCAs - Tricyclic Antidepressants

TGB - Tiagabine

TPM - Topiramate

TTX - Tetrodotoxin

TLE - Temporal Lobe Epilepsy

UGTs - Uridine Diphosphate Glucuronosyltransferase

VFO - Very Fast Oscillations

VGB - Vigabatrin

VGCCs - Voltage-Gated Calcium Channels

VGSCs - Voltage-Gated Sodium Channels

VPA - Valproate

ZNS - Zonisamide

Chapter 1 Introduction

1.1 The Hippocampal Formation

The temporal lobe has a variety of functions and has been implicated with auditory, olfactory, vestibular and visual sensory processing, as well as having roles in speech, memory and emotion (Kiernan, 2012). Pathologically, the hippocampal formation has been associated with Alzheimer's disease, temporal lobe epilepsy (TLE), schizophrenia, depression and anxiety disorders (Small et al., 2011).

Definitions of the neuroanatomical organisation of the temporal lobe structures have varied considerably, depending on whether a cytoarchitectural or hodological viewpoint is adopted. In terms of cytoarchitecture, the cortex can be divided into the allocortex (3-4 cell layers) and the iso- or neocortex (6 cell layers). A translational layer, the mesocortex, exists between the allocortex and isocortex. This mesocortex can further be divided into the periallocortex (bordering the allocortex) and the pro-isocortex (bordering the isocortex). The periallocortex consists of five cell layers and has a densely packed layer two, but the laminar differences are not as clear as those in the pro-isocortex and isocortex (Witter, 1989; Lopes da Silva, 1990). Cytoarchitectonically, the limbic cortex (infralimbic cortex, ventral anterior cingulate cortex, granular retrosplenial cortex, pre- and parasubiculum, medial perirhinal cortex and the entorhinal cortex) belongs to the periallocortex.

From a hodological viewpoint the limbic cortex is strongly connected to the hippocampal formation (Witter, 1989). The hippocampal formation includes the dentate gyrus (DG), hippocampus, subiculum, presubiculum, parasubiculum and the entorhinal cortex (EC), as shown in figure 1-1. This neuroanatomical definition has withstood the test of time, and was first highlighted by Ramon y Cajal (1902) who emphasised the physiological significance of the hippocampus must be related to the EC as they were strongly connected. The EC serves to mediate inputs and outputs to the hippocampus, and hence represents a nodal point in cortico-hippocampal circuits (Canto et al., 2008).

The circuitry of the hippocampal formation, starting from layer 2 of the EC, projects to the DG and CA3 via the perforant pathway and layer 3 of the EC projects to CA1 and the subiculum. The DG gives rise to mossy fibres which terminate in CA3. The CA3 pyramidal cells, in turn, project to CA1 via the Schaffer collaterals. The subiculum and CA1, both project to deep layers of the EC and the deep layers of the EC return projections to cortical areas which originally projected to the EC (Shepherd, 2004).

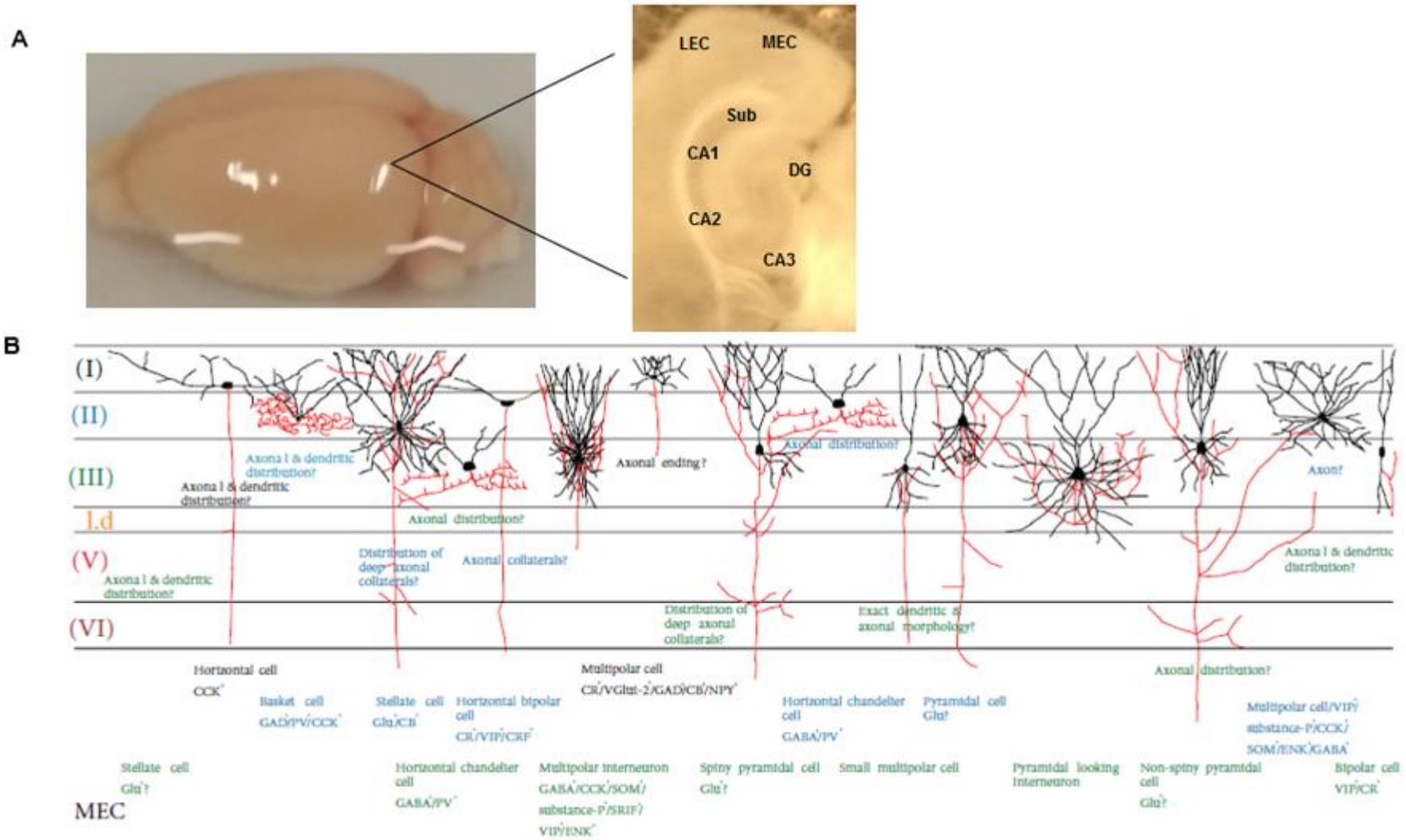

Figure1-1. The hippocampal formation. A. The anatomy and positioning of the hippocampal formation. B. A schematic representation of the morphology MEC cells (adapted from Canto et al., 2008).

1.1.1 The Entorhinal Cortex

The EC plays a pivotal role in the processing of information in cortico-hippocampal interactions. Brodmann (1909) defined the EC as area 28, and subsequently further divided the EC into the lateral entorhinal cortex (LEC) (area 28a) and the medial entorhinal cortex (MEC) (area 28b). Using retrograde and anterograde tracers, it has been demonstrated that the main cortical inputs to the LEC come from the piriform cortex, insular regions and frontal regions (Burwell & Amaral, 1998). The connections are reciprocated back from the LEC, with the strongest output connection to the piriform cortex (Agster & Burwell, 2009). The main cortical input to the MEC comes from the piriform cortex. The MEC also receives cortical inputs from insular, occipital, parietal and cingulate regions. The strongest cortical output connection is to the piriform cortex.

The main subcortical inputs to the LEC and MEC comprise of olfactory areas, the claustrum and the amygdala. These subcortical afferents project equally to the different layers of the LEC but not as equally to the different layers of the MEC. In the MEC, the olfactory areas strongly innervate the intermediate and medial layers, whereas the claustrum strongly innervates the lateral layers and the amygdala strongly innervates the medial layers. Other subcortical inputs to the EC include the septal nuclei, basal ganglia, dorsal thalamus, ventral thalamus and the hypothalamus (Kerr et al., 2007). The LEC provides strong output projections to the amygdala and olfactory areas of the subcortex, and both the MEC and LEC provide strong output connections to the basal ganglia.

1.1.1.1 Entorhinal cortex intrinsic organisation

The EC structure consists of six layers, which are clearly defined by the relative lack of cells in layer 1 and 4 (lamina dissecans). This type of lamination is characteristic of translational structures that connect the three-layered allocortex and the six-layered isocortex. Exploring the electrophysiological functions of different EC laminations, Greenhill et al. (2014) investigated background synaptic activity of the rodent entorhinal cortex via spontaneous inhibitory and excitatory currents, as this activity modulates responsiveness to inputs and outputs, and alterations are perhaps important in synchronization. Using a combination of whole cell patch clamp recordings, VmD and charge transfer (CT) measurements, to account for presynaptic as well as post-synaptic conductances, it was demonstrated, spontaneous excitatory conductances were greatest in L3 and equal in L2 and L5. Inhibitory conductance was greatest in L2, slightly smaller in L3 but substantially lower in L5. However, overall there was a dominance of inhibition over excitation in all three EC layers investigated.

These findings support earlier investigations showing a dominance of inhibition in the superficial EC layers (Gloveli et al., 1997b; Gloveli et al., 2001). Additionally, Woodhall et al.

(2005) demonstrated the frequency of spontaneous inhibitory postsynaptic currents (sIPSCs) in layer II occurred at four times the frequency of those recorded in layer V. The underlying mechanisms for this difference have been attributed to the presence of more inhibitory terminals in layer II cells. In support of this notion other studies have shown that layer II contained the highest number of GABA and GAD immunoreactive neurons in the EC (Kohler et al., 1985). However this observation cannot fully account for differences of inhibition in layer II and V, because the contribution of action-potential driven release was the same in both layers, which would be expected to be greater in layer II due to the larger number of inhibitory cells. Given this evidence, the likelihood of other possibilities such as differences in the release mechanisms or the control release of GABA terminals in the two layers maybe different. Complimentary to these investigations, Berretta and Jones (1996) illustrated layer V cells showed larger spontaneous excitatory postsynaptic currents (sEPSCs) which could occur in high frequency bursts and had a higher contribution from NMDA receptors, in comparison to layer II cells.

Many of the axonal projections from deep to superficial layers have been suggested to be excitatory and serve both excitatory and feed-forward inhibition purposes (van Haeften et al., 2003). There are also, greater morphological and electrophysiological differences between LEC and MEC layers 1 and 2 cells in comparison to layer 3 and 5 (Canto et al., 2008).

1.1.1.2 Layer II

Layer II of the MEC is densely packed with stellate cells (SCs), which are the most abundant cells in layer II, and pyramidal-like or non-SCs (Klink & Alonso, 1997a). Both types of cells have been considered as projection cells. Layer II SCs occasional project to layers III-VI, but their main targets are the DG and CA3 (Tamamaki & Nojyo, 1993). The cell bodies of SCs are ovoid or trapezoid in shape and are preferentially located in the superficial part of layer II. SCs are further characterised by the presence of thick primary apical dendrites (5.6-7.5 μm), whilst the basal dendrites are thinner (2.3- 4.5 μm). Primary dendrites mostly arise from apical and basal locations of the soma, and when they do arise from the sides they tended to curve upwards, giving these cells and hour-glass shape appearance. Dendrites frequently ended with a bouquet of three to six spines.

Non-SCs are smaller than the SCs and project to layers I and III (Klink & Alonso, 1997b). These cells are preferentially located in the deeper part of layer II, and cell bodies are ovoid or triangular in shape. Non-SCs present a radial distribution of dendrites. Many of these cells have one primary thick apical dendrite and several secondary apical dendrites which show a high density of dendritic spines. Basal dendrite projection was equal to apical dendrite projection and basal dendrites could be identified in the superficial part of layer III, this is one third the projection distance of SCs (Klink & Alonso, 1997a).

In terms of functionality, cells in layer II of the MEC display different electro-responsive properties and consequently different integrative properties that project to the hippocampal formation. SCs are highly electro-responsive, and application of subthreshold depolarising or hyperpolarising current pulse injections lead to the membrane potential obtaining an early peak followed by low sag. The presence of inward rectification during depolarising and hyperpolarising ranges indicates SCs are characterised by a non-linear voltage-current relationship. SCs seem to hold strong pacemaker properties which participate in the generation of theta and/or beta oscillations. In comparison to SCs, non-SCs display a less pronounced time-dependent inward rectification. Non-SCs also have longer action potential durations and do not generate subthreshold oscillatory activity, or spike clustering as SCs do (Alonso & Klink, 1993).

Similarly, Jones (1994) also described two cell types (type I: 69 % and type II: 31 %) of the MEC layer II, of SC morphology. In response to hyperpolarising currents, type I cells displayed pronounced time-dependent inward rectification, and rebound depolarisation when stepped back to rest. Inward rectification was abolished by bath application of Cs^+, which also induced a negative shift in membrane potential, thus implying this hyperpolarising inward rectification is activated at resting potential. The rebound depolarisation was diminished by extracellular application of Cs^+, Ca^{2+} channel blockers and TTX, suggesting this activity resulted from multiple conductances.

In response to depolarising currents, type I cells demonstrated early peaks followed by a steady decline, and inward rectification was evident. Type I neurons showed spike frequency adaptation but there was no convincing evidence of intrinsic bursting, as suggested by Alonso and Klink (1993). Finally, rebound hyperpolarisations were apparent in response to positive current steps, even when action potentials were not evoked, and could be blocked by Cs^+ which blocks I_h.

In response to hyperpolarising currents, type II cells did show inward rectification, but only at very large negative deviations from the membrane potential, which would probably not be of significance with regard to normal cellular activity. Type II cells did not show anode break rebound depolarisations, like type I cells. Inward rectification in type II cells, was blocked by Cs^+, but was not accompanied by Cs^+ associated membrane potential changes, as was evident with type I cells, thus indicating this type of inward rectification is unlikely to be activated at resting potential and therefore lacks depolarising influence.

In response to depolarising currents, type II cells displayed inward rectification, which could be reduced by TTX, and to a lesser extent Ca^{2+} channel blockers, indicating mediation by a subthreshold Na^+ and Ca^{2+} currents. If action potentials were evoked, they were followed by slow and fast after-hyperpolarisations. Type II cells did not show intrinsic bursting properties.

Initial studies exploring the morphology, intrinsic membrane properties and pharmacology of layer II MEC cells, have clearly identified the main difference between these two types of principle cells is the presence of a pronounced inward rectification in abundant type I cells (Alonso & Llinas, 1989; Alonso & Klink, 1993; Jones, 1994; van der Linden & Lopes da Silva, 1998). Since inward rectification in type I cells is present at rest, it is likely to drive rebound excitation, hence increased excitability in type I cells in comparison to type II. Jones (1994) further explored the pharmacology of the synaptic response of these two types of cells and found at low frequencies responses were controlled largely by inhibitory inputs, mediated by $GABA_A$ and $GABA_B$ receptors and at higher frequencies responses were dominated by excitatory inputs mediated by NMDA and non-NMDA receptors. Similarly, Gloveli et al. (1997b), demonstrated when layer II cells are stimulated with frequencies less than 5Hz no action potentials are produced, but when stimulated with frequencies more than 10Hz action potentials can be triggered during later stages of the stimulus train. As acetylcholine can modulate intrinsic EC conductances, it was further investigated whether cholinergic transmission effects on potential threshold during stimulation, but the muscarinic antagonist, atropine, had no effect on the frequency dependent induction of action potentials or on the firing threshold. Other explanations for the activation of layer II cells during high-frequency stimulations was suggested to include the down regulation of K^+ currents in response to synaptic input and/or the removal of inactivation of sodium channels as a result of net hyperpolarisation induced by repetitive stimulation, thus lowering the firing threshold. In support of this explanation it has been shown that layer II cells possess K^+ channels that are under strong metabolic control (Eder et al., 1991).

Alonso and Klink (2003) compared layer II MEC and LEC cells, and demonstrated LEC cells had a higher input resistance and MEC cells had a much more pronounced rectification, probably due to larger I_h currents. MEC neurons also displayed larger depolarising after potentials and the ability to fire action potential doublets, presumably from larger low-voltage-activated (LVA) calcium and sodium currents.

More specifically, Tahvildari and Alonso (2005) identified three types of principal cells of layer II of the LEC: fan cells, pyramidal cell and multiform cells. Fan cells had the most depolarised resting membrane potential (-66 ± 0.58 mV), the highest input resistance (57.3 ± 4.9 MΩ) and the shortest action potential duration (1.28 ± 0.03 ms) of the three cell types. Fan cells are the most abundant cells in layer II of the LEC and are relatively similar in morphology to layer II MEC SCs, as they displayed primary dendrites that branched over layers I and II. Like SCs, fan cells also showed time dependent inward rectification, but this was less robust in fan cells. Fan cells displayed inward rectification in the depolarising direction, indicating the presence of a persistent Na^+ current. Despite similarities between fan cells and SCs, fan cells did not develop subthreshold oscillations, as evident in SCs. Pyramidal cells of layer II LEC, display prominent dendritic trees that extend over layers I and II. Layer II MEC

pyramidal-like cells do show a less pronounced inward rectification in comparison to SCs, however, layer II LEC pyramidal cell did not show any presence of inward rectification. Multipolar cells had distinct morphology as dendrites of these cells were orientated in all directions, in comparison to the semi-circle orientation of fan cells. Dendrites of multiform cells extended over cell layers I, II and III. The electrophysiology of multiform cells was not significantly different from fan cells or pyramidal cells, and generally represented an intermediate of the other two types of layer II LEC cells.

Layer II of the EC also consists of a variety of interneurons such as multi-polar-neurons (MPNs) (Kohler et al., 1986), bipolar cells (Germroth et al., 1991), fast-spiking basket-like cells (Jones & Buhl, 1993) and chandelier cells (Soriano et al., 1993). Despite the difficulty associated with obtaining reliable intracellular recordings from EC interneurons, Jones and Buhl (1993) evoked responses from layer II fast-spiking basket cells and further determined they predominantly exhibit prolonged NMDA-mediated synaptic excitation. Assuming these interneurons are GABAergic in nature, they provide inhibitory control over SCs and output to the DG.

1.1.1.3 Layer III

The wider layer III of the EC consists predominantly of loosely arranged pyramidal cells, the morphology and electrophysiological properties, of which, appear to be consistent across the LEC and MEC (Lorente de Nó, 1933; Tahvildari & Alsonso, 2005). Layer III pyramidal cells project to EC layers II and V, and the subiculum.

Gloveli et al. (1997a) distinguished between four types of cell in layer III MEC. In terms of morphology type I and II were considered projection cells. Type I cells were the most commonly identified and displayed large-pyramidal-shaped cell bodies and extensive apical and basal dendrites. The apical dendrites always extended as far as the cortical surface and the basal dendrites extended twice as far in the horizontal direction in comparison to the vertical direction. Basal dendrite axons from this type of cell were detected in the deep layers of the EC and hippocampal CA1 area. Type II cells possessed non-spiny dendrites, whereas type I cells displayed spiny dendrites. Type II apical dendrites also projected to the cortical surface, but the first bifurcation was closer to the cell body. Type II basal dendrites were also presented as projecting to deep EC layers.

Electrophysiologically, both types of projection cells displayed antidromic action potentials with deep layer stimulation and showed slow hyperpolarisation following short subthreshold trains. Differences between type I and type II cells manifested, as type I cells had higher input resistance and slow time constants (estimated time taken to reach 63 % of the steady state voltage deflection during -0.1 nA, 160-250 ms current pulses), whereas type II cells has lower input resistance and faster time constants. These cells differed in synaptic response

too, as type I cells did not present fast IPSPs and type II cells did not present long-lasting EPSPs.

The two other types of cells identified were local circuit cells and could not be activated antidromically, by deep EC layer stimulation, nor did they display prolonged hyperpolarisation with stimulation of the LEC, MEC and subiculum. Type III cells were similar to type I cells; except the apical dendrites of this type did not reach the cortical surface, and apical and basal dendrites were non-spiny. Type III cells possessed local arborisation within layer III but also projected to the deep layers of the EC. Type III cells could further be differentiated from type I cells, by the presence of a lower firing rate and late occurring action potentials in response to current injection. Type IV cells projected predominantly to the superficial layers of the EC, but dendrites had been shown to also reach the cortical surface. Type IV cells possessed few apical dendrites but numerous basal dendrites. Deep layer stimulation did not activate these cells, antidromically, but instead evoke a fast IPSP which was not followed by a slow inhibitory component. Repetitive LEC stimulation did stimulate a depolarising response.

Morphological and electrophysiological properties of layer III cells, clearly demonstrate their suitability in transferring information. The excitability of these cells in the presence of bicuculline ($GABA_A$ antagonist) emphasise the importance of inhibition preserving control within the network, and malfunction of this control may contribute to cognitive impairments associated with Alzheimer's disease (Braak & Braak, 1991), epilepsy (Du et al., 1995) and schizophrenia (Jakob & Beckmann, 1994).

1.1.1.4 Layer V

Layer V of the EC receives input connection from the hippocampus and subiculum (Swanson & Cowan, 1977; Sorensen and Shipley, 1979), and provides long range output back to the neocortex (Insausti et al., 1997). In addition to connections outside the hippocampus, layer V also innervated the superficial layers of the EC (Lorente de No, 1933; Dolorfo & Amaral, 1998). Exploring the morphological and electrophysiological properties of layer V cells, Hamam et al. (2000, 2002) identified three cell types of neurons: pyramidal, horizontal and polymorphic cells. Pyramidal cells were characterised by a primary apical dendrite which mostly branched at the distal extreme end, in layers I and II. Basal dendrites branched radially within layer V and VI. Horizontal cells are similar to pyramidal cells, as both have a primary apical dendrite and prominent basal dendritic tree, but the dendritic tree of horizontal cells projects mainly in the horizontal plane. Horizontal cells did not have a pyramidal-shaped soma, and the apical dendrites branch further down in comparison to pyramidal cell, in layer III and deep layer II. All dendrites of horizontal cells are sparsely covered with spines. In contrast to pyramidal and horizontal cells, polymorphic cells did not have a primary apical

dendrite, and instead displayed numerous primary dendrites that are radially distributed and also consist of short spines along the length of dendrites.

Electrophysiologically, it is difficult to distinguish between the different cell types as they are similar in several parameters including: inward-rectification, spike-frequency adaptation and intrinsic oscillations. Subthreshold membrane potential oscillations were present, but this is unlikely to involve I_h, as layer V neurons displayed subthreshold membrane potential oscillations but not have I_h. Furthermore, time-dependent inward-rectification is minimal in layer V neurons (15 %) compared to layer II SCs (50 %). Subthreshold oscillatory activity is generally associated with pace-maker properties of layer II SC theta generation, but it is known that during prolonged periods of cortical activity the basal forebrain cholinergic system is activated, which significantly innervates layer V of the EC (Milner et al., 1983; Alonso & Kohler, 1984; Lysakowski et al., 1989).

1.2 Epilepsy

The EC is implicated with some of the earliest and most severe pathological changes in Alzheimer's disease (Braak & Braak, 1991; Jansen et al., 1990). Additionally, neuropathological disruption of the EC has also been associated with the development of Parkinson's disease and Down's syndrome (Hirano & Zimmerman, 1962), schizophrenia and manic depression (Jakob & Beckmann, 1986), and temporal lobe epilepsy (TLE) (Rutecki et al., 1989; Deutch et al., 1991; Spencer and Spencer, 1994).

Epilepsy has been recognised as one of the most common neurological disorders, affecting 50 million people worldwide (Kwan & Brodie, 2006). Many different types of epilepsies can be distinguished based on affected areas, but 41 % of cases suffer from temporal lobe epilepsy (Curia et al., 2014). Although significant pharmacological advances have been made, one third of patients remain resistant to two or more antiepileptic drugs (AEDs), and are therefore drug refractory (Kwan & Brodie, 2006; French, 2007). Three main types of drug resistance can be observed clinically: Refractory de novo (patient never enters remission from the onset of epilepsy), progressive refractoriness (patient becomes seizure free initially but seizures recur and become uncontrollable), and wax-and-wane pattern (epilepsy alternates between being controlled and uncontrolled) (Schmidt & Loscher, 2005). Factors that can be used to predict responsiveness to antiepileptic drugs (AEDs) include: the type of epilepsy, aetiology and patient's history of seizure frequency. Environmental factors that affect AED therapy include: trauma, drug exposure, genetics and metabolism. Resistance to AEDs is therefore multifactorial (French, 2007).

Epilepsy is a collection of conditions which have a range of underlying aetiologies and pathologies. The common feature amongst this cluster of conditions is recurrent seizures. Seizures are abnormal, excessive and synchronous excitation of neuronal populations. The Commission on Classification and Terminology of the International League Against Epilepsy (1981), states seizures can be broadly classified into partial seizures, which originate from a focal area of the cortex, or generalised seizures, which are widespread and involve bilateral cortical regions. As illustrated in figure 1-2, partial seizures can be further divided into simple partial (remain conscious) and complex partial seizures (impaired consciousness). There are several subclasses of generalised seizures: tonic, atonic, clonic, tonic-clonic, myoclonic and absence seizures. Generalised seizures which are preceded by partial seizures are known as secondary generalised seizures. The aetiologies of seizures can be broadly classified into several groups too: Idiopathic (mono-or poly-genetic origin), Symptomatic/ Secondary (known cause) and Cryptogenic (unidentified underlying abnormality).

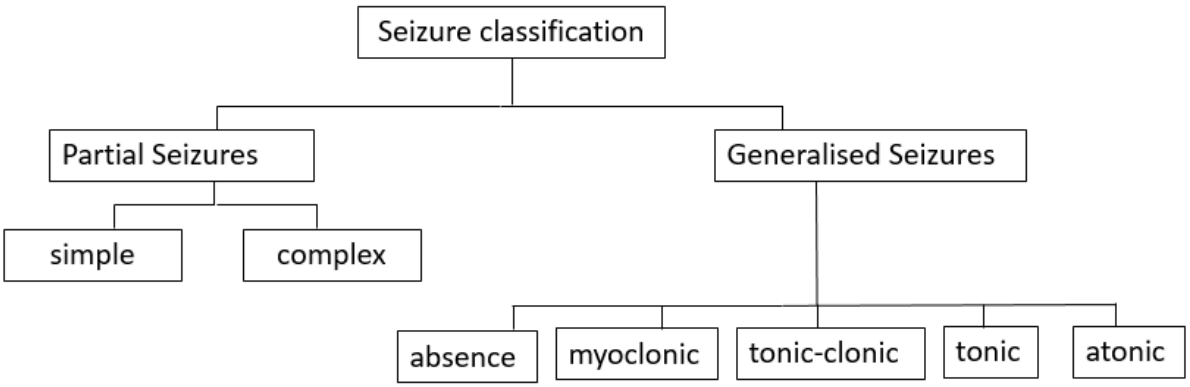

Figure 1-2. Seizure classification. Seizures can be classified into two broad categories partial and generalised seizures depending on brain regions involved. These two broad classes of seizure can be further subdivided depending on level of impaired consciousness and severity of symptoms.

Seizure-like EEG events can vary considerably depending on the age of the individual or animal, the type of epilepsy, the stage of epileptogenesis, and so forth. These complexities make it difficult to temporally define seizure like events (SLE). EEG seizure activity can usually be classified into interictal and ictal like discharges. As illustrated in figure 1-3 inter-ictal discharges (IIDs) consists of a single population burst of approximately 100 ms duration, whereas ictal discharges (IDs) consist of a series of population bursts and often lasts several seconds (Anderson et al., 1986; Avoli et al., 2002). Although seizure activity typically follows from inter-ictal through to ictal discharges, *in vitro* investigations have shown that often tonic-clonic activity that characterises ictal-discharges (IDs) develops into late-recurring-discharges (LRDs) which only display short bursts of tonic activity with prolonged exposure

to seizure inducing agents and extracellular ionic manipulations. In contrast to IDs, LRDs have been shown to be resistant to several AEDs (Drier & Heineman, 1990; Sokolova et al., 1998; Zhang et al., 1995). LRDs have been suggested by Zhang et al. (1995) to mimic status epilepticus (SE) development seen *in vivo*, in humans (Trieman et al., 1990) and animals (Lothman et al., 1989).

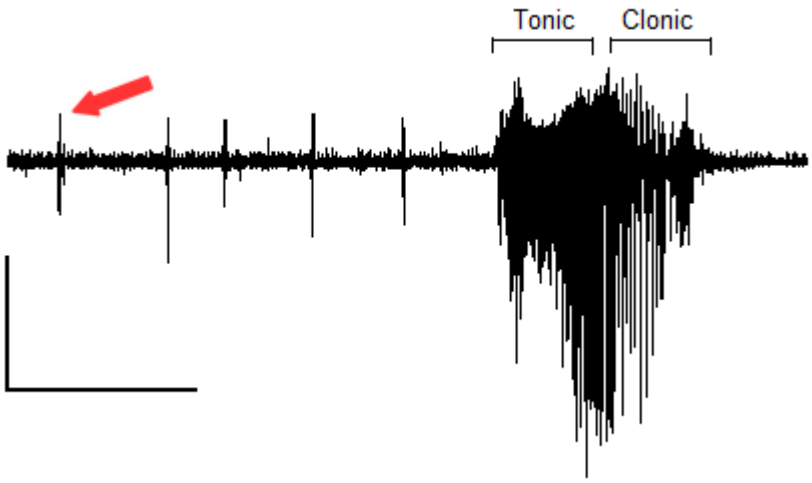

Figure 1-3. Inter-ictal and ictal like discharges recorded in vitro from layer II mEC of a chronically epileptic Wistar rat. Tonic and clonic phases of the ictal event are indicated and the red arrow highlights inter-ictal events. Scale 1000 µV x 20 seconds.

Refractory epilepsy has devastating effects for patients and their families. Many individuals suffer bodily injuries, psychiatric and social impairments that limit employability, likelihood of marriage and significantly decrease the quality of life (Brodie, 2005). Additionally, the cost of treating epileptic patients, and even more so for drug-resistant patients, represents a great burden to the healthcare system (Curia et al., 2014). Given these caveats, it remains an important challenge of epilepsy research to understand the pathophysiogenesis of TLE.

1.2.1 Epileptogenesis

One way in which research has attempted to gain insight into the mechanisms of epilepsy is to employ different kinds of animal models (see section 1.4 for further details). A particular advantage of some types of models is the ability to replicate, to some extent, the development of epilepsy, also known as epileptogenesis. Under these circumstances, researchers can investigate modifications that occur within the network at different stages of pathology which promote seizure activity, rather than just the mechanisms present once recurring seizures are established. Epileptogenesis has been defined as injury initiated, finite changes, which cause surviving neurons to generate abnormal, synchronous and recurring

epileptiform discharges, and which can be precipitated as clinically non-obvious epileptiform event or clinically obvious focal or generalised seizures (Sloviter & Bumanglag, 2013; Curia et al., 2014). The typical time course of epileptogenesis is as follows: initial neuronal injury followed by a seizure free latent period and finally the presence of recurring seizures (Dudek & Staley, 2012; Sloviter & Bumanglag, 2013).

An important question surrounding epileptogenesis, is whether it is a continuous process. The step-function hypothesis suggests the mechanisms responsible for seizure generation are mature at the time of the first seizure, after the latent period. On the other hand, the continuous-function hypothesis suggests the mechanisms responsible for seizure generation are not complete at the time of the first seizure, and seizure frequency increases as epileptogenesis progresses. There is evidence to support both sides of the argument which highlights the complexity surrounding the temporal progression of epilepsy. In support of the continuous-function hypothesis many studies have highlighted that most rats do not reach a steady seizure frequency for many months (Hellier et al., 1998; Nissinen et al., 2000), although not all rodents show progressive activity (Gorter et al., 2001).

Furthermore, it is likely many individuals will demonstrate non-convulsive seizure activity, during the latent period, before the first convulsive seizure (Bertram & Cornett, 1993; Bertram & Cornett, 1994). However, due to the clustering of seizure activity and lack of continuous recording during the latent period these preclinical features are unlikely to be detected. Clearly, some important epileptogenic mechanisms are active during and even after the latent period, with circuit alterations enough to induce epilepsy. Two mechanisms that have been proposed are: death of GABAergic interneurons and axon sprouting with consequential increases in excitation (Ben-Ari & Dudek, 2010).

The latent period has been highlighted as a crucial stage in epileptogenesis, and has taken the focal spotlight of epilepsy research. The idea that “seizures beget seizures” (Gowers, 1881) was one of the earliest proposed mechanisms of epileptogenesis. Since then, numerous supporting studies has shown seizures induce a cascade of events including neuronal damage (Crepel et al., 1989; Cossart et al., 2001; Dinocourt et al., 2003; Dudek & Shao, 2003; Kobayashi & Buckmaster, 2003) and formations of mossy fibres that establish aberrant glutamatergic synapses (Epsztein et al., 2005; Sutula et al., 1988; Tauck & Nadler, 1985) (see Ben-Ari et al., 2008 and Nadler, 2003 for reviews). However, this view of latent period mechanisms of epileptogenesis has been contested by evidence from animal and clinical studies which show the occurrence of a seizure does not influence the longer term predisposition to more seizures, and whilst seizure frequency or seizure density may have some contribution to future predisposition of seizures given that many patients enter remission and discontinue medication other mechanisms must be contributing too (Berg & Shinnar, 1997; Evans et al., 2006; Hauser & Lee, 2002).

Moreover, others have questioned whether the latent period is an obligatory stage of disease progression. Slovitier and Bumanglag (2013) suggest hyper-excitability, disinhibition, loss of interneurons and entorhinal cortex neurons have direct epileptogenic mechanisms which are independent of secondary mechanisms that lead to disease progression. Based on such evidence it would seem apparent that the definition of latent period needs modification rather than the questioning of its existence. Furthermore, the model of epilepsy used and the temporal development span associated with it should be carefully considered when addressing research questions on the epileptogenesis process. As Slovitier and Bumanglag (2013) have suggested research can be divided into disease prevention (immediately after injury) and disease modification (once recurrent seizures begin, attempts are made to silence them).

1.2.2 Seizure initiation and propagation

Many temporal lobe seizure initiation and propagation studies have demonstrated the importance of the EC. In particular, it has been shown that IIDs are initiated in the CA3 hippocampal area, and thereafter propagate to the EC via CA1 and the subiculum, and return to CA3 via the DG (Barbarosie & Avoli, 1997; Barbarosie et al., 2000; D'Antuono et al., 2002). This has been confirmed by the laceration of the Schaffer collaterals which reduces the spread of CA3 initiated IIDs. Conversely, temporal analysis of the effects of 4-AP, pilocarpine, elevated K^+ and Mg^{2+} free medium, alongside laceration of the perforant path, illustrate that IDs are initiated in the EC (Nagao, Alonso & Avoli, 1996; Avoli et al., 1996; Barbarosie & Avoli, 1997; Barbarosie et al., 2000; Walther et al., 1986; Wilson et al., 1988; Dreier & Heinemann, 1991; Bear & Lothman, 1993). More specifically, the deep layers of the EC have been highlighted in the generation of IDs (Lopantsev & Avoli, 1998b; Jones & Heinemann, 1988; Jones & Lambert, 1990). In support of animal electrophysiological research, human studies (Rutecki et al., 1989; Deutch et al., 1991; Spencer and Spencer, 1994) and intrinsic optical signalling studies (D'Arcangelo et al., 2001) have also implicated EC dysfunction.

Jones and Heinemann (1988) further explored the synaptic and intrinsic responses of deep MEC cells in normal and magnesium-free mediums to gain insight into the cellular properties that give rise to the ictogenic region. Three cell types were characterised: non-bursting, bursting and fast spiking cells.

Non-bursting cells displayed minimal late AHPs following repetitive firing, and accommodation of spike firing frequency was not prominent. AHPs and accommodation of spike firing frequency, are presumed to be dependent on a calcium-dependent-potassium conductance (Brown & Griffith, 1983; Hotson & Prince, 1980). Evidently, intrinsic inhibition in the form of spike frequency adaptation during repetitive firing and AHPs are not well expressed in the deep layers of the MEC.

Bursting cells displayed intrinsic bursting properties which was suggested to be due to activation of a persistent Ca^{2+} conductance that is often seen in other brain regions (Deschenes et al, 1982; Wong & Prince, 1978). Synchronous discharges of these cells, promoted by reciprocal excitation could drive epileptic activity in non-bursting cells.

Generally the investigation of current voltage relationships in cells displayed ohmic characteristics (linear voltage-current relationship) but inward rectification in depolarising and hyperpolarising directions was seen. The hyperpolarising rectification was suggested to indicate the presence of inward K^+ or mixed Na^+/K^+ conductance. Depolarising rectification was suggested to be a result of Na^+ and/or Ca^{2+} conductance. Bursting and/or non-bursting cells displayed high threshold Ca^{2+} spikes, which may be synaptically activated, when penetrated with Cs/QX-314 containing electrodes, which blocks K^+ and Na^+ conductances. Such Ca^{2+} or Na^+ inward currents would promote ictal activity. Following subiculum stimulation, deep layer EC cells did not display long-latency, long-duration IPSPs, as seen in neocortical cells, therefore suggesting feed-forward or recurrent inhibition is weak, or masked by powerful excitation. Evidently, the intrinsic and synaptic properties of MEC cells support the generation of seizure activity, and its location and function within the limbic system makes it an ideal candidate in seizure propagation.

Several lines of investigation have further elucidated the active mechanisms during ictal activity. For example, pharmacological investigations have associated NMDA receptors in EC ID generation, as antagonists such as CPP (3,3(2-carboxypiperazine-4-yl)propyl-1-phosphonate) abolish NMDA receptor mediated seizure activity in the EC, but have no effect on slow GABA receptors or CA3 IIDs (Avoli et al., 1996). Although, NMDA independent paths of ID initiation in the EC, do appear to be present in younger rodent brains (Calcagnotto et al., 2000). More specifically, the potent AMPA/Kainate antagonist CNQX (6-cyano-7-nitro-quinoxaline-2, 3-dione) abolishes IIDs and IDs.

Additionally, intracellular recordings from EC cells, during ictal activity have reversal potentials which suggest the involvement of $GABA_A$ receptor mediated conductance (Lopantsev & Avoli, 1998a). Similarly, residual deep EC activity can be extinguished with the application of the $GABA_A$ receptor antagonist, bicuculline methiodide (D'Arcangelo et al., 2001).

The neuronal mechanisms active during ictal and interictal activity are to some extent understood, however the mechanisms underlying the transition from IIDs to IDs remain obscure (Avoli et al., 2002). Nevertheless, IIDs have been postulated to control rather than sustain IDs. When the Schaffer collaterals are cut, ictal activity in the EC and DG is accompanied by large elevation in K^+, which precedes the ictal events. These elevations are not seen in CA3, suggesting this area inhibits IDs (Barbarosie et al., 1997). Moreover, these findings are not replicable in younger animals, further suggesting age-dependent

mechanisms (Calcagnotto et al., 2000). Seizure activity is not only accompanied by extracellular elevation of K^+, but also decreases in Ca^{2+} (Antonio et al., 2016; Gloveli et al., 1995).

1.3 Models of seizures and epilepsy

The pioneering work of Yamamoto and McIlwain (1966) with the development of the brain slice preparation, permitted a more thorough, multidisciplinary, understanding of neurobiological processes and activity. Like many scientific techniques, the *in vitro* brain slice preparation has many advantages and disadvantages (see chapter 3 for further discussion), that researchers should take into consideration when forming a hypothesis. However, in terms of research on seizures and epilepsy, advantages of the *in vitro* brain slice preparation include: the ability to investigate seizure initiation and propagation in combined hippocampal-entorhinal slices, apply pharmacological manipulations to understand the mechanisms of seizure generation and cessation and test the efficacy of different antiepileptic drugs.

In vitro investigations of seizures and epilepsy have typically taken two approaches with the use of either acute or chronic models. Acute models require 'normal' brain slices to be prepared, thereafter manipulations are carried out to induce seizures. In chronic models, brain slices are prepared from chronically epileptic rats which have undergone procedures that initiate the process of epileptogenesis. Slices produced from these processes have the advantage of a modified network architecture which more closely represents the pathological condition. Despite the impressive range of protocols available to carry out such investigations, cautions regarding the interpretation of results should be made. For example, as the brain slice preparation method severs many intrinsic and extrinsic connections, it is not surprising that, generally, seizures do not occur spontaneously and require pharmacological manipulation to induce them. Additionally, acute *in vitro* studies have demonstrated interneurons play a fundamental role in the after-discharge of ictal-like events, but chronic models have demonstrated that interneurons are lost as a result of the epileptic condition, therefore conclusions and generalisations about epilepsy and seizure mechanisms made from such studies, although very insightful, should be treated with caution or only firmly made when combined with other techniques such as *in vivo* studies. Although there a numerous types of acute and chronic models of epilepsy (as illustrated in figure 1-5), only the main acute models (0$[Mg]^{2+}$, low calcium, high potassium, 4-AP and electrical stimulation) and main chronic models (the pilocarpine, kainate and kindling models) shall be considered in further detail.

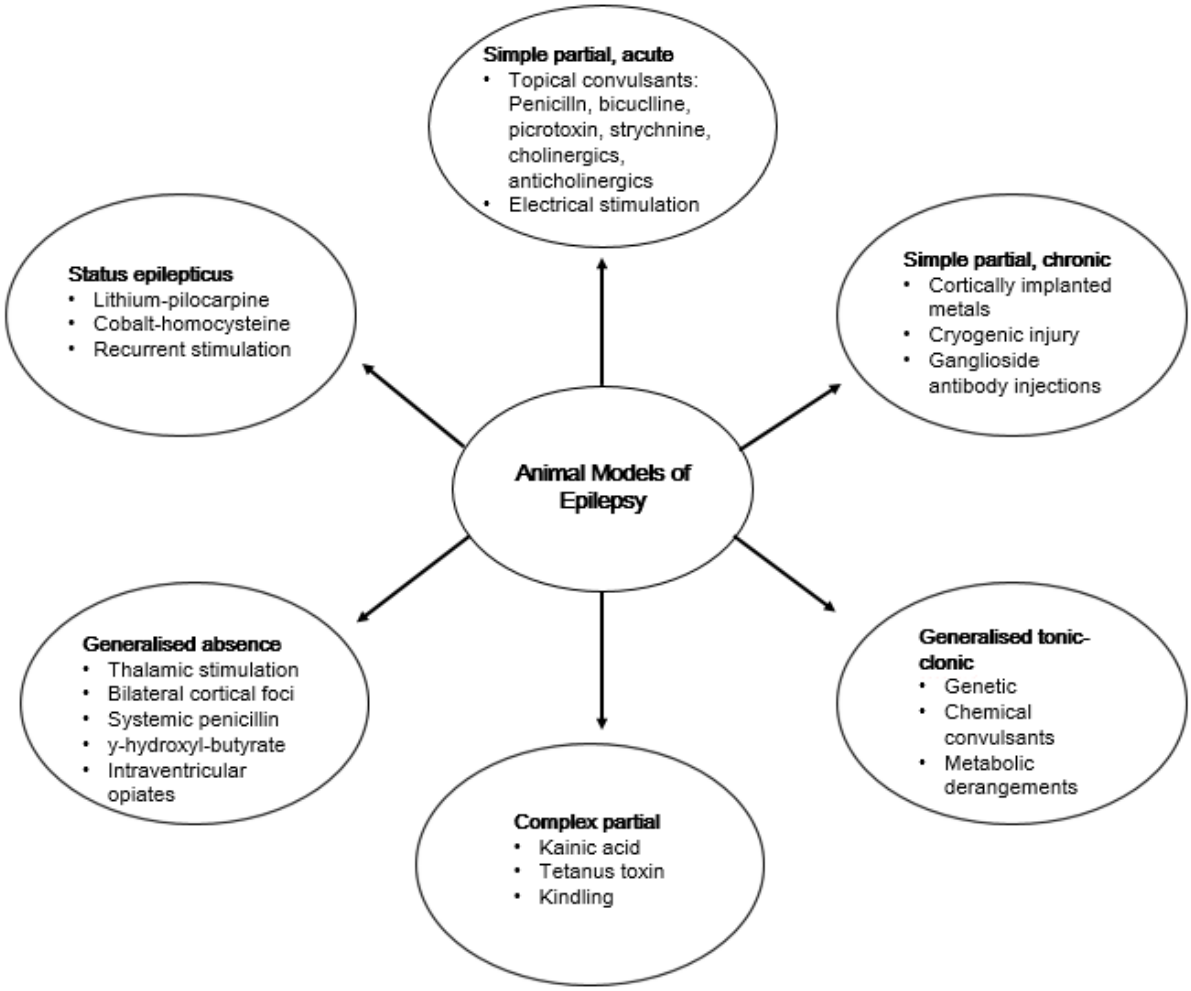

Figure 1-5. Animal models of epilepsy (Adapted from Fisher, 1989). There are many different types of animal models, use is dependent on the hypothesis.

1.3.1 Acute Models

1.3.1.1 Magnesium free model of acute seizures

Since the development of magnesium-free model of seizure induction in the early 1980s, the model has been widely used in the testing of antiepileptic drugs (Albus et al., 2008; Anderson et al., 1986; Coulter & Lee, 1993; Walther et al., 1986). The magnesium-free model of seizures also has clinical relevance as there is evidence that patients with generalised tonic-clonic seizures have Mg^{2+} deficits in serum and cerebrospinal fluid (Afzal et al., 1985; Govil et al., 1981), and has even been suggested as the cause of seizures in some humans (Arnold et al., 1983; Nuytten et al., 1991). Additionally, anticonvulsant properties of Mg^{2+} have been demonstrated by intravenous injection in animal models of epilepsy (Borges & Gucer, 1978).

Under normal physiological conditions Mg^{2+} ions serve to maintain a block on NMDA receptors (see figure 1-6). When extracellular Mg^{2+} is reduced this block is removed and a potent increase in excitability is initiated, and SLEs precipitate. The mechanism through

which the magnesium-free model of seizures works is through the facilitation of NMDA receptors and a decrease in the surface charge screening near voltage activated channels as a result of a reduction of positively charged Mg^{2+} ions in the extracellular solution (Heinemann et al., 2006; Iseav et al., 2012).

There are seven subunits of NMDA receptors: GluN1, GluN2A, GluN2B, GluN2C, GluN2D, GluN3A and GluN3B. NMDA receptors are tetramers which are commonly composed of GluN1 and GluN2 subunits (Candy et al, 2001; Clarke et al, 2013). NMDA receptors play important roles in excitotoxicity, excitatory synaptic transmission and plasticity, as a consequence of their unique features which include: slow activation-deactivation kinetics, high permeability to Ca^{2+} and a voltage-sensitive block by extracellular Mg^{2+} (Candy et al., 2001). The importance of increased NMDA conductance in seizure activity initiation has been demonstrated by SLEs induced by the magnesium-free model, being stopped initially (<2 hrs after first SLE) with application of NMDA receptor antagonists or by restoration of Mg^{2+} in the extracellular solution. However, cessation of SLEs with such agents at later phases often fail to have the same effects, suggesting the presence of a long lasting synaptic potentiation has formed (Anderson et al., 1986; Derchansky et al., 2004; Dichter & Pollard, 2006; Stanton et al., 1987).

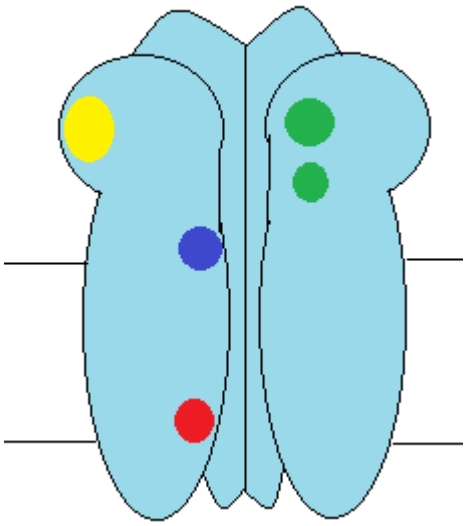

Figure 1-6. A schematic representation of a NMDA receptor. Modulatory (green) and glutamate binding sites (yellow) are shown, where polyamine, glycine and NMDA antagonists are effective, respectively. The Mg^{2+} site is shown in blue. The site where channel blocking agents, such as PCP, are effective is marked in red.

The role of surface charge screening in the magnesium-free model of seizure generation is evident but not as well understood (Isaev et al., 2012). Cell membrane surface charge is produced by phosphates, sialic acid, charged amino acids, charged lipids and other hydrophilic residues of channel proteins (Messner et al., 1985; Iseav et al., 2007). The electrical double layer ‘is the array of charged particles and orientated dipoles which is

thought to exist at every interface' (Grahame, 1947). As illustrated in figure 1-7, the cell surface is predominantly negatively charged, and so attracts a layer of positive ions (Stern layer). As positive ions are large, the cell surface charge is not completely neutralised and more positive ions and a few negative ions are attracted (Gouy-Chapman layer). This additional layer of ions is further from the cell surface and can be displaced. The diffuse double layer theory has traditionally been applied to non-biological substances such as mercury and clay, but a comprehensive description of electrochemical potentials at the surface of biomolecules has been given by Dukhin (1993). There are two types of electrostatic interactions between the negatively charged cell surface and cations. Firstly, the cations can *bind* to cell surface when hydration has been removed. In this instance the cation complex is immobile. Secondly, hydrated cations, found a few angstroms from the cell surface can *screen* the cell surface to repel opposing charges. Here, cations remain mobile and unbound (Bara et al., 1989).

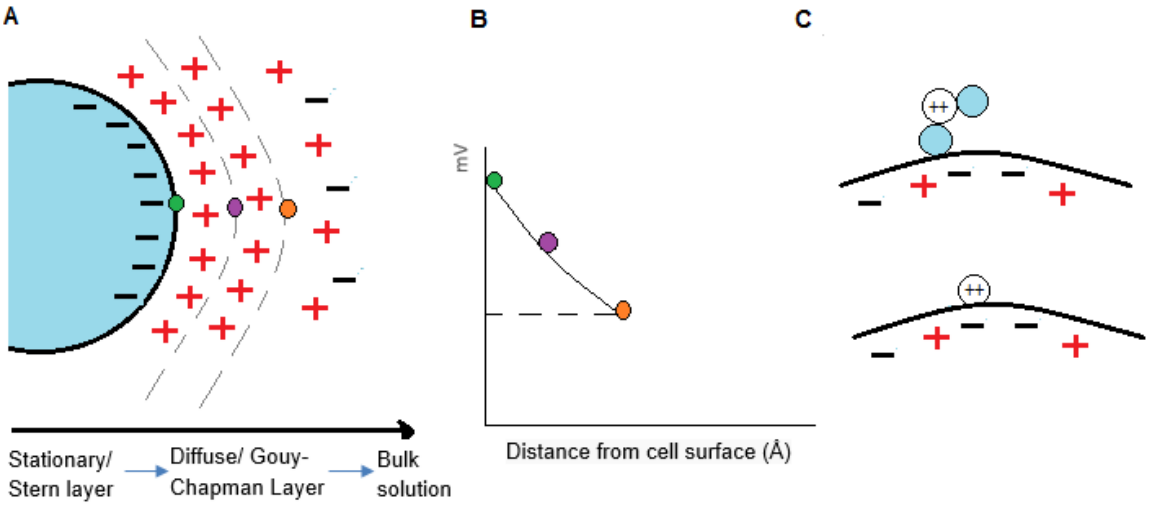

Figure 1-7. A schematic representation of the diffuse double layer theory and surface charge screening and binding. A. The head groups of phosphatidylserine and phosphatidylinositol (two phospholipids of plasma membrane) are negatively charged, hence a predominance of negative charge on the cell surface. This negative charge attracts a well ordered layer of positive ions, also known as the stationary or Stern layer. As positive ions are large they do not fully neutralise the surface charge, and the residual anionic charge attracts more positive ions. Additional positive ions are further away from the cell surface and therefore weakly attracted. This of additional layer of positive ions is known as the diffuse layer or Gouy-Chapman layer and is less ordered and more moveable. B. The graph demonstrates potential differences at the cell surface (green dot), Stern layer (purple dot) and Gouy-Chapman layer (orange dot). The potential at the slipping plane (where the Gouy-Chapman layer and bulk solution meet, is also known as the zeta potential. Ions at the diffuse layer can often be displaced, and the change in potential measured. C. A schematic representation of surface charge screening (top) and binding (bottom).

Whilst the opening of NMDA receptors in low Mg^{2+} is a key determinant of hyperexcitability, so too, is the altered charge screening, which creates conditions where the membrane is much more excitable because the Stern and Gouy layers are not there to screen/shield the

membrane and this can shift voltage dependences (activation and inactivation) of many ion channels toward hyperpolarized potentials (Frankenhaeuser and Hodgkin, 1957; Hille, 2001). Divalent cations such as Mg^{2+} and Ca^{2+} are negative regulators of excitability and so their loss directly excites cells as well as altering NMDAr function.

Early investigations demonstrated how the Mg^{2+} binding effects on the NMDA receptor affects its kinetics. The membrane depolarisation evoked by NMDA receptors was accompanied by an increase in resistance, when neurons were bathed in a Mg^{2+} containing medium. The channel blocking effects provided by the presence of Mg^{2+} is highly dependent on membrane potential, as antagonist effects were strong at -60 mV but practically absent at +20 mV. In contrast, when neurons were bathed in Mg^{2+} free medium, NMDA evoked depolarisation was accompanied by a decrease in resistance (Mayer et al., 1984). Removal of the Mg^{2+} block, promotes the influx of Ca^{2+} and Na^{+} ions (Kandel et al., 2012; Malenka & Nicoll, 1999).

To further investigate the origin of Mg^{2+} conductance effects, voltage-clamp experiments were conducted. Examinations of current-voltage relationships provide insight into conductance of different channels. Typical ohmic conductors display a positive linear I-V relationship. However, many channels have non-linear I-V relations as membrane conductance changes depending on voltage, a property called rectification (Hille, 2001). The current-voltage relationship of the NMDA response has a negative slope over the -70 to -35mV potential range, indicating the conductance activated by NMDA becomes smaller on membrane hyperpolarisation. Conversely this negative slope is not present in a low Mg^{2+} medium, indicating the removal of Mg^{2+} reduces the voltage sensitivity of the conductance activated by NMDA (Mayer et al., 1984). Overall, deviations from physiological concentrations of Mg^{2+} are potent modulators of neuronal excitability, as is evident from differences in conductance curves. Additionally, replacement of depleted Mg^{2+} with Ca^{2+} has been shown to decrease the amplitude of ictal events, thus supporting the notion that reduced surface charge screening contributes to the epileptic-like effects seen in the magnesium-free model (Jones & Heinemann, 1988).

Despite the importance of the EC highlighted by the Mg^{2+} free model, SLEs have been reported to have differential properties in different parts of the brain. For example, the epileptiform pattern seen in the frontal cortex is much shorter and does not include after-discharges commonly seen on temporal lobe regions, and activity seems to spread much faster than in limbic regions (Armand et al., 1998a). SLEs in the EC, subiculum and temporal neocortex are characterised by negative potential shifts of up to 30mV, which are superimposed by high-frequency, small amplitude bursts, after which clonic after-discharges follow. In combined hippocampal-entorhinal slices SLEs can often be seen to start in the EC, but spread to other areas at a slower rate in comparison to low Ca^{2+} induced SLEs

(Buchheim et al., 2000). Additionally, different patterns of seizure spread can be seen when a low Mg^{2+} medium is used to initiate SLEs in chronically epileptic tissue. For example in pilocarpine treated animals, seizure spread can be initiated by the DG or subiculum, whereas in kindled animals seizure spread is preferentially through the DG (Behr et al., 1998). Moreover, SLEs induced by low Mg^{2+} show age-dependent differences as latency to first SLE has been shown to be shorter in immature rodents in comparison to adult rodents (Gloveli et al., 1995). These effects can be explained by less efficacious GABAergic inhibition. In the immature brain, GABA can act in an excitatory rather inhibitory manner. Increased expression of the sodium-potassium-chloride cotransporter, (NKCC2), and reduced expression of the chloride exporter (KCC2) during early development leads to an increased intracellular Cl- concentration, therefore when $GABA_A$ receptors are activated an efflux of Cl^- ions occurs which produces depolarisation rather than hyperpolarisation (Ben-Ari et al., 1989; Rakhade & Jensen, 2009). It has also been highlighted that NMDA expression in the immature brain is higher (Chahal et al., 1998) and less responsive to Mg^{2+} block (Kleckner & Dingledine, 1991). Levels of Na^+, K^+ ATPase levels are sufficiently low in the immature brain, further contributing to increased susceptibility of ictal discharges (Fukunda & Prince, 1992).

1.3.1.1.1 Long Term Potentiation

The induction of LTP fundamentally relies on the activation of postsynaptic NMDA receptors. Glutamatergic activation of postsynaptic AMPA receptors allows intracellular entry of Na^+ and K^+ ions and glutamatergic activation of postsynaptic NMDA receptors promotes influx of Ca^{2+} and Na^+ ions once the Mg^{2+} block has been removed. Metabotropic glutamate receptors have also been shown to enhance LTP via activation of protein kinase C (PKC) (McGuinness et al., 1991).

As described in section 1.4.1.1, the removal of Mg^{2+} from the extracellular environment, removes the NMDA receptor block and facilitates NMDA receptor conductance. The critical rise in intracellular Ca^{2+} as a result of NMDA receptor activation, triggers LTP through a wide range of cellular signal transduction pathways. Despite this number of protein kinases involved in LTP, overwhelming evidence has a suggested an important role of Ca^{2+} activated α-calcium-calmodulin-dependent protein kinase II (CamKII) in initiating LTP effects. CamKII is one the most abundant proteins in neurons and is expressed pre- and post-synaptically, but expression has been found to be higher in postsynaptic cells where it would be ideally located to respond to changes in calcium concentration (Flink & Meyer, 2002; Lynch, 2004). Activation of CamKII leads to AMPA receptor phosphorylation which ultimately increases AMPA receptor conductance (Barria et al., 1997).

Brief, controlled periods of Ca^{2+} elevations occur during physiological processes associated with plasticity changes of LTP in learning and memory (Gnegy, 2000; Malenka & Nicoll, 1999; Tzounopoulos & Stackman, 2003; West et al., 2001). However, at the other of the

spectrum pathological, uncontrolled and irreversible elevations in Ca^{2+} lead to neuronal death. The middle ground, appears to be a characterised by a less severe epileptogenic injury whereby exposure to prolonged but reversible elevations in Ca^{2+} trigger pathological plasticity changes, leading to chronic epilepsy and DRE (DeLorenzo et al., 2005).

1.3.1.2 The low calcium model

The first *in vitro* demonstration of low extracellular Ca^{2+} leading to the induction of SLEs, was carried out by Jefferys and Hass (1982). The CA1 area of the hippocampus seemed to be particularly sensitive to the low Ca^{2+} model. Seizure events become apparent as simultaneous synaptic activity is blocked and elevation in extracellular K^+ (9-10 mM) occurs. The mechanisms through which these effects can be seen are reduced surface charge screening and a reduction in Ca^{2+} dependent K^+ conductance, as indicated by increased input resistance (Haas & Jefferys, 1984; Heinemann et al., 2006). Similar seizure inducing effects can be seen *in vivo* with Ca^{2+} chelating agents, such as EGTA (Feng & Durand, 2003). Furthermore, seizure inducing effects of low Ca^{2+} can be reversed by restoring extracellular calcium. Unlike the Mg^{2+} free model of seizures, which display tonic-clonic firing, SLEs of the low Ca^{2+} model only display tonic activity (Heinemann et al., 2006).

The rate at which seizure activity spreads in the low Ca^{2+} model, is relatively slow (Konnerth et al., 1984). Like the low Mg^{2+} model, the low Ca^{2+} model shows age-dependent effects, with a lower threshold for induction of SLEs in younger rodents (Heinemann et al., 2006). This model is not as effective in human or in other brain regions unless accompanied by further ionic concentration modifications, such as the increase in extracellular K^+ (Schweitzer et al., 1992). Such differences may possibly be a result of less compact cellular spatial arrangement, in comparison to CA1.

1.3.1.3 The high potassium model

As many early studies on seizure activity identified extracellular rises in K^+ accompany seizure activity, it is not surprising such modifications were later used as a model of acute seizure induction. Jensen and Yaari (1988), first, mimicked *in vitro* SLEs by raising the concentration of extracellular K^+ from 3.5 mM to 8 mM. As highlighted by Traynelis and Dingledine (1988), mechanistically high extracellular K^+ causes: a decrease in K^+ mediated AHPs amplitude, a decrease in GABAergic IPSP amplitude as a consequence of increase intracellular Cl^-, depolarisation of pyramidal cells, reduced spike threshold. A reduction in the amplitude of IPSPs and pyramidal cell firing, activate NMDA receptors. As more cells are recruited, extracellular K^+ raises further, producing swelling and reducing the extracellular space, resulting in concentrated effects of high K^+. As IIDs arrive at CA1, and high extracellular K^+ is not effectively removed from the extracellular space, seizures are initiated.

The high K^+ model produces ictal events with durations of 20-90 seconds, at regular intervals of 1-8 minutes. Ictal events showed tonic and clonic patterns of firing (Traynelis & Dingledine, 1988). The high K^+ model has been reported to preferentially induce SLEs in CA1 (Traynelis & Dingledine, 1988), and IIDs initiated in CA3 have been suggested to contribute to the initiation of IDs in CA1. It has been proposed reason for preferential CA1 activity include: increased susceptibility to hypoxia in the CA1 (Aitken & Schiff, 1986), lower Na^+K^+ ATPase in CA1 (Haglund et al., 1985) and higher NMDA receptor density in CA1 in comparison to CA3 (Monaghan & Cotman, 1985). On the other hand, the investigation of SLEs hippocampal and parahippocampal regions have shown both CA1 and the EC are the most susceptible to high K^+ induced SLEs, whereas the DG is least susceptible (Bear & Lothman, 1993). Nevertheless, induced SLEs occur in the DG when high K^+ is combined with low Ca^{2+} (Schweitzer et al., 1992).

1.3.1.4 The 4-Aminopyridine model

4-aminopyridine, has been shown to induce SLEs *in vitro* in the temporal lobe (Avoli et al., 1996; Rutecki et al., 1987; Voskuyl & Albus, 1985) and in humans (Lundh et al., 1984). Mechanistically, 4-AP blocks voltage-gated K^+ channels (Llinas et al., 1976). As a consequence of K^+ blockade, direct or indirect Ca^{2+} influx is facilitated. This has been the proposed explanation for augmentation of inhibitory and excitatory neurotransmitter release by 4-AP (Tapia & Sitges, 1982).

Profile analysis of ictal discharges in combined hippocampal-entorhinal slices shows 4-AP induced ictal events are initiated in the deep layers of EC (Avoli et al., 1996). This finding is congruent with other models such as: low Mg^{2+}, $GABA_A$ antagonists (Jones & Lambert, 1990), high K^+ (Bear & Lothman, 1993) and pilocarpine (Nagao et al., 1996). Ictal discharges in this model have been shown to last between 20-480 seconds, with intervals of 71-1000 seconds and displays both tonic and clonic patterns of firing during IDs (Avoli et al., 1996). Once again, IIDs are initiated in the CA3 and propagate to CA1 but not to the EC. In this model, short recurrent discharges dominate in CA1 and CA3. Short recurrent discharges are thought to be similar to IIDs, but are not associated with increases in extracellular K^+ (Bruckner & Heinemann, 2000; Sokolova et al., 1998). Additionally, short recurrent discharges do not respond as effectively as IDs to AEDs (Bruckner & Heinemann, 2000).

1.3.1.5 The electrical stimulation model

Electrical stimulation trains (60 Hz, 2 seconds) at 10 minute intervals have been applied to combined hippocampal-entorhinal slices and used as an acute model of seizures (Rafiq et al., 1993). Stimulation intensity of trains were four times the voltage required to trigger field potentials, at 8-10V. Initial electrical stimulation of the Schaffer collaterals in CA1 prompted an after-discharge of around 10 seconds. This initial activity was comprised of rapid tonic discharges, but following an additional 2-4 stimulations, elicited activity began to show clonic

discharges too. Additionally, after-discharges increased in duration, with successive stimulation trains, before plateauing at 40 seconds with 5-9 stimulations.

Following the initial after-discharge, secondary discharges began to appear. Both, primary and secondary discharges could be recorded at hippocampal and parahippocampal sites. Latency of secondary discharges was the shortest in the DG, followed by CA3, CA1 and the EC. Lesions of the mossy fibre and subicular region demonstrated secondary discharges were dependent in the hippocampal-entorhinal connections. Discharges were sensitive NMDA antagonists. An advantage this model has over other acute models, is the mechanisms of seizures can be assessed without overtly obvious modifications to the inhibition and ionic medium of slices.

1.3.2 Chronic Models of Epilepsy

1.3.2.1 The pilocarpine model

The pilocarpine model of epilepsy belongs to the SE group of models and was developed shortly after the kainate model (Nadler et al., 1978) and the kindling model (Goddard et al., 1969). These models represent three of the most widely used models in epilepsy research (Pitkänen et al., 2006). The pilocarpine model can be regarded as a model of both, acute and chronic seizures, and was first described by Turski et al. (1983). Turski et al, administered methyl scopolamine nitrate (1 mg/kg s.c) 30 minutes prior to all doses of pilocarpine to reduce peripheral cholinergic effects along with diazepam (10 mg/Kg i.p.). Methyl scopolamine is used to block the peripheral cholinergic effects of pilocarpine, and as it does not cross the BBB, it does not interfere with the development of SE. Diazepam is used to reduce SE duration.

Turski et al. (1983), thereafter, administered different pilocarpine doses (100/200/400 mg/Kg i.p.) into male Wistar rats (200-220 g), and the progression of seizures were classified according to the Racine scale (1972). Behavioural changes were time and dose dependent, with rodents who had received lower doses of pilocarpine, taking longer to show behavioural effects. Similarly, in animals who had received lower doses (100 mg/Kg) of pilocarpine, damage was confined to the piriform cortex and anterior olfactory nuclei, but in animals that had received larger doses of pilocarpine, further profound neuropathological damage was observed.

Animals that received 300-400 mg/Kg of pilocarpine were motionless for 5-10 minutes after pilocarpine administration and displayed oral-facial movements, salivation, eye-blinking, twitching of vibrissae, and yawning. This activity persisted for 18-45 minutes. Thirty minutes after injection limbic motor seizures with intense salivation, rearing, upper extremity clonus, and falling were observed every 5-15 minutes and lasted up to 2 hours. Around 60 % of the rats successfully developed SE (Cavalheiro et al., 1991). Animals entered a post-ictal

unconscious state for 1-2 days following remittance of SE, which occurred 5-6 hours after pilocarpine administration. Following SE, body weight decreased (10–20%), but returned to pre-treatment states after 1 week (Turski et al., 1989). Mortality rates were reported to be around 30–40%.

Since the initial description of the pilocarpine model, various improvements and considerations have been made, consequently leading it to be considered a relevant animal model of the human disease years after its initial characterisation (Curia et al., 2008). Nonetheless, as researchers develop different aims in epilepsy research, differences occur in several variables of the pilocarpine model which ultimately affects findings. For example, route of pilocarpine administration, species, strain, gender and age of animals add variability in the induction and development of epilepsy and findings associated with such changes (Curia et al., 2008). Additionally, different doses of pilocarpine affect the length of the latent period (Cavalheiro et al., 1991; Liu et al., 1994). The length of SE and the induction of various arresting drugs (e.g. diazepam, 10 mg/Kg and pentobarbital, 30 mg/Kg) also affects the length of the latent period with the literature showing considerable variability (Lemos & Cavalheiro, 1995 cf Biagini et al., 2006). Similarly, the extent of damage and activation of epileptogenic mechanisms varies with SE duration (Biagini et al., 2006, 2008; Du et al., 1995; Wozny et al., 2005).

Pilocarpine has also been used in combination with other drugs such as: lithium (Honchar et al., 1983), N omega-nitro-L-arginine methyl ester (Starr & Starr, 1993), picrotoxin (Hamani & Mello, 1997), cycloheximide (Longo & Mello, 1997) and MK-801 (Hughes et al., 1993). However, the lithium-pilocarpine has been the most extensively used. Lithium is administered 24 hours before pilocarpine administration and reduces the dose of pilocarpine (30 mg/Kg) required to induced SE, presumably by increasing sensitivity to pilocarpine (Clifford et al., 1987). The addition of lithium pre-treatment has also increased success of inducing SE. Moreover, further improvement of mortality rates has been made, without affecting electrographic seizures, by administering the central muscle relaxant, xylazine, after SE induction (Yang et al., 2006; Thompson et al., 2007). Evidently, there are many variations within the pilocarpine model of epilepsy; however the refinements have significantly increased the quality and ethical standards of animal research.

In our laboratory, further refinements to the lithium-pilocarpine model have been made, leading to the development of the Reduced-Intensity-Status-Epilepticus (RISE) model (Modebadze et al., 2016). Two major innovations of this model are the use of xylazine during the early development of SE, and the use of a ‘stop’ cocktail which comprised of a mGluR5 antagonist (MPEP), the non-competitive NMDA receptor antagonist (MK-801) and diazepam being administered an hour after SE had been induced. Together these improvements led to a substantial reduction in mortality, below 1 %, and retained epileptogenic morbidity. This

model was developed with the aim to improve the quality of epilepsy research and increase efforts to implement the research ethics principles of 3R's (reduction, refinement and replacement) more effectively. The refinements to the induction procedure led to a reduction in animal suffering and a reduction in the number of animals required, as more animals survived the induction process. These findings exemplify the strengths of this pilocarpine model over others whereby mortality can be as high as 90 % (Curia et al., 2008). Moreover, by limiting the time period of SE, the intensity of SE was reduced which consequently reduced the level of neuronal damage. As highlighted by Sloviter (2005), prolonged periods of SE lead to global cortical damage, inflammation and cerebrovascular damage which do not accurately imitate the natural progression of epilepsy.

Pilocarpine has been suggested to induce SE through activation of M1 and M2 muscarinic receptors subtype. M1 receptor knockout mice do not develop seizures from pilocarpine administration (Hamilton et al., 1997). Additionally, the muscarinic antagonist, atropine, can block pilocarpine induced SE, but only before seizures have been initiated. After seizure initiation the maintenance of seizure activity relies on other mechanisms (Clifford et al., 1987). Activation of these receptors modifies several signalling pathways through a cascade of reactions which ensure excitability and epileptic pathology is initiated and maintained.

Activation of M2 receptors, leads to a decrease of neuronal excitation as adenylate cyclase is inhibited which in turn reduces the release of ACh (Smolders, 1997). The activation of M1 receptors, in contrast leads to a much more complex set of reactions (See Figure 2 from Cavalheiro et al, 2006 for overview). Firstly, phospholipase C is activated with leads to production of diacylglycerol and inositol triphosphate (IP3), this in turn alters Ca^{2+} and K^{+} conductance resulting in increased excitability (Segal et al., 1988).

Additionally, the reduction in ATPase activity alters the homeostasis of ionic environments to promote excitability and seizure activity. For example, Fernandes et al. (1996) noted a reduction of Na^{+} K^{+} ATPase during SE and the latent period but and increase during chronic epilepsy. Glutamate toxicity induces abnormal NMDA conductance which increase intracellular levels of Ca^{2+} which activates the formation of free radicals which in turn alters ATPase activity. Compensatory upregulation of cytosolic Ca^{2+}, through Ca^{2+} ATPases (SERCA and PMCAs) can be seen at initial insult, but 5 hours post SE reductions in ATPase activity is evident, thus promoting loss of Ca^{2+} homeostasis and cell death (Funke et al., 2003). High concentrations of Ca^{2+} lead to increases in glutamate release, which in turn activate AMPA and kainate receptors leading to increased intracellular Na^{+} and Ca^{2+}, which in turn influence the Mg^{2+} block on NMDA receptors, allowing glutamate conductance to further increase and exacerbate excitability (Cavalheiro et al., 2006). Glutamate conductance at NMDA receptors also encourages expression of GAP-43, which leads to mossy fibre sprouting and plasticity (McNamara & Routtenberg, 1995). Status epilepticus has further

been associated with increases in noradrenaline and serotonin, and decreases in dopamine utilisation (Cavalheiro et al., 1994). Metabolic processes associated with the use of these neurotransmitters, release free radicals which have been associated neurodegeneration (Bellissimo et al., 2001). Evidently, all these alternations lead to modified neurotransmission, and cellular sprouting which promote cell death and seizure activity.

1.3.2.2 The kainate model

Kainic acid (KA) is a potent analogue of the excitatory neurotransmitter, glutamate. In the late 1970s it was used to develop a model of epilepsy, as it was demonstrated to induce repetitive seizures and neuronal damage in the hippocampus (Nadler et al., 1978) and amygdala (Ben-Ari et al., 1979). Like the pilocarpine model, the KA model is characterised by three stages of epileptogenesis: 1) an 'initial precipitating incident' which in this model is proposed to be the damage induced during recurring seizures in status epilepticus, following KA administration, 2) the seizure free latent period and 3) the development and increase in the frequency of recurrent spontaneous seizures, which characterises chronic epilepsy. The KA model closely resembles domoic acid poisoning, and in 1987 a severe outbreak of domoic acid poisoning through shellfish was reported. Individuals who had suffered SE from domoic acid poisoning, had a seizure-free period of about a year before developing TLE (Cendes et al., 1995). Superimposed with the temporal progression of recurring seizures, are the anatomical changes which include: neuronal loss, mossy-fibre sprouting (Buckmaster & Dudek, 1997), metabolic (Ebisu et al., 1994) and genetic changes (Nedivi et al., 1993; Hevroni et al., 1998).

Early work of the KA model of epileptogenesis demonstrated preferential neuronal loss and damage to the CA3 area, which probably reflects the greater expression of KA receptors in the CA3, and susceptibility to excitation and damage (Monaghan & Cotman, 1982; Nadler, 1978; Tremblay et al., 1984). However, this pattern of pathology is different to patterns seen in human TLE, whereby CA1 is usually more damaged than CA3. Although, such difference may be explained by the differences in neuroanatomic structure between human and rodent brains. On the other hand, sclerosis is not necessarily required for the generation of seizures, as resected tissue does not always shows neuronal loss in human TLE patients (Williamson et al., 1993).

The KA induction of SE, typically follows one of two protocols, either a single large systemic injections (9-12 mg/Kg) (e.g., IV, SC) (Cronin et al., 1992; Tauck & Nadler, 1985; Tremblay et al., 1984) or single low-volume intracerebral injections (1 - 3.75 nM) (e.g. intraventricular or intrahippocampal) (Bragin et al., 1999; Tauck & Nadler, 1985). Limitations of the former include high mortality rates following treatment, but this has been partially overcome by stopping SE with diazepam after a specified amount of time (Tremblay & Ben-Ari, 1984). However, this led to fewer animals developing recurring spontaneous seizures as

characterised by chronic epilepsy. It is also important to consider the shelf life of drugs such as KA, as differences in the potency of effect is likely to be seen depending on whether it is new or old (Dudek et al., 2006). Furthermore, significant improvements to this protocol have been made by titrating the dose of KA (Hellier et al., 1998).

In contrast to systematic injection, intracerebral injections require anaesthesia and stereotaxic techniques and are therefore time consuming, but importantly are more likely to develop focal injury. Administration of KA intracerebrally has been suggested to not consistently shown as much damage as seen when administered IV (Nadler et al., 1985). Additionally, with intracranial injection, seizures are initiated at the site of injection. In comparison, IV administration of KA initiates seizures in hippocampal and amygdala areas, which propagate to other limbic areas and the neocortex, which drives motor seizures (Nadler, 1981). Evidently such differences in seizure initiation and propagation is the reason why systemic injections are considered to produce more realistic forms of pathology and therefore the preferred method of administration of kainate. Moreover, Bragin et al. (1999) demonstrated through video-monitoring that only 40% of animals who received intracranial KA injections developed spontaneous recurring seizures 3-8 months post SE, presumably such limited progression of epileptogenesis occurring as a result of the focal nature of injury produced by intracerebral injections.

1.3.2.3 The kindling model of epilepsy

The kindling model of epilepsy was first recognised by Goddard et al. (1969) and is commonly initiated by repetitive electrical stimulation which leads to a gradually evolving seizure-induced plasticity which promotes epileptogenesis and the emergence of spontaneous recurrent seizures (SRS) characterised in chronic epileptic states. Following electrode implantation and postoperative recovery, biphasic 1 msec pulses at 60-100 Hz are delivered for a duration of 1-2 seconds. Behaviour is observed and electrographic recordings taken for each daily stimulus. Goddard et al. (1969) noticed there was no obvious behavioural response initially, and no after-discharge present on EEG recordings, but after one week of daily stimulation behavioural response included arrest, closing of the ipsilateral eye and chewing movements. At this time point after-discharges also became apparent. The after-discharge consisted of high amplitude rhythmic spikes which lasted several seconds and altered in frequency. Progression of stimulation induced effects continued thereafter. At the end of two weeks with daily stimulation, the first bilateral clonic convulsion (7 seconds) was observed, 15 seconds after stimulation, and involved rearing, loss of balance, facial contractions and forelimb clonus. Thereafter successive days of stimulation continued to induce convulsions with shorter latencies and longer durations. It is noteworthy to mention, however, whilst consecutive stimulation induced convulsions spontaneous seizures in the absence of stimulation did not occur. On the other hand, Sayin et al. (2003) demonstrated

spontaneous seizures were apparent when paired-pulse inhibition was reduced or lost after 90-100 evoked seizures. The sensitivity to stimulation once induced seemed to be permanent, as intervals of 2, 3, 4 or 6 weeks without stimulation did not reverse kindling effects (Goddard et al., 1969). The slower progression of epileptogenesis in the kindling model has been seen as great advantage over pilocarpine and kainate models whereby SE is induced rapidly, because there is much more control and minimal mortality, along with the ability to investigate more detailed network changes (Galanopoulou & Moshe, 2006).

The timing of intervals between stimulations is also of importance to kindling effects. Goddard et al. (1969) highlighted the 'massed stimulation effect' which refers to a reduction in the response to stimulation when stimulus trains were applied with inter-stimuli intervals less than 24 hours. For optimal kindling effects intervals between stimulation should be 24 hours or longer. This massed stimulation effect shows age dependent effects, as it appears in adult rats but not rat pups (Moshe & Albala, 1982). Moreover, massed stimulation effects can be overcome by increasing the stimulation duration, such as 10 second trains at 30 minute intervals over 6 hours (Lothman & Williamson, 1994) or 20 Hz pulses for 10 seconds delivered per minute over 24 hours (Sloviter, 1983). Such differences in stimulation protocols, undoubtedly, lead to differences seizure activity and progression of epileptogenesis (Nairsmagi et al., 2004; Pitkanen et al., 2002). Additionally, differences in the development of seizures can be seen when kindling is applied to different pathways. Limbic pathways develop stage 5 seizures quicker in comparison to neocortical and sub-cortical areas (Goddard et al., 1969).

In comparison to other chronic models of epilepsy, the kindling model shows similar functional and structural alterations. Following the first evoked seizure, there are reports of increase in NMDA conductance, increased inhibition, apoptosis, neurogenesis and gliosis. In the dentate gyrus, granule cells normally only fire single action potentials, even when inhibition is reduced or blocked, and these properties contributes to the filtering properties. However, when NMDA conductance is increased by kindling induced seizures, filtering properties are reduced and spike discharges are more likely to occur in CA3 (Lynch et al., 2000; Sayin et al., 1999).

Following five evoked seizures axonal sprouting has been shown form recurrent excitatory circuits (Scharfman et al., 2003). Following 30 evoked seizures, presence of hippocampal sclerosis and memory loss is apparent (Sutula et al., 1995), and following 90-100 evoked seizures there appears to be reduced inhibition as a result of interneuron loss (Sayin et al., 2003). Evidently, kindling starts with activation of NMDA receptors, and a further cascade of events leading to functional and structural network changes.

1.3.3 Models of epilepsy as screening tools for antiepileptic drug discovery

The discovery of AEDs originally occurred by serendipity, and later the need for preclinical testing of potential AEDs became apparent, hence the common use of pentylenetrazole (PTZ) and maximal electric shock (MES) screening models. The MES model induces tonic hind limb seizures through corneal or transauricular electrical stimulation. The PTZ model induces generalised clonic and myoclonic seizures via systematic administration of PTZ (Klitgaard, 2005). As early as 1881 Gowers highlighted “abnormal discharges are due to potentiation of excitatory mechanisms or to a failure of intrinsic cerebral inhibitory systems”. Since then epilepsy research has illustrated that the brain’s equilibrium can be disrupted in several ways (e.g. GABA antagonists, 4-AP, alterations to the aCSF concentrations of Na^+, K^+ and Mg^{2+}) to induce seizures (Köhling & Avoli, 2006; Margineanu & Klitgaard, 2009). Additionally, other studies have suggested initial seizure activity promotes recurring seizures by inducing network changes, such as cell loss and formation of novel mossy fibre synapses, which disrupt the balance between excitation and inhibition (Ben Ari & Cossart, 2000). Many AEDs focus on restoring, the excitation-inhibition balance, by reducing excitation or enhancing inhibition (Klitgaard, 2005).

Significant improvements have been made from first generation to second generation AEDs, such as: improved ease of use and tolerability, lowered risk of hypersensitivity and detrimental drug interactions. However, AED discovery investigations have received strong criticism as development of new AEDs have not attenuated drug-refractory epilepsy, or improved treatment in terms of prevention and cure for the disease (Simonato et al., 2012). Evidently, the emphasis of the excitation-inhibition imbalance in epilepsy is of limited value, as this over simplistic view, of a clearly multi-factorial disease, ignores the mechanisms that render an epileptic brain resistance to AEDs (Margineanu & Klitgaard, 2009). Many have recognised the most important step in AED discovery is the choice of animal model, which should accurately mimic human drug-refractory epilepsy in order to elucidate the mechanisms that lead to resistance and screen for new AEDs which are superior to currently available AEDs (Kupferberg, 2001; Potschka, 2012).

Intractable epilepsy has been defined as a failure to respond to two or more drugs, with more than one seizure per month, for a specified time period (e.g. 12-18 months) (Berg et al., 2001; Cowan, 2002). Given the various types of epilepsy, drug resistance is likely to be multifactorial, hence the need for several types of animal models. Nevertheless, several suggestions as to the mechanism of drug-refractory epilepsy have been indicated. For example the target (or pharmacodynamics) hypothesis suggests alterations of cellular targets leads to reduced sensitivity to AEDs (Remy et al., 2003; Loup et al., 2000). On the other hand the transporter (or pharmacokinetics) hypothesis proposes that cerebral endothelium of

the blood brain barrier (BBB) overexpress efflux drug transporters, hence reduces the AEDs at intended sites (Loscher & Potschka, 2002; Sisodiya, 2003). Chronic models that imitate the epileptogenesis process have been recognised as adding significant value to the discovery of AEDs and elucidating the mechanisms of drug-refractory epilepsy, but protocols are often elaborate and time consuming, therefore the need for improved acute models also exists.

1.4 Antiepileptic Drugs

The first effective AED was potassium bromide, introduced by Charles Locock in 1957 (Eadie, 2012; Sieveking, 1861). Locock was an obstetrician, who described patients suffering from 'hysterical epilepsy', a disorder of seizures in women which were prominent around menstruation. Locock reported 93% of patients obtained seizure control following treatment with potassium bromide. Sixty years later, Alfred Hauptmann (1912) described the antiepileptic effects of phenobarbital given to epileptic patients for sedation. Preclinical evaluation of bromides and barbiturates were not conducted; nevertheless, the importance of screening antiepileptic agents was recognised leading to the development of the MES and PTZ models (Kupferberg, 1989; Loscher and Schmidt, 1988).

Since the advent of bromides and barbiturates, several other first generation AEDs became available, such as benzodiazepines (BZD), ethosuximide (ESM), carbamazepine (CBZ), phenytoin (PHT), and valproate (VPA) (Klitgaard, 2005). After 1965, several second generation AEDs became available, such as lamotrigine (LTG), felbamate (FBM), vigabatrin (VGB), tiagabine (TGB), topiramate (TPM), gabapentin (GPT), zonisamide (ZNS) and levetiracetam (LEV). Second generation AEDs have been associated with fewer side effects and interaction effects, therefore are more tolerable than classical AEDs, but have not made significant advances in terms of antiepileptic efficacy; therefore there remains a continued need for new medical therapies (Loscher and Leppik, 2002). To maintain ethical standards new AEDs have been tested as add-on treatments in drug-resistant epileptic patients, consequently this trend has been maintained once drugs have been licenced (Perucca, 1996). The variety of AEDs available has allowed greater propensity to tailor treatment to the characteristics of the patient. Drug selection requires professionals to evaluate type of seizures, pharmacokinetic properties, co-morbid conditions, age, ease of use and cost (Perucca, 2005).

Ideally, AEDs should decrease the spread of excessive abnormal neuronal firing, without interfering with normal physiology. AEDs are typically not categorised based on mechanistic action due to several reasons. Firstly, the molecular mechanisms of AEDs are not fully understood and many have more than one mode of action. Secondly, knowledge of the pathophysiology of epilepsy is incomplete, therefore categorising AEDs is of limited value (Peruuca, 2005). Despite these limitations many AEDs have been demonstrated to control

seizures by modulating voltage-dependent Na^+ channels, Ca^{2+} channels, and potentiating GABAergic inhibition (Macdonald & Kelly, 1995; Perucca, 2005; Rogawski & Loscher, 2004), as illustrated in Table 2.

Table 1.2 Mechanisms of first and second generation antiepileptic drugs (Adapted from Perucca (2005) and Rogawski & Loscher (2004))

	Abbrev.	Sodium channels	Calcium channels	GABA system	Other	Partial Seizures	GTC seizures	Absence seizures	Myoclonic Seizures	Infantile Spasms	Lennox-Gastaut
First Generation AEDs											
Benzodiazepines	BDZ			++		+	+	+	+		+
Carbamazepine	CBZ	++	+ (L-type)	?	+	+	+	-	-		
Ethosuximide	ESM		++ (T-type)			-	-	+			
Phenobarbital	PHB		?	+	++ (Cl-)(AMPA)	+	+	-			
Phenytoin	PHT	++	?		+	+	+	-	-		
Valproic Acid	VPA	?	+ (T-type)	?	++	+	+	+	+		+
Second Generation AEDs											
Felbamate	FBM	++	+ (L-type)	+	+ (NMDA)	+	+	+			+
Gabapentin	GPT	?	++ (N-,P/Q-type)	?		+	+	-	-		
Lamotrigine	LTG	++	++(N-,P/Q, R-,T-type)	+	+	+	+	+	?		+
Levetiracetam	LEV		+ (N-type)	+	++ (SV2A)	+	+	+	+		
Oxcarbazepine	OXC	++	+ (N-, P-type)		+	+	+	-	-		
Pregabalin	PGB		++(N-, P/Q-type)								
Tiagabine	TGB			++							
Topiramate	TPM	++	+(L-type)	+	+ (KA/AMPA)	+	+	+	+	+	+
Vigabatrin	VGB			++		+	+	-	-	+	
Zonisamide	ZNS	++	++(N-,P-,T-type)	?	+	+	+	+	+	+	+

1.4.1 Voltage-gated sodium channels

Voltage-gated sodium channels (VGSCs) play an important role in regulating the electrical excitability of many cells. VGSCs can rapidly cycle through resting, open and inactivated states, promoting high-frequency actions potentials required for normal physiological function and epileptic activity. As a result serval AEDs target the modulation of VGSCs (Rogawski & Loscher, 2004).

VGSCs consist of an α-subunit which consists of four homologous domains and each domain is made of six transmembrane segments (S1-S6). Between segments S5 and S6 there is a P-loop which comprises part of the channel pore. The α-subunit is covalently linked with an accessory β subunit (β1, β2, β3) in some tissues (Ragsdale & Avoli, 1998; Catterall, 2000; Goldin, 2003). The physiological functions of VGSCs are fundamentally dependent on voltage, which controls the opening and closing or inactivation of the channel. Once a threshold voltage has been reached within the cell the channel will become activated and the channel will open allowing positive ions to flow into the cell (depolarisation). At the peak of the action potential, the channel closes or becomes inactivated and the cellular potential decreases back to its resting potential as the neuron repolarises and thereafter hyperpolarises itself. During hyperpolarisation the membrane's voltage becomes sufficiently low and removal of inactivation or de-inactivation occurs, rendering the cell ready to participate in another action potential.

Inactivation closes the channel until sufficient recovery time has elapsed, hence inactivation determines the refractory period, which determines the frequency of firing (Goldin, 2003). There are two main kinetic classes of inactivation, fast and slow. Inactivation that is fast, has been described as a 'ball-and-chain' mechanism, in which the inactivating particle (IFMT (a four amino acid stretch consisting of isoleucine, phenylalanine, methionine and threonine)), which consists of a cytoplasmic linker between domains 3 and 4, and docks at domains 3 (between S4 and S5) and 4 (between S4 and S5 and at the end of S6) (Rohl et al., 1999; Goldin, 2003). The molecular mechanism governing slow inactivation is not clearly defined, but it has been suggested to involve structural rearrangement (Mitrovic et al., 2000; Ong et al., 2000; Vilin et al., 2001; Goldin, 2003).

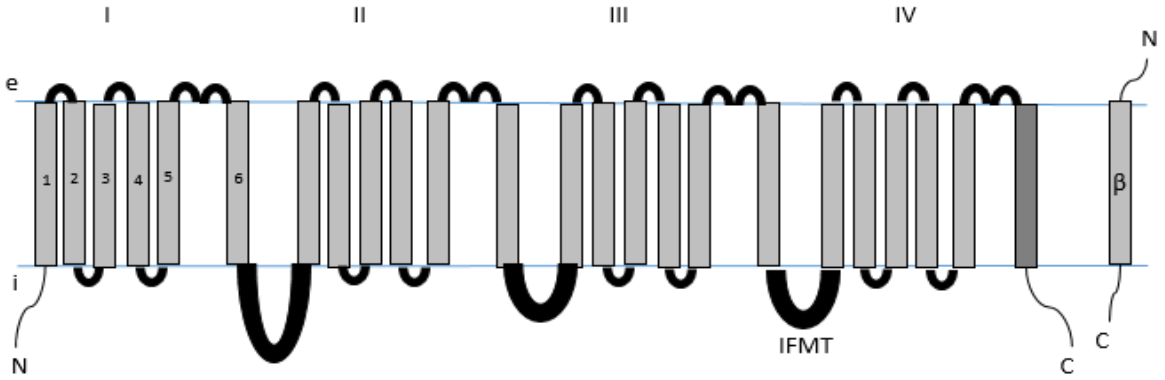

Figure 1-8. A schematic representation of voltage-gated sodium channels.

Many AEDs block high-frequency firing and the spread of seizure activity by enhancing sodium channel inactivation, such as: PHT (Quandt, 1988), LTG (Leach et al., 1986; Kuo & Lu, 1997), CBZ (Willow et al., 1983; Kuo et al, 1997), OXC (Schmutz et al., 1994), ZNS (Schauf, 1987), and possibly FBM (Taglialatela et al., 1996), TPM (Taverna et al., 1999) and VPA (Van den Berg et al., 1993). The inhibitory potency of these drugs is strongly use-dependent; therefore at pronounced levels of depolarisation, the block is strengthened. This mechanism, explains why normal neuronal activity is spared and only pathological seizure discharges are inhibited. AEDs which exert antiepileptic effects by enhancing sodium channel inactivation have been suggested to be useful in treating partial and secondarily generalised tonic-clonic seizures (Perucca, 2001) but not very effective and possibly aggravating in treating myoclonic and absence seizures (Perucca et al., 1998).

Additional subtle differences can be identified when affinity and drug kinetics are explored. For example, Kuo (1998) found that, mechanistically, CBZ and LTG are only effective extracellularly and only one drug molecule can bind to one channel; hence there are no significant additive effects by using two or more of these types of drugs. Despite striking similarities in modes of action, AEDs acting on sodium channels are structurally rather different, but PHT, CBZ and LTG do all contain two phenyl groups separated by one or two C-C or C-N single bonds, which are probably essential for binding (Kuo, 1998; Rogawski & Loscher,2004). Additionally, Kuo et al. (1997) found the affinity of CBZ to sodium channels was three times less than that of PHT, but the binding rate of CBZ was five times faster. These differences have been suggested to explain treatment efficacy differences, as CBZ is likely to be better at treating shorter ictal events.

There has also been further debate regarding whether certain AEDs effect fast or slow sodium channel inactivation. The normal firing of action potentials triggers fast inactivation, but sustained firing triggers slow inactivation. Xie et al. (1995) proposed LTG works by enhancing slow inactivation. This conclusion was based on findings that LTG did not show

inhibition of Na^+ currents when a train of short pulses were applied, which would activate fast inactivation not slow inactivation. Additionally, when the duration of pulse applied was increased, to activate slow inactivation, the inactivation curve shifted with LTG application. In contrast, Kuo and Lu (1997) demonstrated Xie et al's findings could also be explained by LTG binding to fast inactivated Na^+ channels but with slower binding kinetics, by comparing recovery kinetics. If recovery from fast inactivation of Na^+ channels is 1 ms and recovery from slow inactivation is 10 ms or more then if LTG is bound to slow inactivating Na^+ channels the recovery time would be expected to increase more than 10ms however this was found not be the case, and instead recovery time of LTG-bound Na^+ channels was faster than non-LTG-bound slow inactivating Na^+ channels, supporting the notion LTG binds to fast inactivating Na^+ channels but at a slower rate.

1.4.2 Voltage-gated calcium channels

Voltage-gated calcium channels (VGCCs) influx in response to action potentials and sub-threshold depolarisation signals. Calcium ions are also important second messengers that initiate intracellular events such as secretion, contraction, synaptic transmission and gene expression (Catterall, 2000). VGCCs were first reported by Hagiwara et al. (1975). VGCCs can be broadly divided into two categories: high-voltage activated (HVA) (-40 to -10 mV) and low-voltage activated (LVA) (-60 to -70 mV). HVA Ca^{2+} channels can further be divided into L-type, N-type, P/Q-type and R-type and LVA Ca^{2+} channels only consist of T-type channels (Yamakage & Namiki, 2002). Seven genes have been identified for HVA calcium channels and three for LVA calcium channels (Moreno, 1999). As demonstrated in figure 1-9, calcium channels consists of an α1 protein that forms the channel pore and voltage sensor, and additional β and γ subunits (Curtis & Catterall, 1984,1985). Later, a α2δ subunit was also discovered (Takahasi et al., 1987).

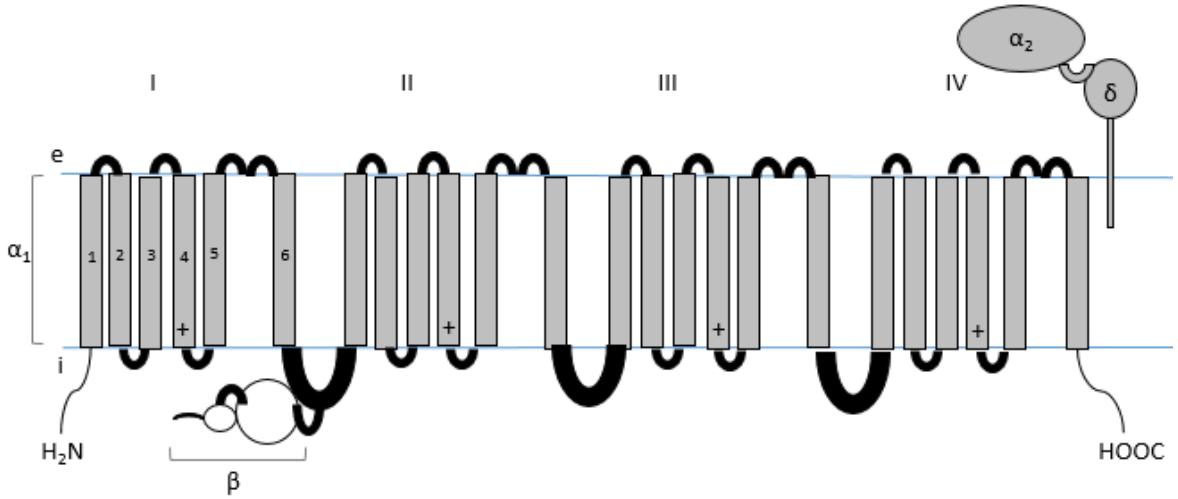

Figure 1-9. A schematic representation of the structure of VGCC.

Several studies using acute models of seizure initiation, through application of various convulsants or stimulation, have demonstrated decreases in extracellular Ca^{2+} just before the onset of an ictal event, indicating Ca^{2+} entry into the intracellular environment (Heinemann et al., 1977; Heinemann & Louvel, 1983; Heinemann et al., 1984; Pumain & Heinemann, 1985; Hablitz & Heinemann, 1987). In support, chronic models of epilepsy have also suggested seizure activity is associated with rises in extracellular K^+ and decreases in Ca^{2+} (Heinemann et al., 1981; Pumain 1981; Pumain et al., 1985; Heinemann et al., 1986). Calcium plays a pivotal role in maintaining neuronal homeostasis therefore, decreases in extracellular Ca^{2+} further promote neuronal excitability by reducing the threshold for activation of inward current (Frankenhaueser and Hodgkin, 1957). Also, as IPSPs fail to generate at extracellular Ca^{2+} concentrations of 0.7mM and EPSPs are blocked at extracellular Ca^{2+} concentrations of 0.4mM so the balance between inhibition and excitation is shifted in support of excitation (Konnerth & Heinemann, 1983; Jones & Heinemann, 1987).

Several studies have utilised different models of chronic epilepsy to demonstrate that calcium levels are altered during epileptogenesis (Rice & DeLorenzo, 1998; Pal et al., 2000; Raza et al., 2004). Based on these studies the Ca^{2+} hypothesis of epileptogenesis suggests at the time of initial brain insult, intracellular calcium concentrations are elevated, but not sufficient to cause cell death. During the latency phase of epileptogenesis intracellular Ca^{2+} levels remain elevated and cause long-term-plasticity changes which later aid the maintenance of SRS during the chronic phase (Meyer, 1989; DeLorenzo et al., 2005).

Considering the prominent role of Ca^{2+} in seizure initiation and maintenance it is not surprising many AEDs target Ca^{2+} channels. GABA analogues, GPT and pregabalin (PGB) were originally synthesised in the hope of acting at GABA sites, but have been shown to exert AED effects by binding to the Ca^{2+} channel auxiliary subunits, α2δ-1 and α2δ-2 (Suman-Chauhan et al., 1993; Gee et al., 1996; Piechan et al., 2004). Although the functional effects of this binding are not fully understood, it has been demonstrated that GPT inhibits HVA Ca^{2+} current at clinical concentrations (Bryans et al., 1998; Marais et al., 2001; McClelland et al., 2004) which ultimately reduces excitation through regulation of neurotransmitter release. In support, studies from rat neocortex (van Hooft et al., 2002) and resected human epileptic tissue (Fink et al., 2002) have shown GPT and PGB reduce presynaptic Ca^{2+} influx. In addition to producing Ca^{2+} dependent reductions in glutamate release, GPT and PGB have also been shown to act on other neurotransmitters such as norepinephrine (Dooley et al., 2000, 2002). Furthermore, GPT and PGB have also been implicated in modulating Ca^{2+} independent neurotransmitter release, potentially through interactions with P/Q type VGCCs (Cunningham et al., 2004). Other AEDs such as LTG have also been shown to inhibit N- and P/Q-type HVA Ca^{2+} channels (Stefani et al., 1996; Wang et al., 1996). LEV also acts on inhibits N-type Ca^{2+} channels (Lukyanetz et al., 2002).

Phenobarbital (PB) primarily acts at $GABA_A$ receptors, but has also been shown to inhibit HVA Ca^{2+} channels too (Ffrench-Mullen et al., 1993).

In contrast to HVA calcium channels, LVA T-type Ca^{2+} channels regulate activity by participating in bursting and intrinsic oscillations (Perez-Reyes, 2003). These channels play a significant role in seizure activity of the thalamus (Suzuki & Rogawski, 1989; Steriade, 2006). Two AEDs which are effective in treating absence seizures, by inhibiting T-type Ca^{2+} channels are ESM (Coulter et al., 1989; Gomora & Daud, 2001) and ZNS (Kito et al., 1996; Matar et al., 2009). Interestingly, LTG is also effective at treating absence seizures, but does not affect T-type Ca^{2+} channels (Rogawski & Loscher, 2004). Additionally, ESM has a ureide ring structure, like PHT, which acts on Na^+ channels, but ESM is not effective in partial or generalized tonic-clonic seizures (McLean & McDonald, 1986). Evidently, AEDs cannot be easily compartmentalised according to structure, site of activity and type of seizure.

1.4.3 GABA

The major inhibitory neurotransmitters are γ-aminobutyric acid (GABA) and glycine. Inhibitory neurons make up around 20% of CNS neurons. GABA activates two types of receptor, $GABA_A$ (ligand-gated ion channels) and $GABA_B$ (G-protein coupled) (Farrant & Nusser, 2005; Bettler et al., 2004). The most common subunit stoichiometries are α1β2γ2, α2β3γ2 and α3β1-3γ2 subunits.

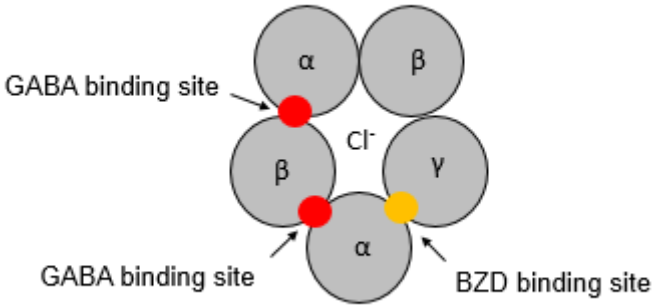

Figure 1-10. The structure of $GABA_A$ receptors.

Despite the imbalance between number of inhibitory and excitatory neurons, inhibitory neurons play a vital role in preventing epileptiform discharges and maintaining neuronal homeostasis as demonstrated by Galaretta and Hestrin (1998). In this study, dual recordings from excitatory pyramidal and fast-spiking inhibitory neurons were taken in response to stimulation. It was demonstrated that excitatory synaptic currents showed a stronger depression in comparison to inhibitory ones. As a consequence of this difference, synaptic impact will peak at higher frequencies at inhibitory synapses in comparison to excitatory synapses, and therefore promote stability.

Genetic studies (Kang & Macdonald, 2004) have further demonstrated the role of GABA receptor mutations supporting neuronal instability and promotion of seizure events. Likewise, pharmacological studies have shown $GABA_A$ blockade, with bicuculline, leads to pathological discharges (Jones & Lambert, 1990a, b). In contrast, when excitatory transmission is blocked, the convulsant, 4-AP, induces GABA-mediated ictal discharges by raising extracellular K^+ (Avoli et al., 1996).

Seizure events can also initiate long term circuitry changes which impact inhibitory control. For example, hilar GABAergic cells are much more susceptible to seizure-induced damage, in comparison to dentate basket cells (Houser, 1999; Obenaus et al., 1993). Furthermore, rather than loss of inhibitory neurons, seizures may lead to the disconnection of inhibitory and excitatory neurons, as proposed by dormant interneuron hypothesis (Sloviter, 1987; Bear et al., 1996; Fountain et al., 1998; Sloviter, 2003). However, support for this hypothesis is questionable as reviewed by Bernard et al. (1998). Moreover, Ascady et al. (1997) demonstrated granule cells of the fascia dentate provide strong excitatory drive onto hilar interneurons. Additionally, it has also been found that anatomical and electrophysiological markers of inhibition are increased and pyramidal cells are hyperexcitable (Prince et al., 1997). These paradoxical findings demonstrate the role of inhibition is by no means straight-forward to decipher.

AEDs that aim to enhance inhibition by acting on GABA receptors, do so by affecting GABA transport, metabolism or receptor kinetics. One of the earliest AEDs discovered to work by acting at $GABA_A$ receptor are BZDs (Gastaut et al., 1965). The BDZ receptor forms part of the $GABA_A$ receptor complex and exists in number of forms (e.g. BZD1, BZD2 and BZD3). BZDs bind to its $GABA_A$ receptor complex binding site to increase channel opening frequency (Vicini et al., 1987; Rogers et al., 1994). Similarly, TPM has also been shown to increase $GABA_A$ opening frequency, but appears to have a different binding site in comparison to BZDs, as the BZD receptor antagonist, flumazenil, failed to inhibit TPM induced activity (White et al., 1995). Alternative modulatory $GABA_A$ binding sites for TPM include: butyrolactone, neurosteroid, inverse agonist site or another novel site (Turner et al., 1989; Majewska et al., 1990). Barbiturates also impact $GABA_A$ receptor kinetics but instead of effecting opening frequency like BZD and TPM, barbiturates act by increasing the duration of time the channels is open (Study & Barker, 1981; Macdonald et al, 1989). Two enzymes that control GABA levels are glutamate decarboxylase (GAD) and GABA transaminase (GABAT).VGB affects GABA metabolism by irreversibly binding to the enzyme responsible for GABA metabolism, GABAT, leading to increased levels of GABA and consequently increased inhibitory activity (Jung et al., 1977; Schecter et al., 1977). TGB affects GABA transport by inhibiting neuronal GABA uptake (Braestrup et al., 1990).

Besides benzodiazepines, many AEDs that influence GABA inhibition, also promote antiepileptic effects by acting through other mechanisms. For example, TPM also has effects on Na^+ channels (White, 1997) and PB inhibits HVA Ca^{2+} channels (Ffrench-Mullen et al., 1993). Additionally, FBM has shown to be effective by inhibiting Na^+ channels (Taglialatela et al., 1996), glycine binding site of the NMDA receptor (McCabe et al, 1993; Wamsley et al., 1994) but also enhancing GABA currents (Rho et al, 1994). Similarly, VPA has been shown to reduce Na^+ currents (Zona & Avoli, 1990) and Ca^{2+} currents (Kelly et al., 1990), but also elevate GABA levels (Loscher, 2002). AEDs that are multi-mechanistic in modes of action are favoured among clinicians as drug interactions are less likely and tolerability is better (White, 1997). Generally, multi-modal AEDs are useful in treating a wide range of types of seizures however, there are some exceptions. For example, PB is not effective in treating, and may even provoke, absence seizures (Rogawski & Loscher, 2004; Perucca, 2005). Other AEDs acting on GABA, such and VGB and TGB are also not effective in treating absence seizures. Additionally, VGB is also not effective in treating myoclonic seizures.

Whilst, AEDs acting on GABA maybe useful in treating a wide variety of seizures there are also detrimental side effects to consider. For example, serious side effects of aplastic anaemia and fatal liver toxicity have been associated with FBM (Leppik & Wolff, 1995). VGB is also associated with detrimental irreversible visual defects (Kalviainen & Nousiaine, 2001). As a result, these AEDs are only used as a last resort or when treatment is justified, as in the case of VGB in the treatment of infantile spasms due to tuberous sclerosis (Chiron et al., 1997).

1.5 Aims and Objectives

The EC has been implicated with some of the earliest and most severe pathological changes in a variety of diseases, including temporal lobe epilepsy. Several studies have demonstrated the intrinsic and synaptic properties of MEC cells support the generation of seizure activity, and its location and function within the limbic system makes it an ideal candidate in seizure propagation. Despite significant pharmacological advances in the treatment of epilepsy, one third of patients remain resistant to two or more AEDs, and are therefore drug refractory (Kwan & Brodie, 2006; French, 2007). One fundamental possibility for the lack of understanding and treatment options for drug refractory patients, is due to the focus on the excitation-inhibition balance within the brain which has been suggested to be a secondary effect of underlying pathology. For example investigations of epileptogenesis mechanisms often focus on network circuitry changes as a result of death of GABAergic interneurons and axonal sprouting. Similarly, many AEDs focus on enhancing inhibition and/or reducing excitation.

It remains an important challenge of epilepsy research to understand the pathophysiogenesis of TLE. Several acute and chronic models have been developed in order to explore the

mechanisms of seizures and epileptogenesis, however these models have also been criticised on the basis they induce severe damage and do not accurately imitate human pathology. Based upon these issues within epilepsy research the following project aims and objectives are proposed:

- To explore how improved brain preparations act as acute models of drug-resistant seizures, and mechanisms behind resistance.
- To explore excitability and AED response during epileptogenesis in the refined lithium pilocarpine model of epilepsy, *in vitro*.
- To compare the excitability and AED response of resected human tissue to chronically epileptic rodent tissue, *in vitro*.
- To explore the role of LTP induced effects in chronic epileptogenesis.

Chapter 2 Methods

2.1 Brain slice preparation

Transverse hippocampal-EC slices (450 µm) were prepared from male Wistar rats (45-600 g). Each rat was anaesthetised with 3% isoflurane in N_2 and O_2 for 2-3 minutes for smaller animals and up to 5 minutes for larger animals. Once the heartbeat had reduced until it was no longer visible, a subcutaneous (s/c) injection of pentobarbital (600 mg/Kg) was administered along with an intramuscular (i/m) injection of combined ketamine (100 mg/Kg) and xylazine (10 mg/Kg). Once the pedal reflex had ceased to be active, smaller animals (45-150 g) were held in an ice bath for 30-45 seconds to reduce metabolic rate. Thereafter, animals were transcardially perfused with ice-cold sucrose aCSF, which contained (mM): 206 sucrose, 2 KCl, 1.6 $MgSO_4$, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 2.25 $CaCl_2$, 10 glucose, and 5 sodium pyruvate. Neuroprotectants (mM): 0.04 indomethacin, 0.4 uric acid and 0.19 ketamine were added and the solution was saturated with carbogen (95% O_2, 5% CO_2) (see EC preparation in Table 2.1).

Following the perfusion, the brain was carefully extracted and placed into the sucrose aCSF. Thereafter, the brain was prepared for slicing by dissecting and fixing the dorsal surface onto a platform using cyanoacrylate adhesive (super-glue, RS components, UK) before being placing it into a small bath of cold sucrose aCSF which was continuously bubbled with carbogen. Transverse hippocampal-EC slices (450 µm) were then cut using a Vibroslicer (Campden Instruments, UK). Slices were then placed into an interface holding chamber, which contained a NaCl based aCSF (mM): 126 NaCl, 3 KCl, 1.6 $MgSO_4$, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 2 $CaCl_2$, 10 glucose. Neuroprotectants, indomethacin (0.4 mM), uric acid (0.4 mM) and ketamine (0.19 mM) were also added. The holding chamber solution was stored at room temperature and also continuously bubbled with carbogen. After slices had been prepared they were transferred into an interface chamber (Scientific System Design Inc., Canada), and left to rest for 1 hour. The perfusate was temperature maintained to 30-31 °C using a PTCO3 proportional temperature controller (Scientific System Design Inc., Canada) and was bubbled with carbogen. Carbogen was also bubbled through distilled water underneath the interface recording chamber in order to maintain humidity between 95-100 %.

The majority of slices were prepared using the method outlined above (see chapter 4 and 5), but as chapter 3 explored the effects of brain slice preparation differences on excitability in 0$[Mg]^{2+}$, different brain slice preparation methods were employed, the main differences are outlined below. In chapter 3 transverse hippocampal-EC slice (450 µm) were prepared from male Wistar rats (100 g) in one of two ways:

Firstly, animals were anaesthetised as highlighted above and decapitated, without transcardial perfusion. The brain was carefully and swiftly extracted and placed in ice-cold NaCl based aCSF, and cut and stored in the same solution. The only difference between

cutting and holding solutions being the temperature. No neuroprotectants were added. This method is a standard method of slice preparation and still used by many laboratories.

Secondly, animals were anaesthetised as explained above, and transcardially perfused with and cut in a sucrose based solution, which comprised of (mM): 180 sucrose, 2.5 KCl, 10 $MgSO_4$, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 0.5 $CaCl_2$, 10 glucose, 1 L-ascorbic acid, 2 N-acetyl-L-cysteine, 1 taurine, 20 ethyl pyruvate, 0.04 indomethacin, 0.4 uric acid, 0.01 aminoguanidine, 0.19 ketamine and saturated with carbogen. The holding chamber contained the NaCl based aCSF with neuroprotectants, indomethacin (0.4 mM) and uric acid (0.4 mM) (see M1 preparation in Table 2.1).

All experiments were carried out in accordance with the Animals (Scientific Procedures) Act 1986, European Communities Directive 1986 (86/609/EEC) and Aston University ethical review documents. Male Wistar rats were housed in groups of 1-10 depending on size, in temperature and humidity controlled cages with a 12/12 light/dark cycle. Access to food and drink was provided ad libitum.

Finally, all human tissue and piriform cortex slices (see chapter 4) were cut in a choline based aCSF, which contained (mM): 110 choline chloride, 26 $NaHCO_3$, 10 D-Glucose, 11.6 ascorbic acid, 7 $MgCl_2$, 3.1 sodium pyruvate, 2.5 KCl, 1.25 $NaHPO_4$ and 0.5 $CaCl_2$. The solution had neuroprotectants: indomethacin (0.4 mM), uric acid (0.4 mM) and ketamine (0.19 mM) added to the ice-cold solution which was saturated with carbogen. An agar ‘seat’ was fixed onto the standard metal platforms. Resected human tissue was then glued onto the agar, to prevent the tissue from becoming detached during slicing.

Table 2.1. Summary of different brain slice preparation methods

	EC Preparation	M1 Preparation	Standard Preparation	Piriform and Human Tissue Preparation
Perfusion	Yes	Yes	No	PC: Yes, HT: No
cutting solution	206 sucrose, 2 KCl, 1.6 $MgSO_4$, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 2.25 $CaCl_2$, 10 glucose, and 5 sodium pyruvate	180 sucrose, 2.5 KCl, 10 $MgSO_4$, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 0.5 $CaCl_2$, 10 glucose, 1 L-ascorbic acid, 2 N-acetyl-L-cysteine, 1 taurine, 20 ethyl pyruvate	126 NaCl, 3 KCl, 1.6 $MgSO_4$, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 2 $CaCl_2$, 10 glucose	110 choline chloride, 26 $NaHCO_3$, 10 D-Glucose, 11.6 ascorbic acid, 7 $MgCl_2$, 3.1 sodium pyruvate, 2.5 KCl, 1.25 $NaHPO_4$ and 0.5 $CaCl_2$
cutting solution neuroprotectants	0.04 indomethacin, 0.4 uric acid and 0.19 ketamine	0.04 indomethacin, 0.4 uric acid and 0.19 ketamine	N/A	0.04 indomethacin, 0.4 uric acid and 0.19 ketamine
storage solution	126 NaCl, 3 KCl, 1.6 $MgSO_4$, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 2 $CaCl_2$, 10 glucose	126 NaCl, 3 KCl, 1.6 $MgSO_4$, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 2 $CaCl_2$, 10 glucose	126 NaCl, 3 KCl, 1.6 $MgSO_4$, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 2 $CaCl_2$, 10 glucose	126 NaCl, 3 KCl, 1.6 $MgSO_4$, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 2 $CaCl_2$, 10 glucose
storage/experimental solution neuroprotectants	indomethacin (0.4 mM), uric acid (0.4 mM) and ketamine (0.19 mM)	Indomethacin (0.4 mM) and uric acid (0.4 mM).	N/A	indomethacin (0.4 mM), uric acid (0.4 mM) and ketamine (0.19 mM)
Chapters	4, 5	3	3	4

2.2 Reduced intensity lithium pilocarpine model of epilepsy

2.2.1 Model protocol

The reduced intensity lithium pilocarpine model (Modebadze et al., 2016) was employed in experiments aimed at exploring effects of epileptogenesis on neuronal network excitability. One to three days post weaning (postnatal day 21 - 24) rats were marked for identification and a dose of LiCl (127mg/Kg) was administered s/c, 24 hours before the protocol was fully initiated. Pre-treatment with LiCl has been shown to increase sensitivity to pilocarpine (Clifford et al., 1987). The following day, the rats were weighed and the protocol was initiated with a dose of α-methyl scopolamine (1 mg/Kg, s/c), which reduces the peripheral effects of muscarinic cholinergic receptor activation. Thirty minutes later, a low dose of pilocarpine (25-30 mg/Kg) was administered s/c. Thereafter rats were carefully monitored for signs of seizure activity, which was classified according to the Racine scale (see Table 2.2). After 45-60 minutes, if animals had not developed stage 4 seizures, they were given another dose of pilocarpine. A maximum of 3 doses were administered. Once two stage 4 seizures were observed, the central muscle relaxant, xylazine (2.5 mg/Kg, i/m) was administered, to reduce seizure severity. Rats were permitted to stay in this SE state for 1 hour, after which a ‘stop’

solution (1 ml/Kg, s/c) was administered to halt intense repetitive seizures. The 'stop' solution was comprised of MK801 (0.1 mg/Kg), diazepam (2.5 mg/Kg) and MPEP (20 mg/Kg). Seizures ceased to occur around thirty minutes after the stop solution had been given, and animals remained in a sleep-like state for 3-4 hours.

Table 2.2. The Racine Scale.

Stage	Activity
1	Facial/mouth movement, chewing and salivation
2	Head nodding and eye twitching
3	Forelimb clonus
4	Clonic rearing (sitting up on hind limbs, forelimbs shaking)
5	Clonic rearing and falling over with loss of postural control

Animals were kept on heat pads and carefully monitored and given 0.5 ml (saline/glucose) s/c every 2 hours for 8 hours after induction. Whilst the animals were sleeping, their nails were also trimmed to prevent cutaneous damage at later stages from excessive grooming which is often seen in epileptic behaviour. Regular checks were made for 3 days, whereby animals were weighed daily to ensure dramatic weight loss had not occurred, and break-through seizures were not severe (e.g. running-bouncing-seizures) or prolonged. An end-point limit of 20 % weight loss from pre-induction weight was set to ensure prolonged suffering was not endured. Minimal weight loss was overcome by giving rats treats (e.g. sweetcorn, peanut butter, muesli and milkshake) or oral gavage in certain cases. Following full recovery animals were returned to temperature and humidity controlled environments with a 12-12 light-dark cycle.

2.2.3 Experimental timeline and SRS development

Animals were killed at 24 hours, 1 week, 5 weeks and 12 weeks+ post induction. As illustrated in figure 2-1, 1 week and 5 weeks after SE induction are representative of latent period time points in epileptogenesis, whereas 12 weeks+ post SE induction is typically when SRS is observed, characterising chronic epilepsy. Often comparisons with age-matched controls were made, whereby weight was used as an indication of age.

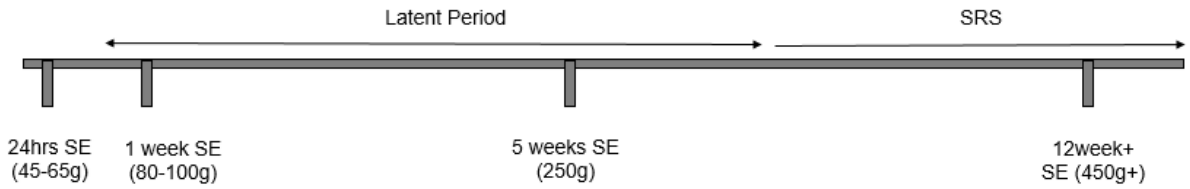

Figure 2-1. A schematic timeline representation of epileptogenesis. The time points at which SE and aged matched controls were sacrificed is noted.

2.2.4 The post-seizure behavioural battery test

The post-seizure behavioural battery (PSBB) test, developed by Moser et al. (1988) and adapted by Rice et al. (1998), was employed, to confirm the development of the SRS that characterises the chronic epileptic stage. The test involved a touch task and a pick-up task. The touch task involved the animal being gently prodded in the rump with a blunt object (e.g. permanent marker). The pick-up task required an attempt to pick-up the animal. Behavioural response to both tasks were rated on a scale from 1-7 for the touch task and 1-6 for the pick-up task, as highlighted in Table 2.3. The two scores were then multiplied together. Once an animal had obtained 4 consecutive scores higher than 10 it was confirmed as epileptic. It has previously been demonstrated that animals who have undergone variations of the pilocarpine model of epilepsy induction show behavioural modifications such as hyperexcitability and aggression in comparison to controls (Huang et al., 2012; Modebadze et al., 2016; Polascheck et al., 2010).

Table 2.3 Post seizure behavioural battery test responses and scores

Touch task		Pick-up Task	
1	No Reaction	1	Very easy pickup
2	Rat turn toward instrument	2	Easy pickup with vocalisation
3	Rat moved away from instrument	3	Some difficulty in pickup (rears and faces hand)
4	Rat freezes	4	Rat freezes
5	Rat turns toward the touch	5	Difficult pickup (rat moves away)
6	Rats turns away from the touch	6	Very difficult pickup (defensive/attacks hand)
7	Rat jumps (with or without vocalisation)		

The PSBB task was initiated 1 week post SE induction, and was completed twice a week (Tuesday and Friday) by three individuals on a rotational basis, to avoid bias. Some animals that had been confirmed epileptic by the PSBB task, were video recorded in the 'big brother system'. The 'big brother system' was set up by Astra Zeneca, and recordings helped in the development of software to detect seizures. As illustrated in figure 2-2, animals that had undergone SE induction scored consistently higher on the PSBB task in comparison to control animals. The majority of SE animals had been confirmed epileptic, with 4 consecutive scores above 10, by 28 weeks (~6 months).

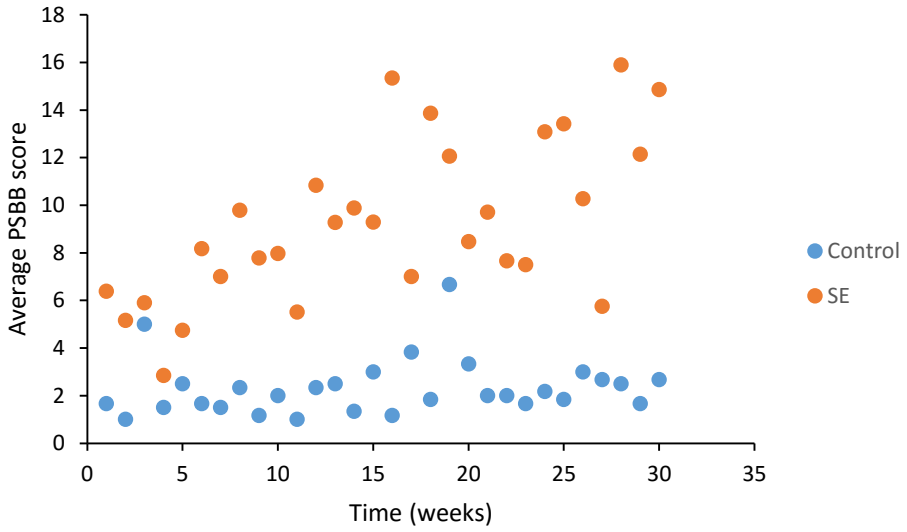

Figure 2-2. PSBB scores over time in SE (n= 44) and control (n=6) animals.

2.3 Human tissue electrophysiology

A collaboration with Birmingham Children's Hospital allowed informed consent to be obtained from parents and paediatric patients who were undergoing resective brain surgery due to intractable epilepsy. Ethical approval specified by the Black Country LREC (protocol 'Cellular studies in epilepsy' 10/H1202/23), and the Birmingham Children's Hospital NHS Trust (RECREF 10/H1202/23) can be found in Appendix 1. Pre-surgery electrocorticography methods determined site of resection. Brain tissue was surgically removed and placed into cold choline-based aCSF, which had been saturated with carbogen, as described in section 2.1. Patient data, from which human tissue experiments are based, is outlined in Table 2.4.

Table 2.4. Site of resection in human tissue

Patient	Human tissue with induced IDs
1	Motor cortex
2	Temporal gyrus and amygdala
3	Sensory motor cortex
4	Medial temporal gyrus and amygdala
5	Inferior frontal gyrus
6	Left parietal gyrus
7	Sensory motor cortex
8	Temporal lobe
9	Medial temporal gyrus
	Human tissue unable to induce IDs
10	Frontal cortex
11	Temporal lobectomy
12	Frontal cortex
14	Left temporal gyrus and amygdala

2.4 Extracellular recording

Extracellular recording techniques were used to assess the neural network activity of LFPs. Microelectrodes were prepared from borosilicate glass which was pulled using a Flaming/ Brown micropipette puller (P-97, Sutter instrument Co, U.S.A.). The resistance of electrodes was 1-3MΩ. Microelectrodes were then filled with a NaCl based aCSF and inserted into the microelectrode holder, which has attached a silver piece of wire which was coated with silver chloride. Once the microelectrode was securely fitted to the holder it was mounted on the manually operated micromanipulators (Narishige MM-3, Japan). As discussed in detail in section 1.2, LFP emerge from multiple sources but are mainly generated by the summation of current sinks and sources distributed along neuronal aggregates, which when geometrically aligned often give rise to oscillations. Microelectrodes can detect activity within 250 μm of the electrode tip (Xing et al., 2009).

Using an optical stereomicroscope Olympus SZX16 (Leica Wild M3Z, Leica UK), 4 microelectrodes were placed in layer II of the MEC of 4 different slices, and simultaneously recorded from. Signals were passed through an EXT amplifier headstage (NPI electronics GMBH, Germany) and then further amplified x100 and low-pass filtered at 1 KHz using an EX10-2F amplifier (NPI Electronics GMBH, Germany). Low amplitude 50 Hz noise was reduced using Hum Bug noise eliminators (Quest Scientific, North Vancouver, Canada) and the signal was filtered between 1 Hz and 1000 Hz. Thereafter, signals were digitized to a PC, at a 10 KHz sampling rate, using an analogue to digital converter (CED micro-1401 mkII;

Cambridge Electronic Design, UK). On-line recordings were made using Spike2 software (CED, UK).

Different types of noise (e.g. vibrations and electrical noise from lights and power supplies) can interfere with the accurate recording of biological signals. As mentioned, the use of Hum Bug noise eliminators reduces some of the electrical noise, by cancelling out interferences in real time. Electrical noise is further reduced by the presence of a Faraday cage around the experimental set-up, which is connected to a common ground (e.g. the microelectrode amplifier). Vibrational noise is reduced by the vibration isolation table, on which the experimental set-up is located.

2.5 Drugs

Stock solutions of drugs were prepared at known concentrations by diluting drugs with water, DMSO or ethanol depending on solubility and stored at -20 °C before use, for no longer than 6-9 months (all drugs were purchased from Sigma, Abcam or Tocris, see table 2.5). The drugs were directly applied to the perfusing aCSF, and the effects on neuronal activity observed for 30-45 minutes depending on experimental design.

Table 2.5 Drugs used in this thesis

Drug	Purchased from	Stock concentration and solution	Concentration used
(-)-scopolamine methyl bromide	Sigma	1 mg/ml water	1 mg/kg
7-nitraindazole	Abcam	20 mM ethanol, 100mM DMSO	200 µM
D-AP5	Abcam	100mM, water	50 µM
bicuculline	Abcam	5 mM ethanol, 100mM DMSO	20 µM
cannabidiol	GW Pharma	60 mM, DMSO	30 µM
carbachol	Abcam	50 mM, water	Variable
carbamazepine	Abcam	100 mM, DMSO	50 µM
CNQX	Abcam	100 mM, DMSO	20 µM
cycloheximide	Abcam	25 mM water	60 µM
diazepam	Hameln	2.5mg/ml, ethanol	2.5 mg/kg
gabapentin	Abcam	100mM, water	20 µM
gabazine	R&D systems	2.5 mM, DMSO	250 nM & 2.5 µM
GF109203X	Abcam	100 mM, DMSO	100 µM
kainate	Abcam	1mM water	variable
lamotrigine	Abcam	20 mM DMSO	20 µM
MK-801	Abcam	20 mM, water, *in vitro*, 0.1 mg/ml in RISE model	0.01 - 30 µM *in vitro,* 0.1mg/kg in RISE model
MPEP	Abcam	100mM, DMSO, *in vitro*, 20 mg/ml, ethanol, RISE model	20 µM *in vitro,* 20mg/kg in RISE model
pentobarbital	R&D systems / JML	10 mM *in vitro*, 200 mg/ml in terminal procedure	10 µM *in vitro,* 600mg/kg in terminal procedure
pilocarpine	Sigma	20-25mg/ml water	20-75 mg/kg (repeated doses)
tiagabine	Abcam	25 mM, water	50 µM
valproate	Abcam	100 mM water	500 µM
xylazine	Bayer	20 mg/ml terminal procedure, 2.5 mg/ml, water, RISE	100 mg/kg terminal procedure, 2.5mg/kg RISE
zonisamide	Abcam	100 mM, DMSO	100 µM

2.6 Data collection and analysis

2.6.1 Seizure activity data

All data were analysed in Spike2 (version 7.0). To be considered for analysis seizure-like events (SLEs or 'seizures') had to be between 15-500 seconds in length, and have a characteristic shape which was accompanied by an increase in the power of activity, and the IEI between seizures needed to be less than 1000 seconds for accurate monitoring of AED effects. For accuracy, data regarding the following variables was manual extracted and input in matrices in Excel (2013): percentage of slices showing IDs, latency to first seizure, inter-event-intervals (IEIs), duration of IDs and frequency of IDs. Many of these variables are illustrated in figure 2-4. For the analysis of power of seizures 90 seconds epochs of 3 seizures per experimental manipulation was extracted and pooled together. For each seizure

activity segment, the highest power value at each frequency band was extracted by a model built in Excel (2013).

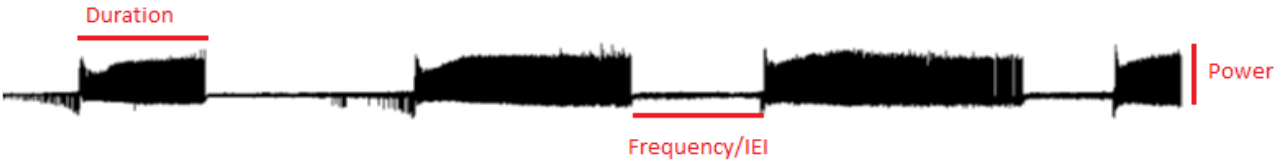

Figure 2-3. Representation of ID variables: frequency, IEI, duration and power.

In addition, to exploring how different ID parameters change in different acute and chronic models of epileptogenesis, resistance to AEDs was also explored using two different definitions. The measurement of resistance to AEDs in clinical studies as well as physiological *in vivo* / *in vitro* investigations have often been flawed. For example, in many clinical trials the 'last observation carried forward' measure includes patients who completed and dropped out of the trial, leading to the false inflation of seizure-free rates as patients who become seizure free and then drop-out are included in the seizure-free population (Gazzola et al., 2007). Similarly, many studies assess resistance towards a single AED treatment, but the definition of DRE clearly identifies a failure to respond to two or more AEDs (Berg et al., 2001; Cowan 2002; Kwan & Brodie, 2006).

The first definition of DRE clearly identifies a failure to respond to two or more AEDs (Berg et al., 2001; Cowan 2002; Kwan & Brodie, 2006) and so resistance was calculated using the criteria of percentage of slices which continue to show seizures following application of 2 AEDs. Secondly, healthcare providers acknowledge a 50 % reduction as a measure of efficacy, but the clinical relevance over the patient's health status is thought to be limited as patients are not seizure-free and therefore cannot drive (Beyenburg et al., 2010; Birbeck et al., 2002). Although seizure-freedom is the ultimate goal, it is not currently a realistic goal in patients with refractory epilepsy and a 50 % reduction can be seen as a significant improvement. The second analysis of resistance uses the clinical criteria in which a less than 50 % reduction in seizure frequency is evidence of resistance and a 50 % reduction, or more, in seizure frequency is evidence of no-resistance.

Depending on the complexity of questions hypothesised and the type of data collected (e.g. categorical vs continuous) a range of statistical analyses were adopted such as: t-tests, chi-square analyses, log linear analyses, regressions and ANOVAs (Field, 2009; Gravetter & Wallnau, 2009; Pallant, 2010). As hypotheses were often multifactorial, different types of ANOVA and regression analyses were carried out for which there are limited non-parametric alternatives. Before analysis the distribution of data for different variables was determined. If data significantly deviated from normal distribution, log10, box-cox transformations or ranking operations were applied to make sure assumptions of normality were not violated and

conclusions drawn from analyses were accurate. Additional assumptions of homogeneity of variances and multicollinearity were checked before results were interpreted. All statistical analyses was carried out using SPSS and R. All data are expressed in terms of mean and standard error of the mean (SEM). Where analysis has been conducted on transformed data, this data is presented, but illustrations of the raw data can be found in appendices.

2.6.2 GABAergic LFP activity

The presence of unitary inhibitory field potentials (UIFPs) has been illustrated by Bazelot et al., (2010). To explore the effects of various manipulations on UIFPs) data were imported from Spike2 to the Mini-Analysis programme (Synaptosoft, U.S.A.). At least 50 events were taken from each recording, and the amplitude and frequency of events was calculated. Statistical analysis was carried out as described in section 2.6.1.

Chapter 3 Brain slice preparations as acute models of seizure-like events and the differential effects of antiepileptic drugs

3.1 Introduction

3.1.1 The history of the *in vitro* brain slice preparation

The pioneering development of the brain slice preparation by Henry McIlwain in the 1950s, was one of the most important historical landmarks in the neuroscience discipline. To validate the preparation, electrophysiological techniques were used to demonstrate that slices were metabolically viable (McIlwain et al., 1951), neurons displayed healthy resting membrane potentials (Li & McIlwain, 1957) and synaptic responses could be recorded from electrical stimulation (Yamamoto & McIlwain, 1966). Since these demonstrations, popularity of the technique has attracted researchers from multiple disciplines (e.g. biologists, pharmacologists, physiologists and anatomists) to investigate physiological and pharmacological properties of the different brain areas.

The brain slice preparation is an excellent method of exploring local neuronal networks and has led to insights on the dynamics of physiological and pathological neuronal activity that would otherwise be difficult due to practicalities and ethical considerations. However, it is important to recognise some of the limitations of this technique. For example, many input and output connections are severed through the process of slicing and such stress inducing processes can trigger a cascade of excitotoxic and neuro-inflammatory reactions. As a result of network modifications from these processes the translational abilities of findings from *in vitro* research is not completely compatible with *in vivo* findings.

Since the innovation of the brain slice preparation, various improvements have been made to reduce the trauma induced effects of the slicing process and increase oxygenation of the tissue. For example, considerations to the way in which the brain is removed, how it is sliced, what sort of equipment is used to glue the brain, what angle the brain is cut at, thickness of slices, oxygenation and temperature of chambers and the length of the preparatory process have been made to increase slice viability and thus improve the quality of research (Dingledine et al., 1980). Various tests have been utilised to assess the healthiness of slices, such as: metabolic tests (e.g. measuring levels of energy-rich phosphates), histological assessment of cell live/dead ratio and the response measurement to electrical stimulation, as the presence of multiple population spikes in response to weak stimulation indicates the slice has become anoxic (oxygen deprived).

In the original description of brain slice preparation a hand-held razor blade was used, but since then mechanical choppers and vibratomes have been employed for greater precision and control. In addition, some potential improvements have actually been found to decrease viability, but have nonetheless improved our knowledge of the neuronal tissue. For example, it was previously thought using higher concentrations of K^+ (5-6 mM) reduces cell swelling and maintains intracellular concentration of K^+ to a similar level as found *in vivo* (Franck,

1972). However, subsequent research actually demonstrated increased extracellular K^+ concentration depolarised neurons and reduced input resistance (Schofield, 1978). Additionally, profound epileptiform activity was also demonstrated to occur in hippocampal slices with elevated extracellular K^+ (Schwartzkroin & Prince, 1978). Evidently, the composition of the extracellular environment has important implications in maintaining neuronal homeostasis (Nicolson, 1979; Schmitt & Samsom, 1969), and it therefore unsurprisingly remains a target of research to improve slice viability.

One particular improvement to aCSF during slice preparation, has been the replacement of NaCl with sucrose or glycerol, which prevents cell swelling and lysis (Aghjanian & Rasmussen, 1989; Ye et al., 2006). The use of NaCl promotes passive Cl^- entry and associated water transport which leads to neurotoxic effects (Aghajanian & Rasmussen, 1989). This substitution for a sucrose-based aCSF has been shown to be particularly useful in cellular electrophysiology studies of the perigeniculate nucleus (Kim and McCormick, 1998), inferior colliculus (Li et al., 1998) and hippocampus (Urban et al., 1998; Xiang and Brown, 1998).

Moreover, within our laboratory the addition of several neuroprotectants (NAC, aminoguanidine, taurine and ascorbate) to aCSF have been shown to considerably improve motor cortex slice viability (Prokic, 2012). Furthermore, Prokic (2012) also demonstrated, whilst small improvements could be seen with the addition of such neuroprotectants to the aCSF, the biggest improvement to slice viability was actually evident when decapitation of the animal was replaced by transcardial perfusion with the modified sucrose based aCSF.

Despite many improvements being suggested since the late 1980's, many laboratories continue to prepare slices with decapitation, followed by slicing with NaCl based aCSF (standard methods). Whilst, differences in oscillatory activity and intracellular properties have been demonstrated between standard and modified methods of brain slice preparations (Kuenzi et al., 2000; Modebadze, 2014; Prokic, 2012), it is important to question how this brain slice preparation affects results when used in acute models of seizure-like-events in comparison to more viable slices.

3.1.2 Effects of antiepileptic drugs in acute models

Many studies which have used standard slicing methods and promoted SLEs with the application acute models (e.g. 0$[Mg]^{2+}$, 4-AP, KA etc), have often found sensitivity to a wide range of antiepileptic drugs (AEDs) (e.g. CBZ, VPA, PHT, BZDs and barbiturates) in the entorhinal cortex (EC) (Drier & Heinemann, 1990; Zhang et al., 1995). Although there are variations in response to AEDs from model to model (Sokolova et al., 1998) these types of findings have provided the fundamental basis of criticism of AED discovery studies, as they simply promote the development of 'me too' drugs that do not offer insight into the

mechanisms of drug resistance or effective treatment. Furthermore, this point has been exacerbated by the findings that demonstrate drugs such as LEV do not show anticonvulsant activity in acute MES and PTZ models, but show potent protection against seizures in a variety of chronic animal models of epilepsy (Loscher & Honack, 1993).

Tonic-clonic activity that characterises ictal-discharges (IDs) has been demonstrated to transition (> 2 hours) into late-recurring-discharges (LRDs) which only display short bursts of tonic activity with prolonged exposure to seizure inducing agents and extracellular ionic manipulations. This transitional behaviour is thought to be due to a reduction in synaptically available GABA (Mody et al., 1994).

In contrast to IDs, LRDs have been shown to be resistant to several AEDs (Drier & Heinemann, 1990; Sokolova et al., 1998; Zhang et al., 1995). LRDs have been suggested by Zhang et al. (1995) to mimic status epilepticus (SE) development seen *in vivo*, in humans (Trieman et al., 1990) and animals (Lothman et al., 1989). As LRDs have been shown to be sensitive to NMDA receptor antagonism, but resistant to AEDs, it has been suggested drug resistance may be dependent on long-term-potentiation (LTP) (Drier & Heinemann, 1990; Zhang et al, 1995). However, others have suggested LRDs are similar to inter-ictal-discharges (IIDs), but are not associated with increases in extracellular K^+ (Bruckner & Heinemann, 2000; Sokolova et al, 1998). There is also the possibility that LRDs occur more prominently as a result of poor viability of slices and harsh seizure inducing modifications.

Whilst it has been recognised that chronic models will undoubtedly provide the best insight into mechanisms of drug-resistant epilepsy, these models are often elaborate and time consuming. Moreover, the efforts of implementing such models could be argued to be wasted by poor brain slice preparation, which promotes neurotoxic effects and so may not accurately capture network alterations induced by epileptogenesis. Adding such confounds through inaccuracies produced by poor brain slice preparation, further raises ethical issues surrounding the use of animals.

As outlined above, slice preparation could potentially act as a confounding variable when investigating AED efficacy and mechanisms. Our primary investigation aimed to investigate AED responsiveness and drug-refractory epilepsy (DRE) in the reduced-intensity-status-epilepticus (RISE) pilocarpine model of chronic epilepsy. However, before this can be accurately investigated, the effects of slice preparation acting as a confounding variable on the susceptibility to IDs needed to be evaluated.

The 0$[Mg]^{2+}$ model is a commonly used, subtle *in vitro* model of epileptiform activity in entorhinal-hippocampal slice used to establish the effects of drugs in seizure activity and mechanisms of antiepileptic activity. Using the 0$[Mg]^{2+}$ model, the current study aimed to investigate the effects of 3 different brain slice preparations on the excitability and viability of layer II of the MEC. Layer II of the MEC is highly sensitive to epileptogenesis (see section

1.3.2). The current study additionally aimed to investigate the possibility of DRE as a consequence of slice preparation. The method which produces the most viable slices from these investigations can then be used in the preparation of slices from chronically epileptic animals, to accurately assess AED efficacy and resistance.

3.2 Results

3.2.1 Excitability and parameter characteristics of two different brain slice preparations

Transverse hippocampal-entorhinal slices were prepared using standard and modified sucrose brain slice preparation methods. Standard methods required the animal to be anaesthetised, decapitated and then the brain was extracted and sliced in a cold NaCl based aCSF. Modified methods required the animal to be anaesthetised and transcardially perfused with a modified sucrose based aCSF, which contained neuroprotectants: taurine, aminoguanidine, ethyl pyruvate, uric acid and ketamine (See section 2.1 for more details). Acute SLEs were modelled in layer II of the MEC in both types of slice preparations, by applying a 0$[Mg]^{2+}$ aCSF perfusate.

There was a significant difference between the two preparations, in terms of the number of slices that displayed IDs χ^2 (1, n = 189) = 7.17, $p < 0.01$. As illustrated in figure 3-1, there was a greater number of standard prepared slices (57/87) that showed IDs in comparison to sucrose prepared slices (47/102).

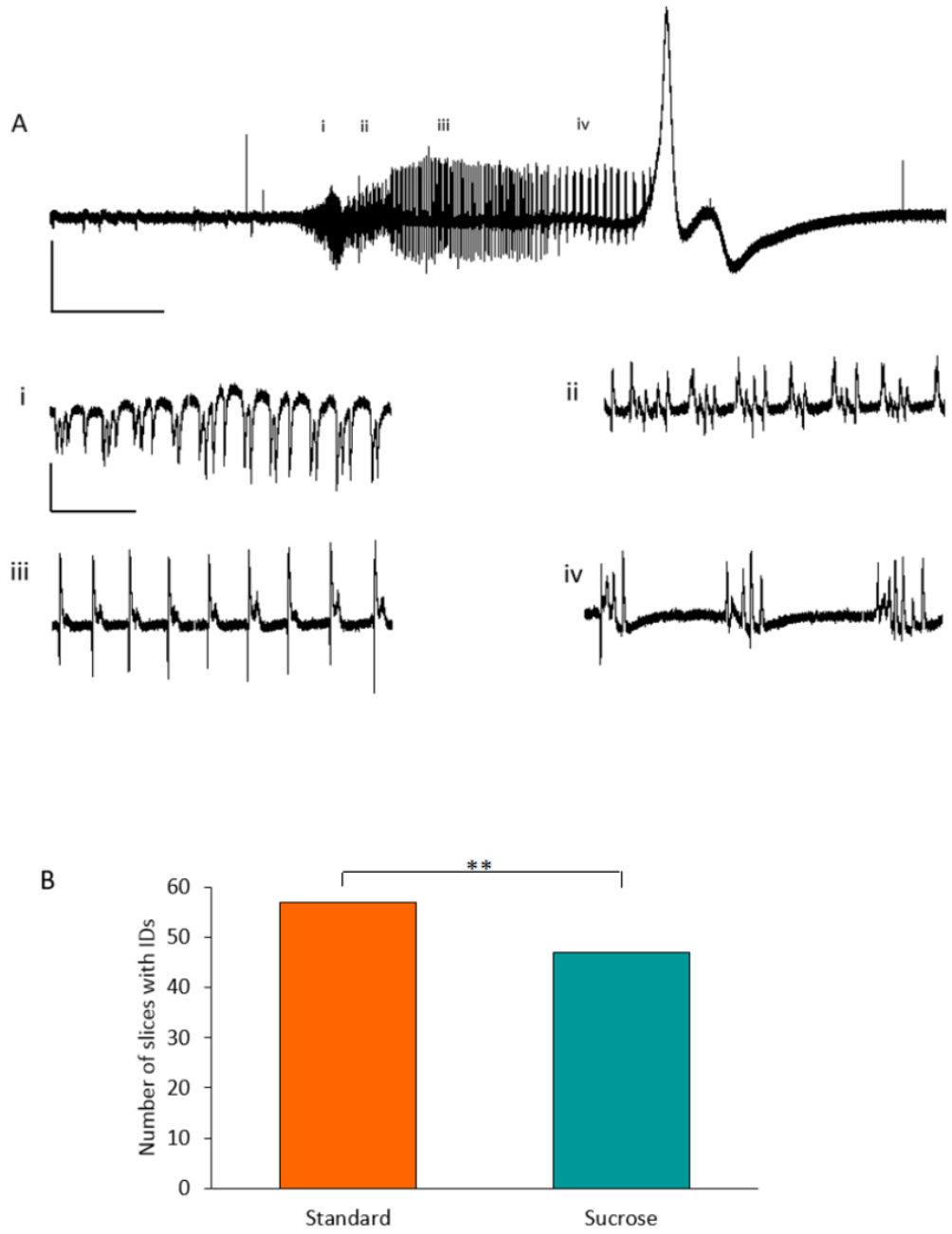

Figure 3-1. Percentage of slices which displayed IDs in two different slice preparations. A. A typical ID from a sucrose prepared 100 g control rat (Scale: 1000 µV x 25 secs), i, ii, iii and iv correspond to different time points of the ID on a smaller scale (500 µV x 1 sec). Expansion of the tonic portion of the ID is illustrated in Ai and Aii. Expansion of clonic phase bursts illustrated in Aiii and Aiv. B. Number of slices showing IDs following 0$[Mg]^{2+}$ application in standard and sucrose prepared slices.

Additionally, there were significant differences between the two types of brain slice preparation in terms of latency to first ID, t (51.67) = -4.41, $p < 0.01$. As illustrated in figure 3-2, sucrose slices had a significantly longer latency to first ID (3824.53 ± 316.46 seconds) in comparison to standard (2268.87 ± 155.91 seconds).

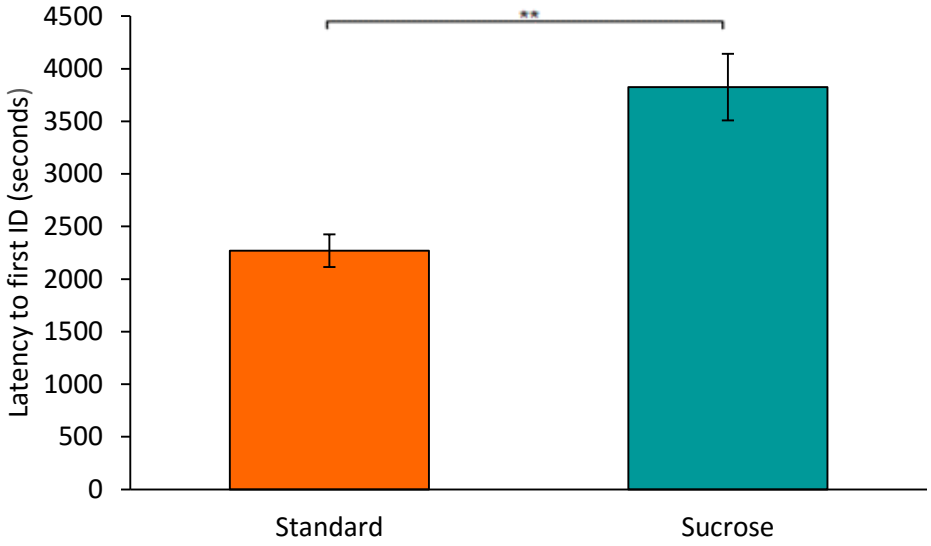

Figure 3-2. Latency to first ID in two different brain slice preparations. Latency to first ID in sucrose slices in comparison to standard slices.

Different parameters of seizure activity, such as: frequency of IDs, the inter-event-intervals (IEIs) between IDs and the duration of IDs were characterised in the different preparations (Figure 3-3). In terms of frequency of IDs there were significant differences between the two slice preparations t (68.73) = 3.89, $p < 0.01$. The frequency of IDs in the sucrose preparation (3.38 ± 0.33 frequency per 45 minutes) was significantly lower in comparison to standard slices (5.48 ± 0.43 frequency per 45 minutes). IEIs showed the converse relationships.

There were significant differences between the two slice preparations in terms of IEIs between IDs, t (71) = - 3.74, $p < 0.01$. Specifically, IEIs between IDs in the sucrose preparation (1106.68 ± 106.58 seconds) were significantly longer in comparison to standard (608.02 ± 82.75 seconds).

There were also significant differences between the two slice preparations in terms of duration of IDs, t (75) = - 4.07, $p < 0.01$. Specifically, the duration of IDs in the standard preparation (65.62 ± 3.38 seconds) was significantly shorter in comparison to sucrose (89.85 ± 4.79 seconds) prepared slices, $p < 0.01$. There were no significant differences between the two slice preparations in terms of power of IDs, $p > 0.05$.

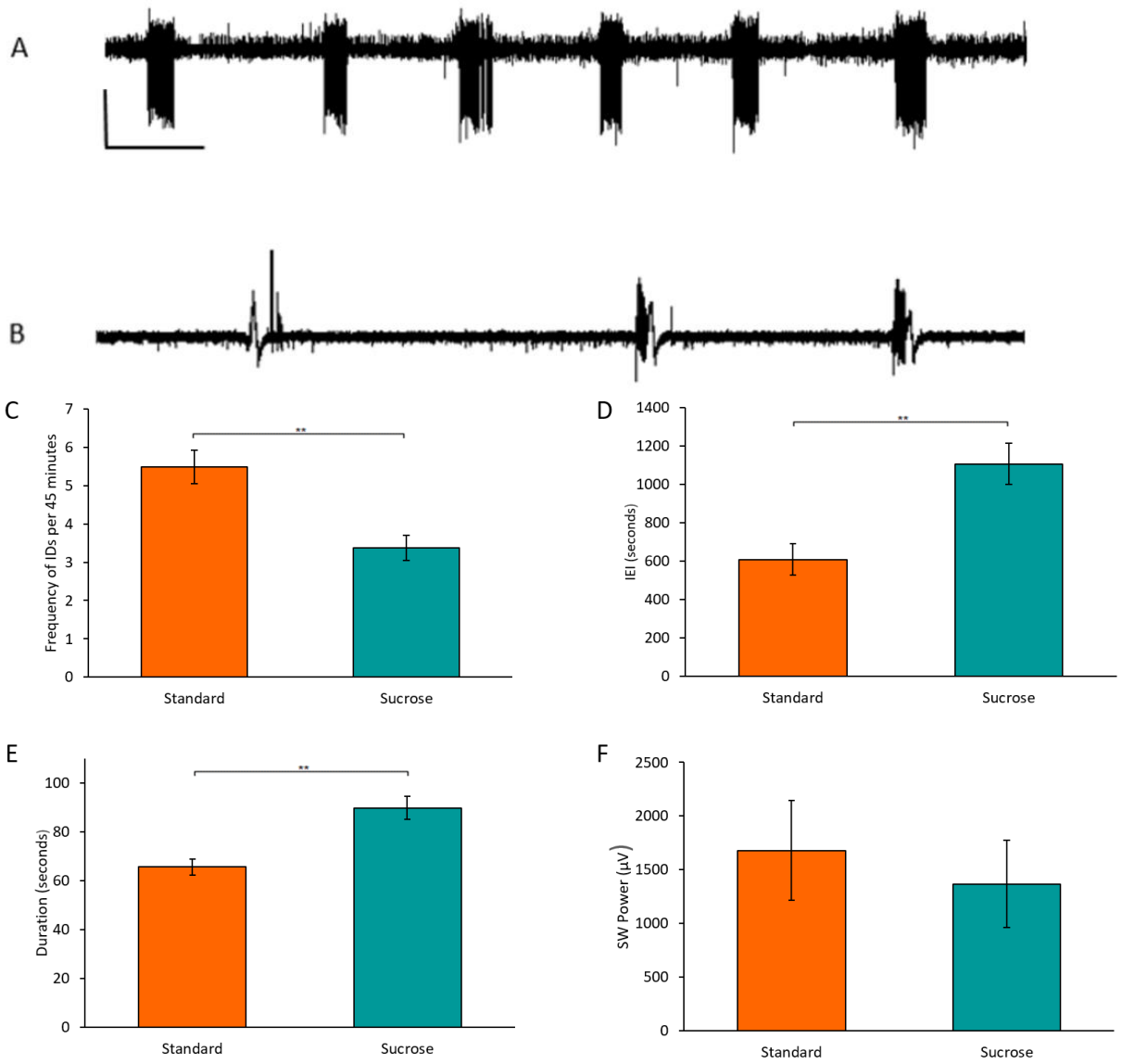

Figure 3-3. Parameter characteristics of induced IDs in two brain slice preparations. A. IDs of standard preparation (Scale: 500 µV x 200 secs). B. IDs of sucrose preparation. C. Comparison of frequency of IDs in two slice preparations (frequency per 45 minutes). D. Comparison of IEIs between IDs in two slice preparations. E. Comparison of durations of IDs in two slice preparations. F. Comparison of power (SW frequency: 0.7-2 Hz) of IDs in two slice preparations.

3.2.2 Expression of parvalbumin interneurons in the entorhinal cortex of standard and sucrose slices

The results presented thus far demonstrate increased excitability of standard prepared slices in comparison to sucrose prepared slices. These findings led us to question what may be causing heightened excitability in standard prepared slices.

Interneurons have profound effects on the excitability of slices, and abnormalities in the GABAergic circuitry of animals and humans has been proposed to underlie the hyper-excitability seen in epilepsy (de Lanerolle et al., 1989; Kumar & Buckmaster, 2006; Peterson & Ribak, 1989). Moreover, levels of inhibition *in vitro* and *in vivo* are not always consistent

(Buckmaster & Schwartzkroin, 1995). Whilst such differences can, to some extent, be accounted for by the removal of inhibitory axon collaterals induced during slicing procedures, another source of this variability in inhibition maybe due to poor slice preparation. It has been previously demonstrated that LTP, induced by field excitatory potentials (fEPSPs), is significantly reduced in the CA1 area of sucrose prepared slices in comparison to standard aCSF prepared slices. This LTP deficit in sucrose slices was reversed by blocking $GABA_A$ receptor function with picrotoxin, thus suggesting sucrose aCSF prepared slices better preserve GABA mediated transmission (Kuenzi et al., 2000).

To investigate the possibility that interneurons in the EC are poorly preserved in standard preparations in comparison to sucrose preparation, and therefore potentially lead to increased excitability, our colleagues at Newcastle University (Hazra et al., 2015) carried out parvalbumin (PV) interneuron staining on the two types of slices. As shown in figure 3-4, there are appears more PV interneurons in slices which have been sucrose perfused in comparison to slices which have been prepared by standard protocols. Additionally, the presence of IDs may further reduce PV interneurons in both types of slices, supporting the notion that seizure-like activity promotes loss of GABAergic network input, which could potentially lead to increased excitability and increased likelihood of more SLEs (Kumar & Buckmaster, 2006).

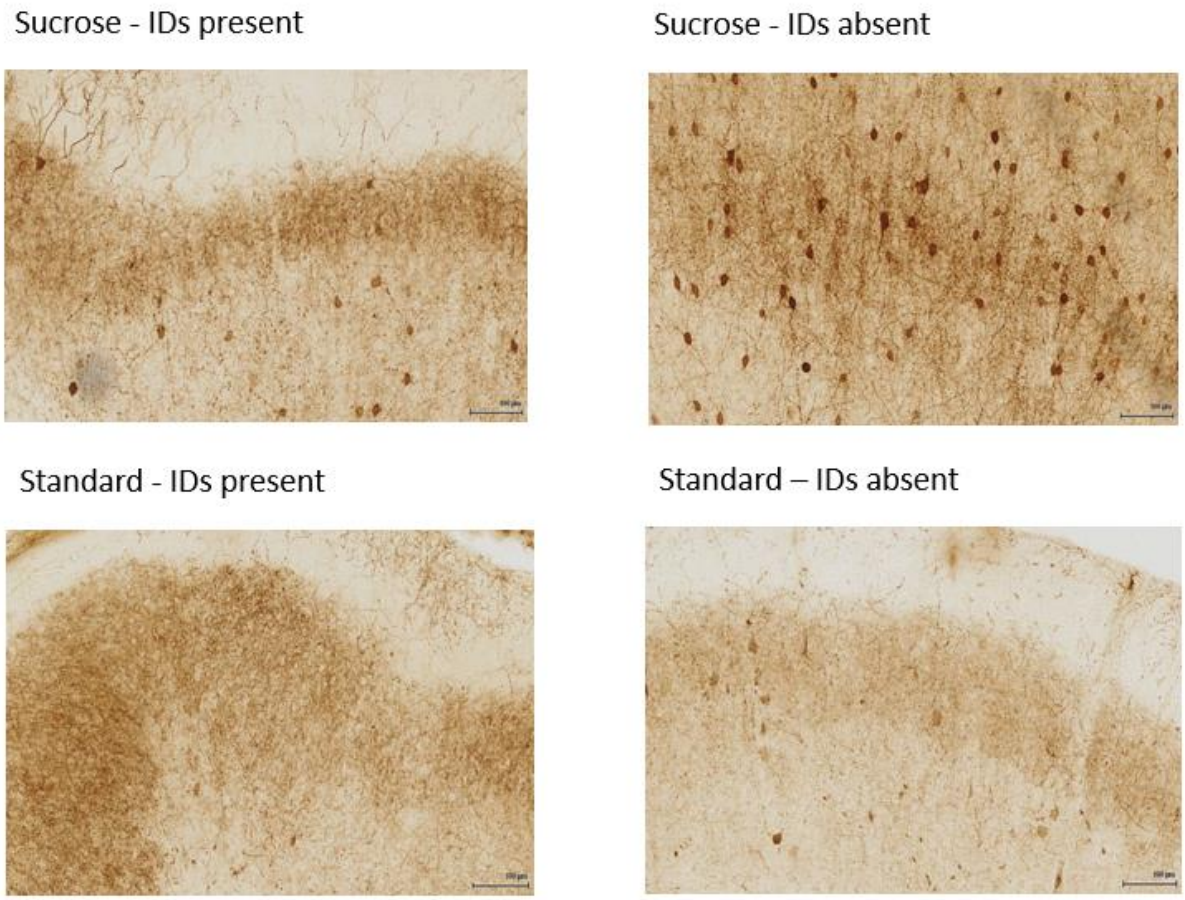

Figure 3-4. Preservation of parvalbumin interneurons in the entorhinal cortex of sucrose perfused and standard prepared slices. (Scale: 100 μm) (See Hazra et al., 2015 for further analysis)

3.2.3. Persistence of ictal-like events

As a result of network architecture differences between standard and sucrose aCSF prepared slices, consequent differences may also exist in the efficacy and resistance of AEDs. However, before this can be effectively tested, the persistence of IDs needed to be accounted for to ensure AED effects seen over the course of such experiments are true and not confounded by instability in excitability. It has been well documented, for example, that slices prepared from standard protocols exhibit the presence of LRDs after prolonged exposure to seizure inducing agents, and have often been shown to be resistant to AEDs (Drier & Heinemann, 1990; Sokolova et al., 1998; Zhang et al., 1995). LRDs have been suggested to represent more IID activity (Bruckner & Heinemann, 2000; Sokolova et al, 1998).

As shown in figure 3-5, both types of brain slices had the capacity to display persistent ID activity. However, both types of slices also showed the capacity to transform regular ID to recurring IIDs/LRDs, as illustrated in figure 3-6. Whilst this raises some concerns over the reliability of results, it was reassuring to find the occurrence of this transformation in sucrose slices was relatively low, and generally occurred when: the flow rate of the perfusate was

more than 2.5ml/minute, when IDs took 2.5 hours or more to be induced and when slices were placed directly into the 0$[Mg]^{2+}$ aCSF perfusate rather than being gradually washed out. On the few occasions, this transformation of activity occurred in sucrose slices, it arose during the initial few IDs and could consequently be disregarded.

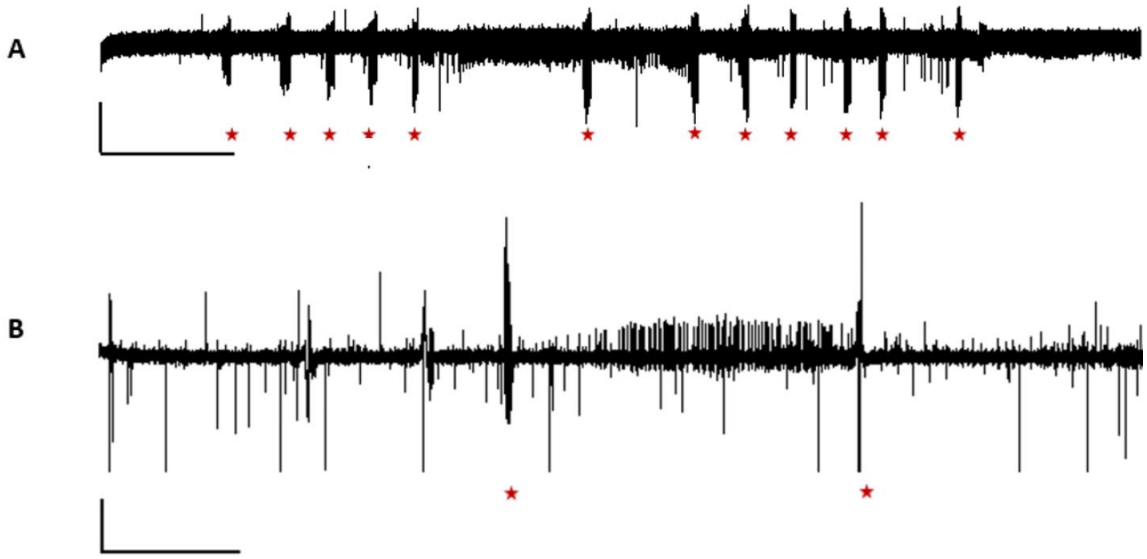

Figure 3-5. The persistence of ID activity in two brain slice preparations. A. The persistence of ID activity standard brain slice (scale: 200 µV x 1000 secs). B. The persistence of ID activity sucrose brain slice (scale: 200 µV x 1000 secs).

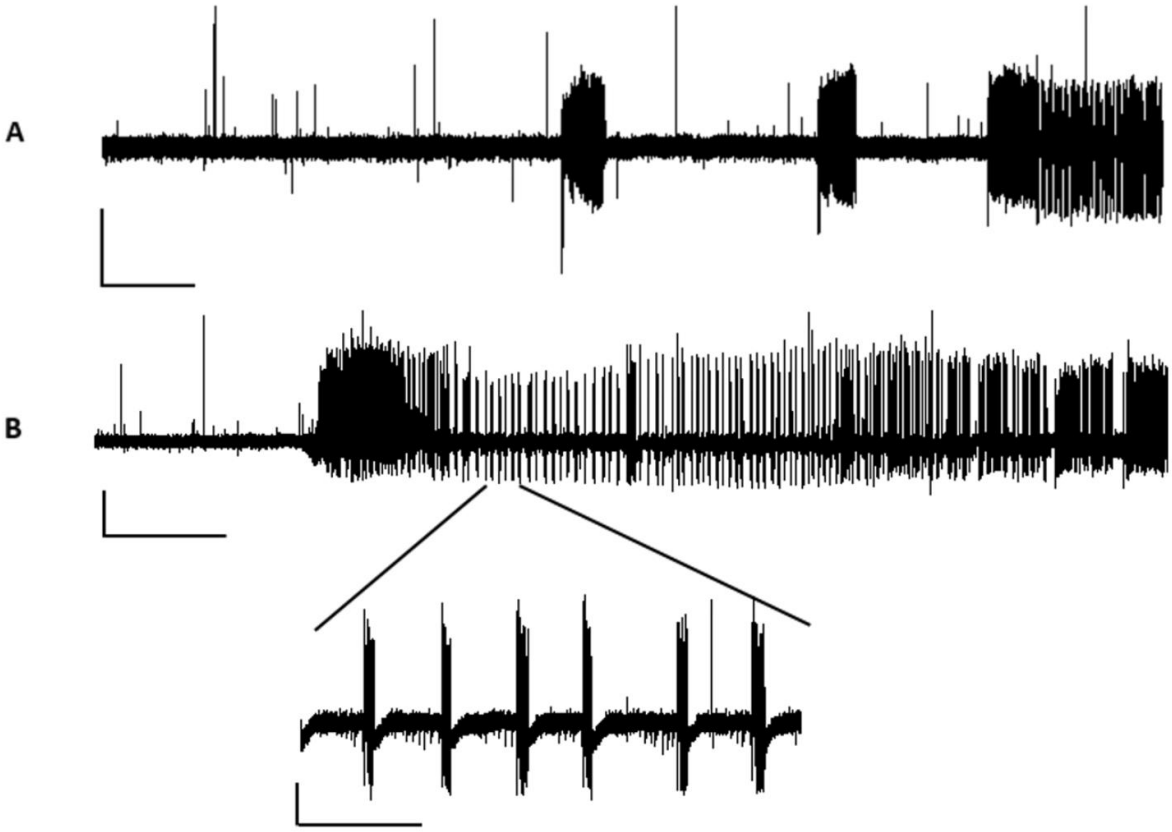

Figure 3-6. The transformation from ID activity to IID/LRD activity in two brain slice preparations. Tonic-clonic activity that characterises ictal-discharges (IDs) has been demonstrated to transition (> 2 hours) into late-recurring-discharges (LRDs) which only display short bursts of tonic activity with prolonged exposure to seizure inducing agents and extracellular ionic manipulations. This transitional behaviour is thought to be due to a reduction in synaptically available GABA (Mody et al., 1994). A. The transformation of ID activity to IID/LRD activity in a standard prepared slice (scale: 500 µV x 250 secs). B. The transformation of ID activity to IID/LRD activity in a sucrose prepared slice (scale: 500 µV x 250 secs). Underneath IID/LRD type activity at shorter time scale (scale: 250 µV x 20 secs).

3.2.4 Efficacy of antiepileptic drugs in two brain slice preparations

Here, the antiepileptic activity of several combinations of AEDs was characterised in standard and sucrose prepared slices. The combinations of AEDs used are presented in table 3.1. A combination of two AEDs were used to assess resistance, as the definition of DRE is defined as a failure to respond to two or more drugs, with more than one seizure per month, for a specified time period (e.g. 12-18 months) (Berg et al., 2001; Cowan, 2002; Kwan & Brodie, 2006). As poly-therapy is generally not endorsed amongst patients, due to complications surrounding drug interactions, and relatively few studies use combinations AEDs, the choice of combination of AEDs was generally based on the use of a primary AED followed by a secondary AED as is commonly done with patients, as secondary AEDs have been tested and approved as add-on therapy.

Table 3.1 Combinations of antiepileptic drugs. Combination of AEDs was generally based on the use of a primary AED followed by a secondary AED as is commonly done with patients, as secondary AEDs have been tested and approved as add-on therapy.

1st AED		2nd AED	
Tiagabine	20 µM	Carbamazepine	50 µM
Valproate	500 µM	Carbamazepine	50 µM
Carbamazepine	50 µM	Zonisamide	100 µM
Carbamazepine	50 µM	Gabapentin	20 µM
Lamotrigine	20 µM	Gabapentin	20 µM
Cannabidiol	30 µM	Carbamazepine	50 µM

3.2.4.1 Resistance to AEDs in two brain slice preparations.

The following analysis explores changes in resistance to AED combinations in different slice preparations, according to two measurements of resistance.

Firstly, to test the difference between the two slice preparations and resistance to several combination AEDs, a chi-square test for independence was conducted, and showed no significant difference χ^2 (1, n = 69) = 0.94, $p > 0.05$, as illustrated in figure 3-7A.

Based on the first definition of AED resistance (continued presence of IDs following application of two AEDs), chi square tests of independence were conducted for each AED combination to assess differences between the two brain slice preparations. As illustrated in figure 3-7B, the only significant difference is between the two brain slice preparations when treated with CBD + CBZ χ^2 (1, n = 14) = 4.67, $p < 0.05$. The percentage of resistance was the highest in standard slices (85.71 %) in comparison to sucrose slices (28.57 %).

Based on the second definition of resistance (< 50 % reduction of IDs following application of 2 AEDs), chi square tests of independence were conducted for each AED combination to assess differences between the two brain slices preparations. An initial comparison of overall resistance between the brain slice preparations, showed no significant differences χ^2 (1, n = 77) = 0.61, $p > 0.05$. As illustrated in figure 3-7C, there were no significant difference in resistance between standard and sucrose slices in response to different AED combinations, $p > 0.05$.

Table 3.2 The resistance of different brain slice preparations and combined resistance to AEDs.

	Standard	Sucrose	Combined
Number of slices	20/37	21/40	41/77
Proportion (%)	54.05	52.5	53.25

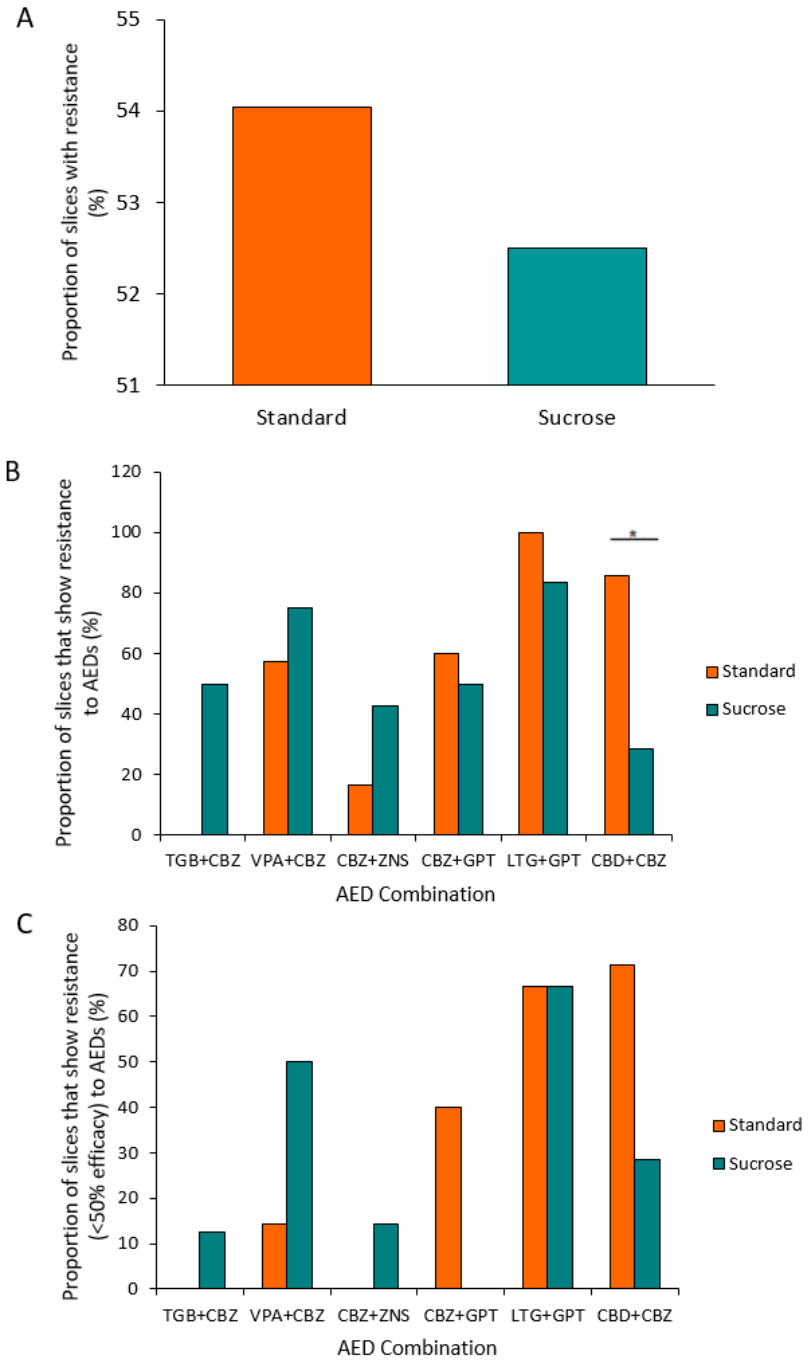

Figure 3-7. Resistance to combination AEDs in layer II of the MEC in two brain slice preparations. A. Percentage of slices from two different preparations that show resistance to combination AEDs. B. Percentage of slices from two different preparations that show resistance to specific combinations of AEDs where resistance is the presence of IDs after 2 AEDs. C. Percentage of slices from two different preparations that show resistance to specific combinations of AEDs where resistance is < 50 % reduction in ID frequency after 2 AEDs.

3.2.4.2 The effects of AEDs on different ID parameters in two brain slice preparations

To assess AED efficacy, the frequency of IDs and latency to first ID are often used in clinical settings (for examples see Brodie et al., 1999b; Buchanan, 1994; de Silva et al., 1996; Sivenius et al., 1994). However, there is a need to consider other parameters (Baker et al.,

1991) such as: the IEI between IDs, the duration of IDs and the power of IDs. Further assessment of such parameters when investigating AED effects could provide useful insights, and was therefore conducted here to further assess responses to AEDs in the two brain slice preparations (see Appendices 3-6 for representation of raw data).

3.2.4.2.1 Frequency

In terms of the frequency of IDs, a mixed model ANOVA demonstrated there was no significant difference between different brain slice preparations and the effects of AED combinations, $p > 0.05$. There was a main effect of drug combination F (5, 65) = 4.70, $p <$ 0.01. Irrespective of brain slice preparation, the TGB+CBZ combination showed a lower frequency of IDs in comparison to CBD+CBZ and LTG+GPT, $p < 0.01$.

3.2.4.2.2 Inter-event intervals

Changes in the frequency of IDs is a rather crude measure, therefore changes in IEIs were also explored. Whilst IEIs are the reciprocal measure of frequency this type of measure provides more information as to the spread of seizures over time. For example, frequency of IDs may suggest three IDs occurred over 45 minutes *in vitro*, but whether these three IDs occurred in the last 20 minutes of recording or equally spread over 45 minutes is not captured. This information is provided by exploring IEIs.

In terms of IEI between IDs, a mixed model ANOVA demonstrated there was a significant difference between the response to different AED combinations in different brain slices preparations, F (10, 112) = 2.51, $p < 0.01$ (see figure 3-9). Specifically, post hoc analysis showed sucrose slices (3.28 ± 0.15 seconds Log10) had significantly longer IEIs following VPA application, in comparison to standard slices (2.78 ± 0.12 seconds Log10), $p < 0.05$. Additionally, sucrose slices also had significantly longer IEIs (3.48 ± 0.12 seconds Log10) following CBD+CBZ application, in comparison to standard slices (2.47 ± 0.14 second Log10), $p < 0.01$. These results suggest IDs displayed in sucrose slices were better controlled by AED combinations VPA+CBZ and CBD+CBZ.

There was also a main effect of drug combination F (5, 56) = 3.53, $p < 0.01$. Post hoc analysis showed irrespective of brain slice preparation method, CBD+CBZ combination displayed significantly lower IEIs in comparison to CBZ+ZNS, $p < 0.05$, suggesting CBD+CBZ was not as an effective AED combination in comparison to CBZ+ZNS.

3.2.4.2.3 Duration

In terms of durations of IDs, a mixed model ANOVA demonstrated there was no significant difference in the response to AED combinations in both brain slice preparations, F (10, 126) = 1.64, $p > 0.05$ (see figure 3-10). There was a main effect of drug combination, F (5, 63) = 2.36, $p <$ 0.0.5. Post hoc analysis showed, irrespective of brain slice preparation, durations of

IDs following LTG+GPT application were longer in comparison to CBZ+ZNS and TGB+CBZ, $p < 0.05$.

3.2.4.2.4 Power

As the greatest power of IDs was concentrated at the lower frequency ranges, analysis of AED effects was conducted on SW (0.7-1.9 Hz) and delta (2-4 Hz) ranges, but a full breakdown of AED effects on the power of IDs at different frequency bands, please see Appendix 6.

With regards to the power of IDs at SW (0.7-1.9 Hz) frequencies, a mixed model ANOVA, demonstrated there was no significant difference between method of brain slice preparation and power of IDs with different AED combinations, $p > 0.05$ (see figure 3-11). Irrespective of brain slice preparation, there was a significant main effect of AED combination F (5, 58) = 3.39, $p < 0.01$.

In terms of power of IDs at delta (2- 4Hz) frequencies, a mixed model ANOVA demonstrated there was no significant role for method of brain slice preparation and power of IDs with different AED combinations, $p > 0.05$ (see figure 3-12). There was a main effect of brain slice preparation method F (1, 58) = 25.05, $p < 0.05$. Post-hoc analysis showed, specifically, irrespective of drug combination the power of sucrose slice IDs at the delta frequency was significantly lower in comparison to standard slices, $p < 0.05$. There was also a main effect of pooled drug combinations, F (5, 58) = 5.13, $p < 0.01$. Post-hoc analysis showed, irrespective of brain slice preparation method, TGB+CBZ reduced the power of IDs significantly more than CBD+CBZ, LTG+GPT, $p < 0.01$.and CBZ+ZNS

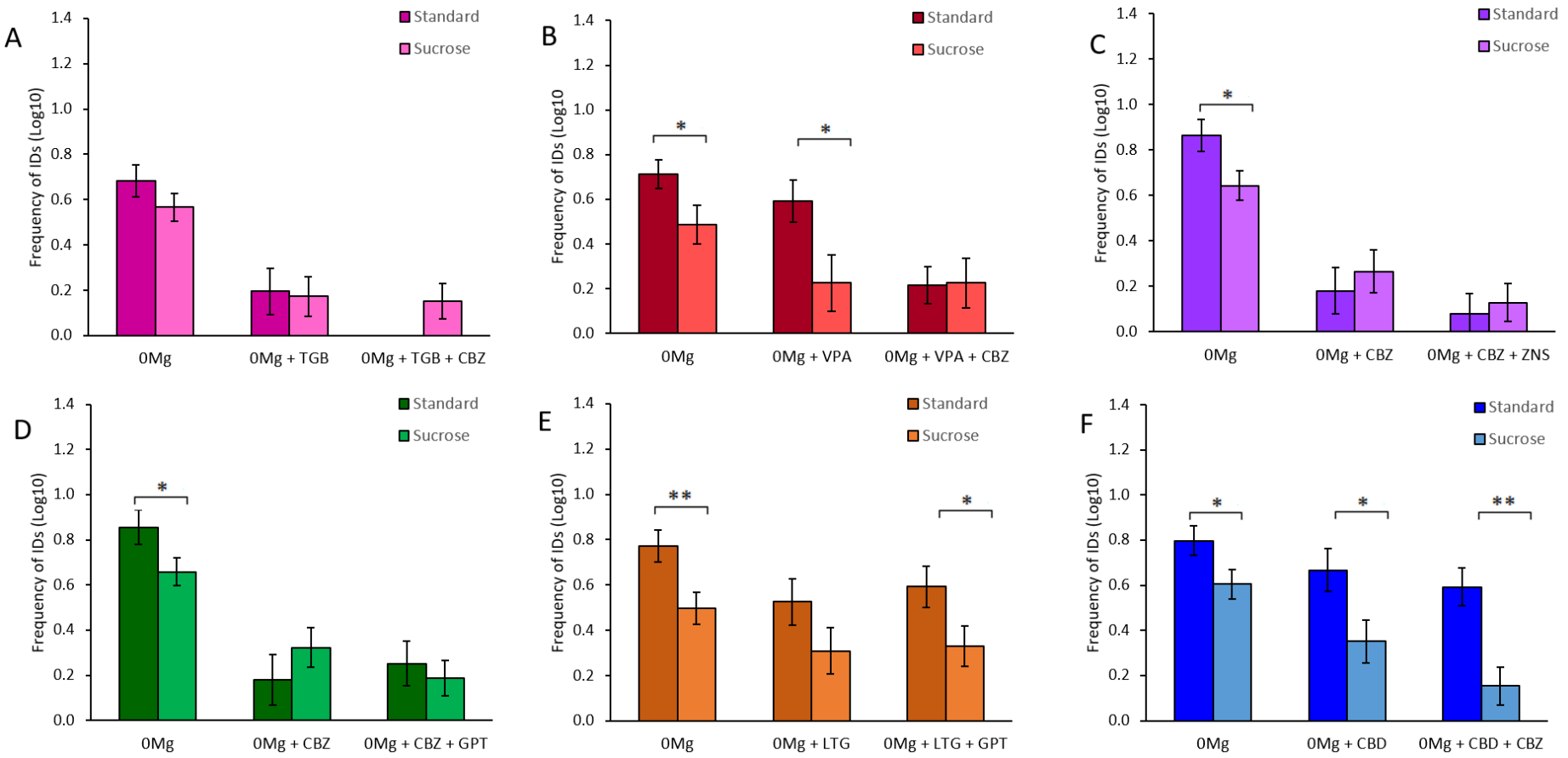

Figure 3-8. The effects of AEDs on the frequency of IDs in two brain slice preparations. A. The effects of TGB+CBZ on frequency of IDs in two slice preparations B. The effects of VPA+CBZ on frequency of IDs in two slice preparations C. The effects of CBZ+ZNS on frequency of IDs in two slice preparations D. The effects of CBZ+GPT on frequency of IDs in two slice preparations E. The effects of LTG+GPT on frequency of IDs in two slices preparations F. The effects of CBD+CBZ on frequency of IDs in two slice preparations.

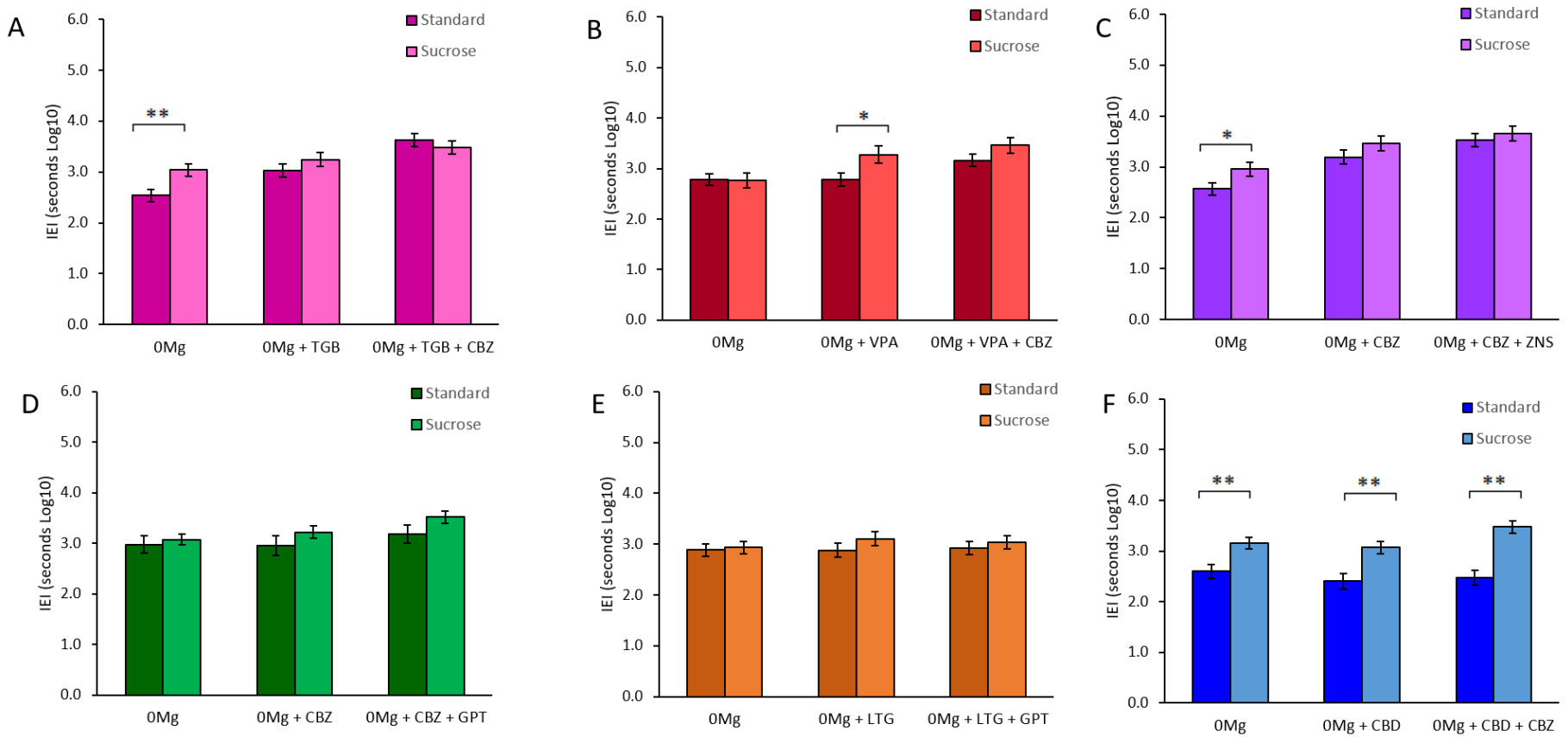

Figure 3-9. The effects of AEDs on the IEIs between IDs in two brain slice preparations. A. The effects of TGB+CBZ on IEIs between IDs in two slice preparations B. The effects of VPA+CBZ on IEIs between IDs in two slice preparations C. The effects of CBZ+ZNS on IEIs between IDs in two slice preparations D. The effects of CBZ+GPT on IEIs between IDs in two slice preparations E. The effects of LTG+GPT on IEIs between IDs in two slices preparations F. The effects of CBD+CBZ on IEIs between IDs in two slice preparations.

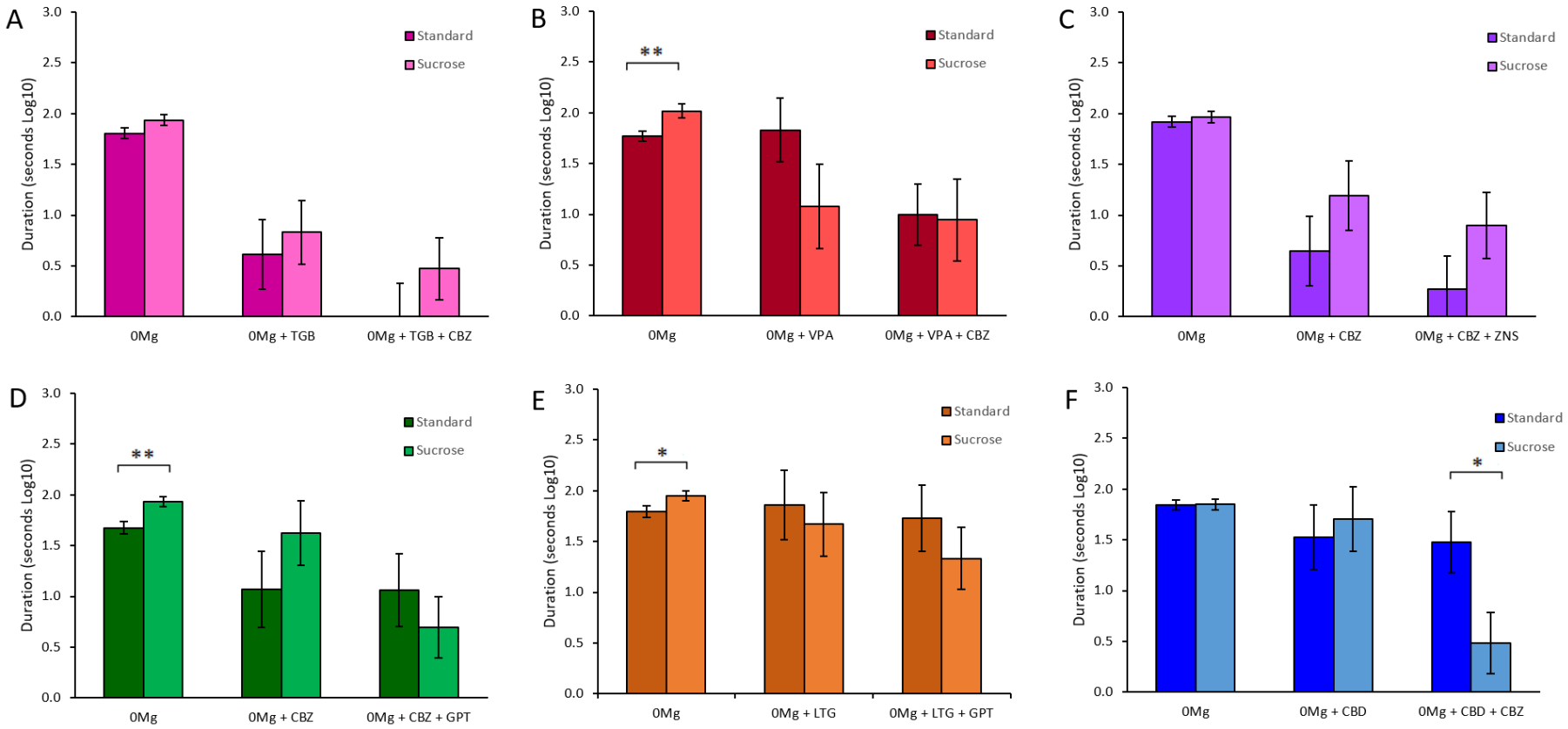

Figure 3-10. The effects of AEDs on the duration IDs in two brain slice preparations. A. The effects of TGB+CBZ on the duration of IDs in two slice preparations B. The effects of VPA+CBZ on the duration of IDs in two slice preparations C. The effects of CBZ+ZNS on the duration of IDs in two slice preparations D. The effects of CBZ+GPT on the duration of IDs in two slice preparations E. The effects of LTG+GPT on the duration of IDs in two slices preparations F. The effects of CBD+CBZ on the duration of IDs in two slice preparations.

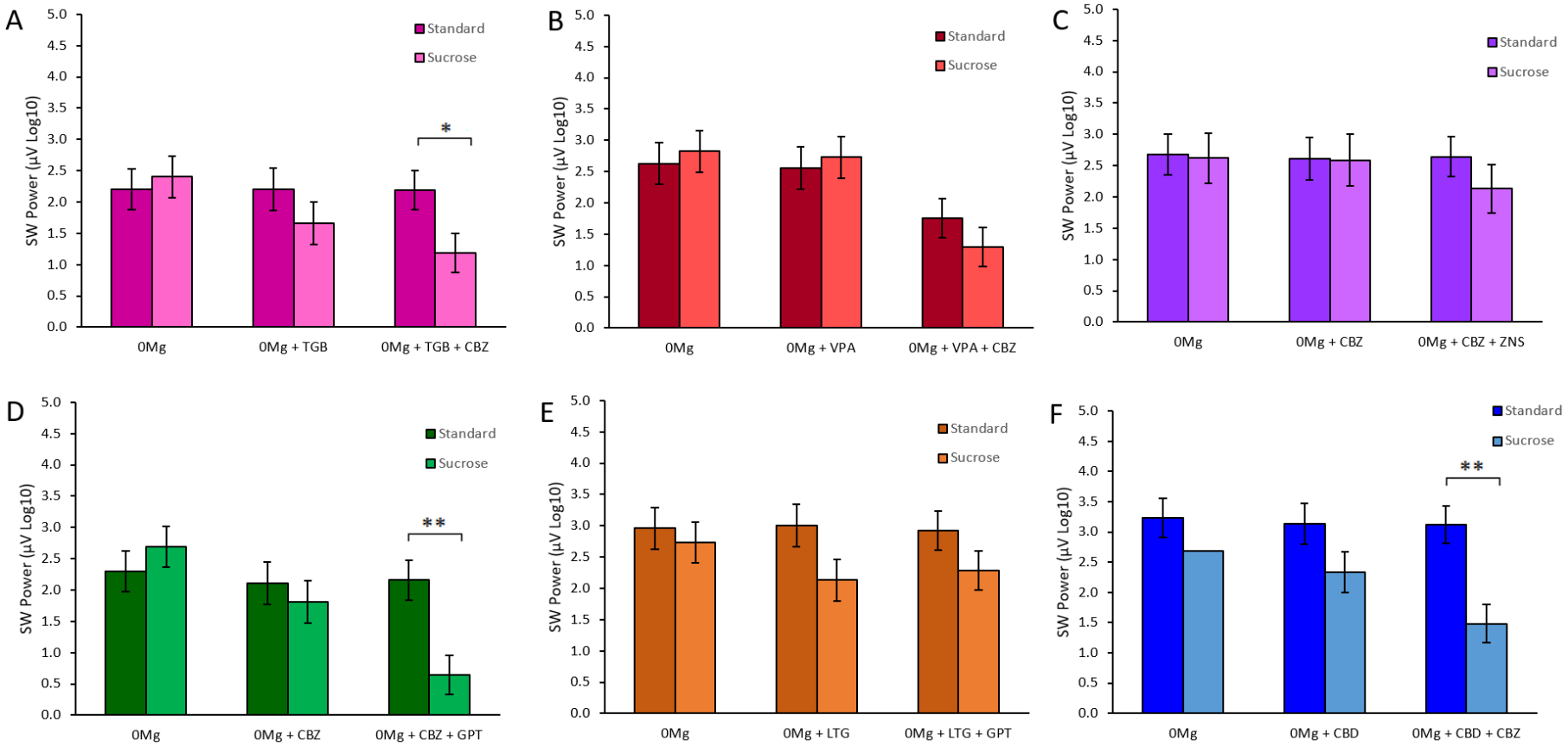

Figure 3-11. The effects of AEDs on the SW (0.7-1.9Hz) power of IDs in two brain slice preparations. A. The effects of TGB+CBZ on the SW (0.7-1.9 Hz) power of IDs in two slice preparations B. The effects of VPA+CBZ on the SW (0.7-1.9 Hz) power of IDs in two slice preparations C. The effects of CBZ+ZNS on the SW (0.7-1.9 Hz) power of IDs in two slice preparations D. The effects of CBZ+GPT on the SW (0.7-1.9 Hz) power of IDs in two slice preparations E. The effects of LTG+GPT on the SW (0.7-1.9 Hz) power of IDs in two slices preparations F. The effects of CBD+CBZ on the SW (0.7-1.9 Hz) power of IDs in two slice preparations.

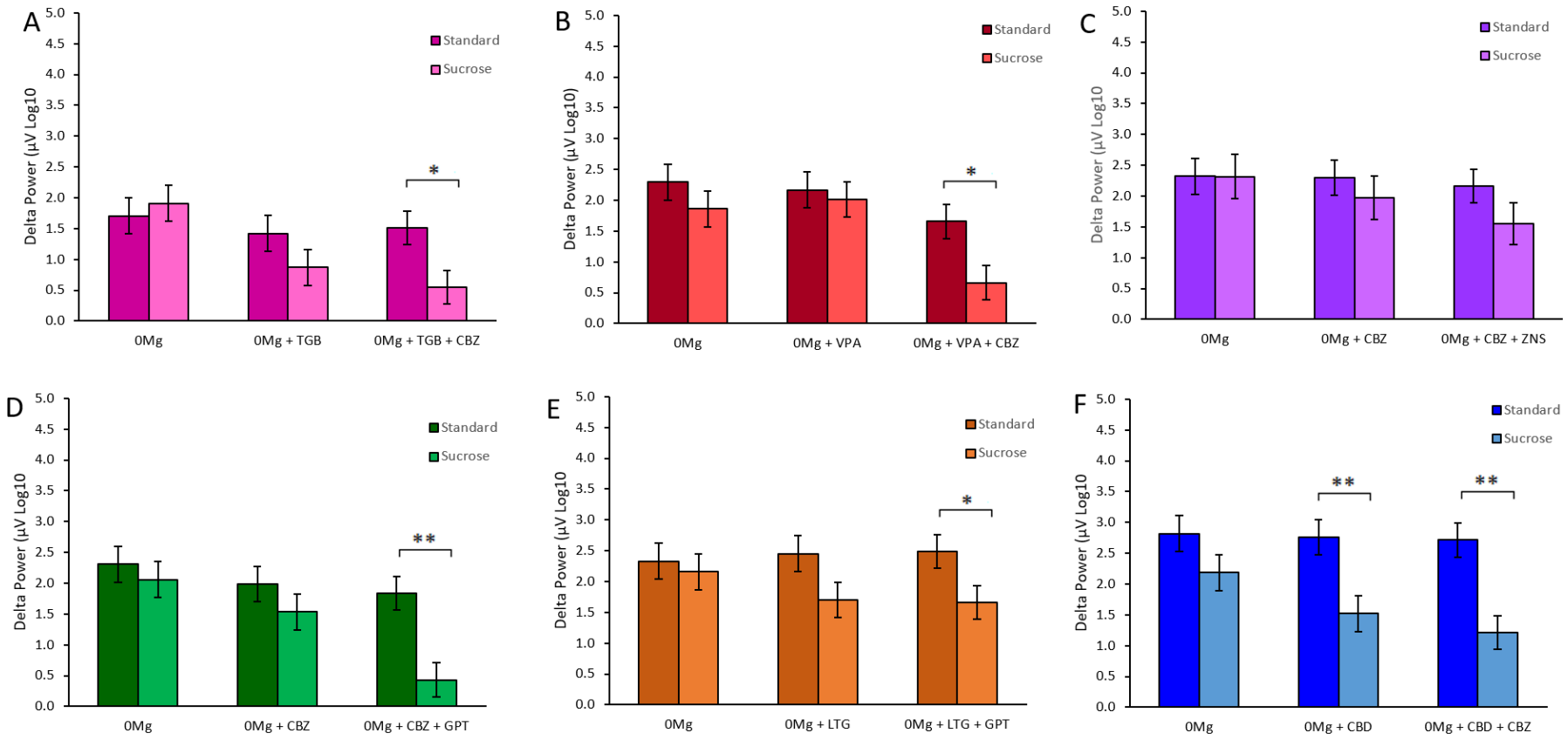

Figure 3-12. The effects of AEDs on the delta (2-4 Hz) power of IDs in two brain slice preparations. A. The effects of TGB+CBZ on the delta (2-4 Hz) power of IDs in two slice preparations B. The effects of VPA+CBZ on the delta (2-4 Hz) power of IDs in two slice preparations C. The effects of CBZ+ZNS on the delta (2-4 Hz) power of IDs in two slice preparations D. The effects of CBZ+GPT on the delta (2-4 Hz) power of IDs in two slice preparations E. The effects of LTG+GPT on the delta (2-4 Hz) power of IDs in two slices preparations F. The effects of CBD+CBZ on the delta (2-4 Hz) power of IDs in two slice preparations.

3.2.4.2.5 Predictive power of different ID parameters

The analyses of AED effects on different parameters of IDs in the two brain slice preparations is complex. Only the IEI parameter was able to discriminate AED efficacy between the two slice preparation methods. Sucrose slices were shown to respond better to VPA and CBD+CBZ. However, all parameters consistently showed, irrespective of brain slice preparation, TGB+CBZ were the most effective AED combinations whereas LTG+GPT and CBD+CBZ were the least effective.

To assess which parameter held the most predictive power of slice preparation a logistic regression was conducted. The regression model contained four predictor variables of slice preparation: percentage change from 0$[Mg]^{2+}$ aCSF to application of two AEDs in the frequency of IDs, percentage change of the IEI between IDs, percentage change in the duration of IDs and percentage change in the power of IDs at the SW frequency range. The full model containing all predictors was not statically significant, χ^2 (9, n= 67) = 13.23, p > 0.05, indicating the model was not able to distinguish between different brain slice preparations (see figure 3-13). The model as a whole explained between 17.9% (Cox and Snell R square) and 23.9 % (Nagelkerke R squared) of the variance in brain slice preparation methods, and correctly classified 73.1 % of cases. Although this analysis was unable to highlight which out the four parameters hold the most predictive power, it is evident from analyses investigating the efficacy of AEDs in different brain slice preparations that IEI and duration parameters hold a great deal of predictive power and shall therefore be the continued primary parameters of investigation.

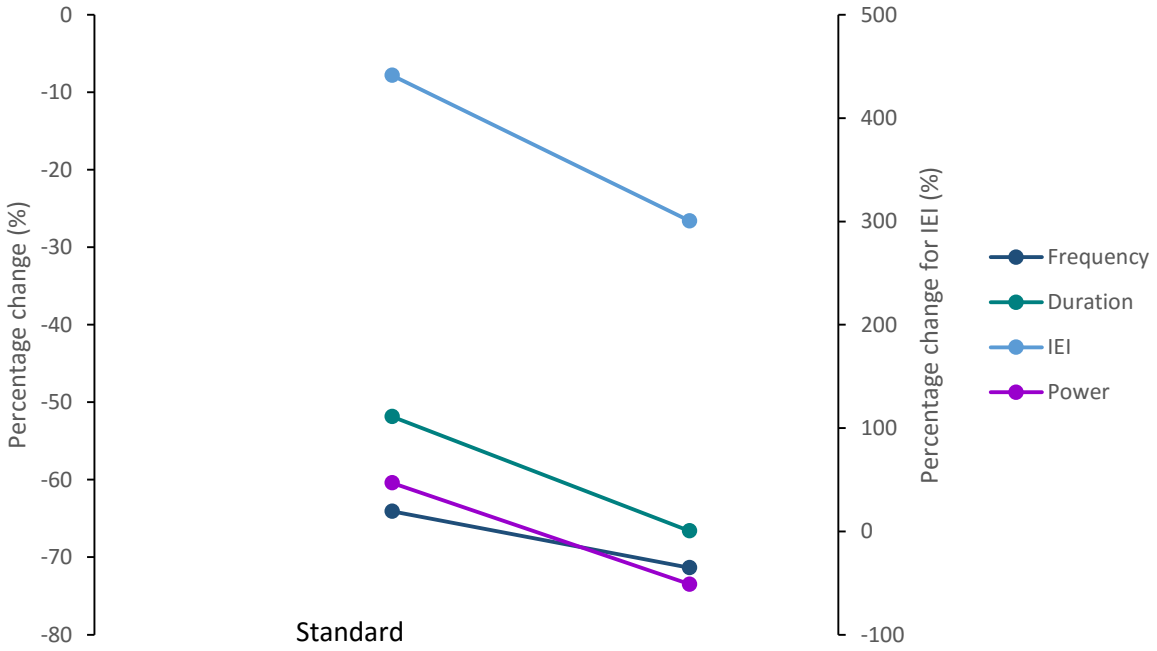

Figure 3-13. The percentage change of ID parameters with application of AEDs in two brain slice preparations.

3.2.5 The role of LTP in the resistance to AEDs in sucrose slices

From the analyses presented thus far, it is evident that sucrose prepared slices are less excitable in comparison to standard prepared slices, and show little or no difference in the response and resistance to many combination AEDs. Given that sucrose slices have a better preserved inhibitory network and consequently better control over network activity, it would be expected that these slices show significantly less resistance. What is the mechanism behind this degree of persistent resistance in sucrose prepared slices? We have shown that sucrose slices display a greater latency to first ID time period compared to standard slices, the period of time when LTP processes are activated.

Brief, controlled periods of Ca^{2+} elevations occur during physiological processes associated with plasticity changes of LTP in learning and memory (Gnegy, 2000; Malenka & Nicoll, 1999; Tzounopoulos & Stackman, 2003; West et al., 2001). However, at the other of the spectrum, pathological uncontrolled and irreversible elevations in Ca^{2+} lead to neuronal death. The middle ground, appears to be a characterised by a less severe epileptogenic injury whereby exposure to prolonged but reversible elevations in Ca^{2+} trigger pathological plasticity changes, leading to chronic epilepsy and DRE (DeLorenzo et al., 2005).

To test the hypothesis that sucrose slice AED resistance was due to longer duration of exposure to LTP inducing 0$[Mg]^{2+}$ aCSF, sucrose slices were incubated with the protein synthesis inhibitor, cycloheximide, for 30 minutes prior to 0$[Mg]^{2+}$ aCSF application**.** Application of cycloheximide would be expected to inhibit the expression of LTP, and consequently reduce synaptic strength.

As demonstrated in figure 3-14, there were no significant differences in the number of cycloheximide treated slices which displayed IDs in 0[Mg]$^{2+}$ aCSF, in comparison to sucrose perfused slices, $p > 0.05$. On the other hand, cycloheximide slices (6910.2 ± 676.70 seconds) showed a significantly longer latency to first ID, in comparison to sucrose slices (4753.8 ± 476.55 seconds)., $p < 0.01$.

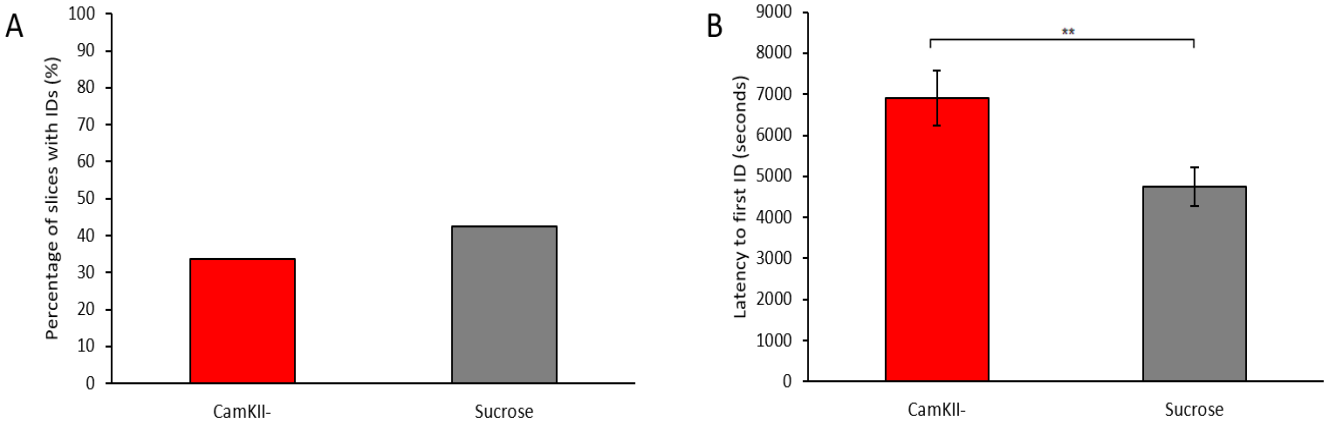

Figure 3-14. Excitability of sucrose perfused and cycloheximide treated slices. A. Percentage of cycloheximide treated and sucrose perfused slices that displayed IDs. B. Latency to first ID in in cycloheximide treated and sucrose perfused slices.

Differences in ID parameters between cycloheximide treated and sucrose slices in 0[Mg]$^{2+}$ aCSF were assessed. As shown in figure 3-15, an independent-sample t-test showed there no significant differences between the duration of IDs in cycloheximide treated (95.53 ± 9.03 seconds) and sucrose slices (87.45 ± 7.21 seconds), t (33) = 0.69, $p > 0.05$. A Mann-Whitney- U test revealed no significant difference in the IEI between IDs in cycloheximide treated (1000.5 seconds ± 264.94) and sucrose slices (1074.8 ± 159.18 seconds), $U = 84.00$, $Z = -1.16$, $p > 0.05$.

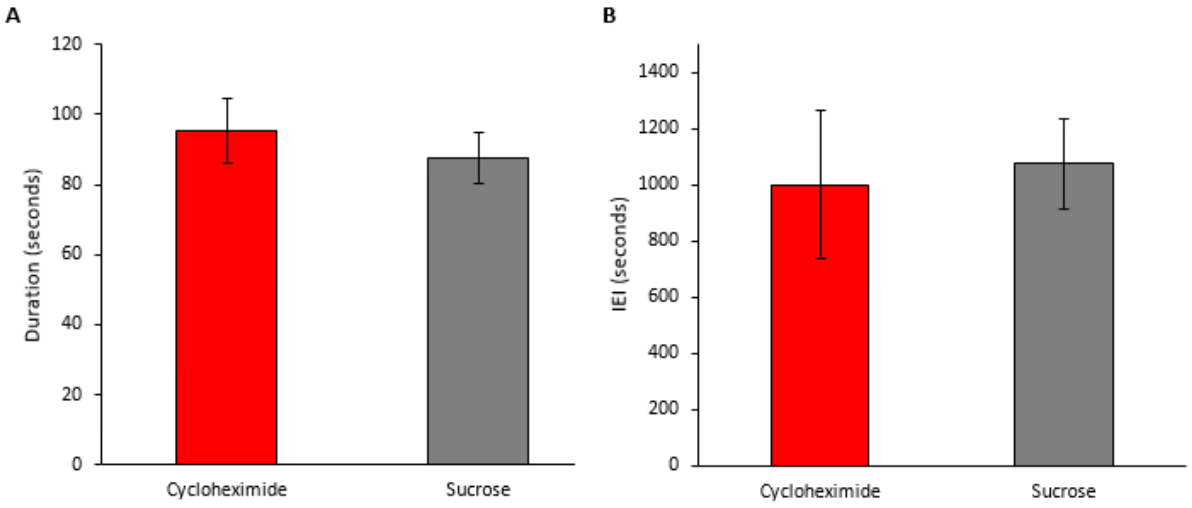

Figure 3-15. ID parameters of cycloheximide treated and sucrose perfused slices in 0[Mg]$^{2+}$ aCSF. A. The duration of IDs in cycloheximide treated and sucrose perfused slices. B. The IEIs between IDs in cycloheximide treated seconds and sucrose perfused slices.

To assess the effects of LTP inhibition on AED resistance, CBZ+ZNS, LTG+GPT and CBD+CBZ were applied to cycloheximide treated slices and sucrose perfused slices. These drug combinations were chosen based on the premise that they displayed the highest levels of resistance in sucrose slices. The differences in duration was not considered as part of this analysis as very few cycloheximide slices showed IDs following AED applications.

As illustrated in figure 3-16, a chi-square test for independence indicated no significant association between the percentage of cycloheximide treated and sucrose slices with resistance to CBZ+ZNS, χ^2 (1, n = 13) = 1.04, $p > 0.05$. There was a significant difference between the percentage of cycloheximide treated (20 %) and sucrose slices (83.33 %) with resistance to LTG+GPT, χ^2 (1, n = 11) = 4.41, $p < 0.05$. There was no significant association between the percentage of cycloheximide treated and sucrose slices with resistance to CBD+CBZ, χ^2 (1, n = 12) = 0.11, $p > 0.05$.

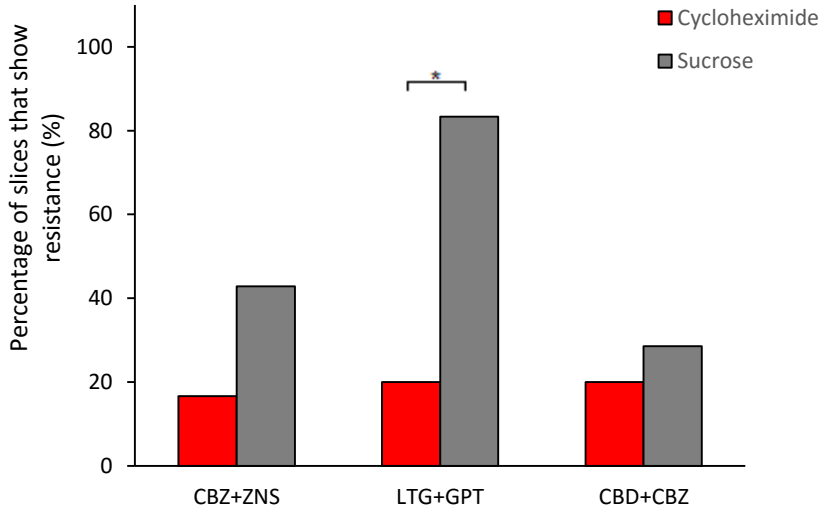

Figure 3-16. The resistance of cycloheximide treated and sucrose slices to AEDs. Significantly more sucrose slices than cycloheximide treated slices showed resistance to LTG+GPT AEDs.

As demonstrated in figure 3-17, a mixed model ANOVA demonstrated, irrespective of drug combination, the IEIs of cycloheximide treated slices were significantly higher than sucrose slices overall, $p < 0.05$. There was no significant interaction effect between cycloheximide treated and sucrose slices with different drug combinations, $p > 0.05$.

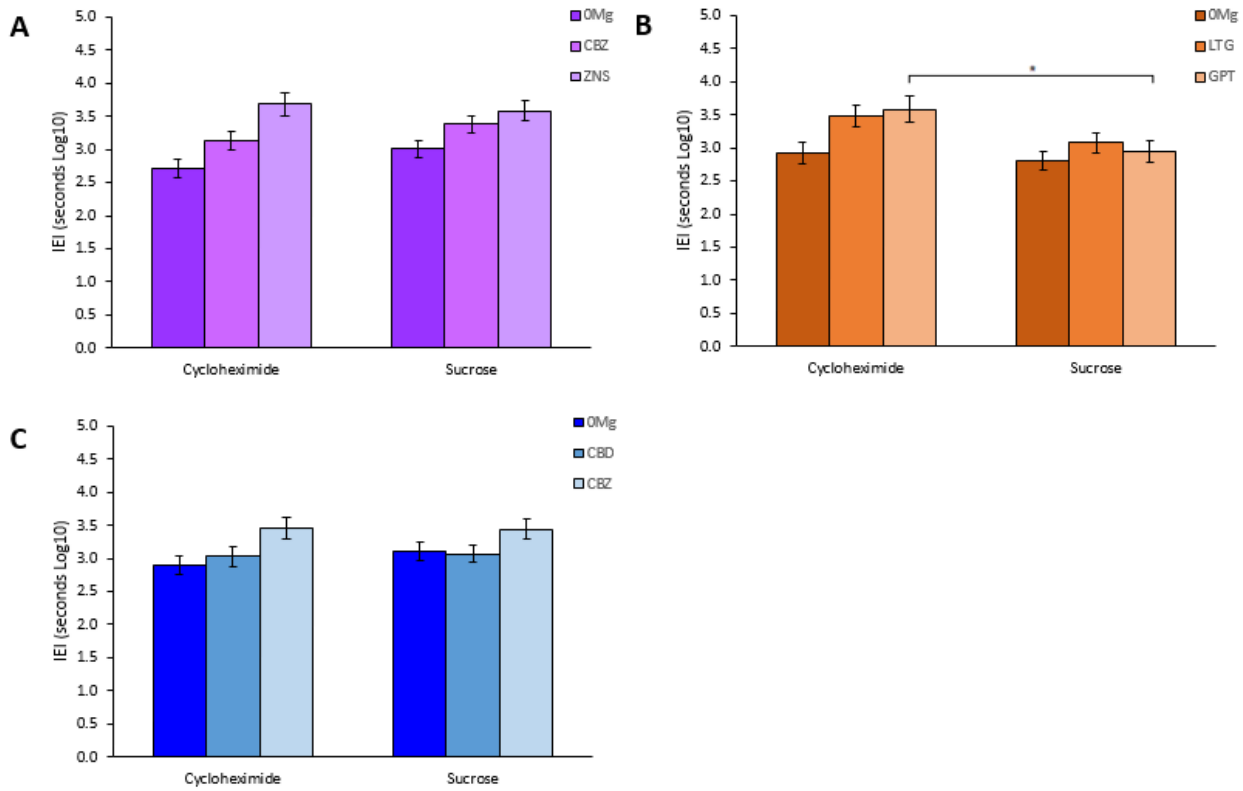

Figure 3-17. The effect of AEDs on the IEIs between IDs in cycloheximide treated and sucrose slices. A. The effects of CBZ (50 µM) and ZNS (100 µM) on IEIs between IDs in cycloheximide treated and sucrose slices. B. The effects of LTG (20 µM) and GPT (20 µM) on IEIs between IDs in cycloheximide treated and sucrose slices. The effects of CBD (30 µM) and CBZ (50 µM) on IEIs between IDs in cycloheximide treated and sucrose slices.

As differences in the resistance of AEDs between cycloheximide treated and sucrose slices were only evident in one out of three AED combinations, and in latencies to first ID analysis, it was attempted to further test the role LTP in sucrose slice AED resistance by treating slices with a different LTP inhibitor, GF109203X, which inhibits PKC. However, this treatment considerably inhibited the induction of IDs, as only 2 out of 20 slices consistently displayed more than one IDs after prolonged exposure to 0$[Mg]^{2+}$. Only 4 out of 20 slices (20 %) displayed more than one ID, but these IDs occurred inconsistently with very long IEIs. As illustrated in figure 3-18, the latency to first seizure or total time recorded in 0$[Mg]^{2+}$ without an ID was significantly longer in slices pre-treated with GF109203X (10,441.75 ± 698.47 seconds) in comparison to cycloheximide treated slices (6910.20 ± 676.70 seconds), t (33) = 3.49, $p < 0.01$. These results suggest PKC plays a role in the induction of IDs.

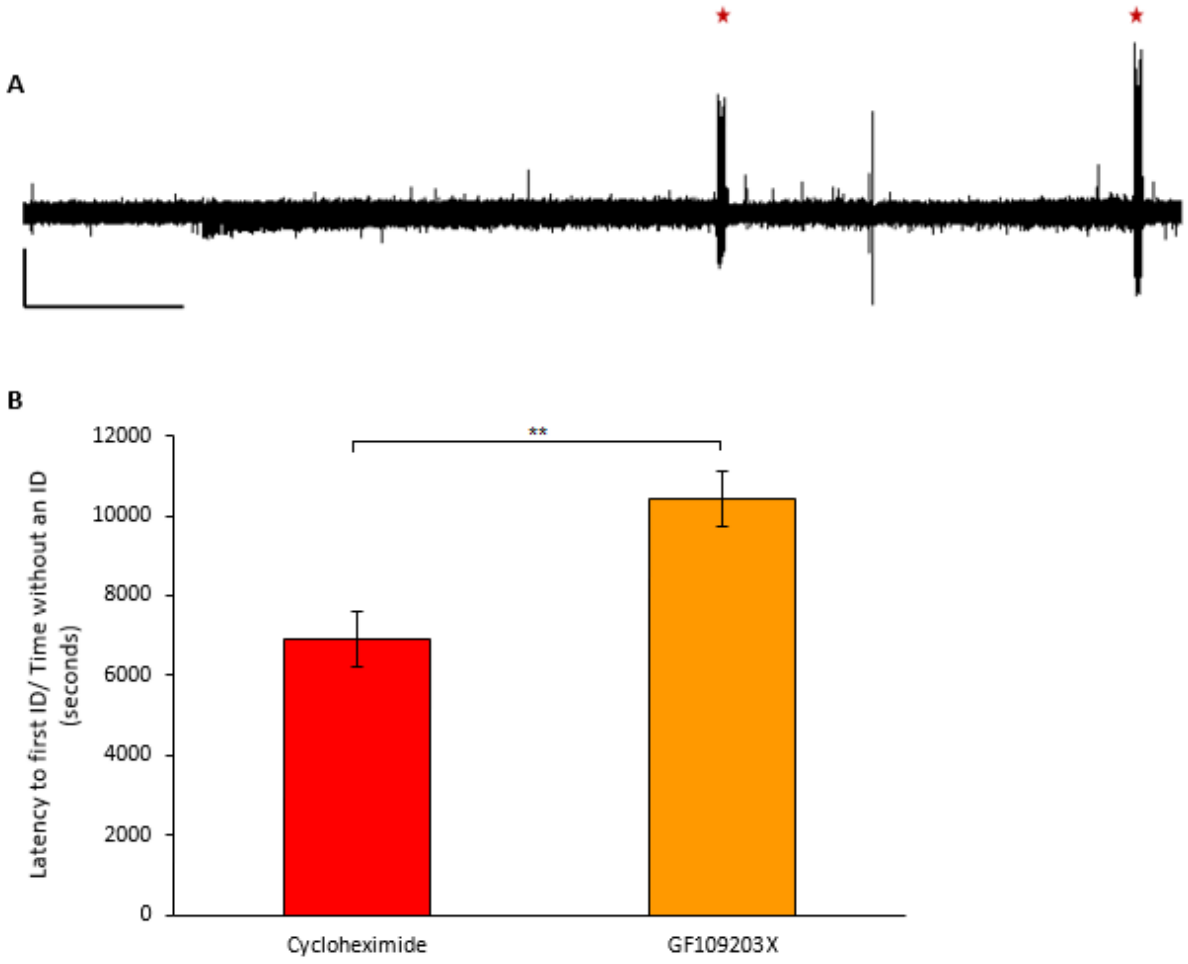

Figure 3- 18. The excitability of GF109203X treated slices. A. A representative trace illustrating the long latency to first ID in GF109203X treated slices. The first ID occurred at 9145 seconds followed by another ID 5380 seconds later, as indicated by the asterisks. Scale: 500 µV x 2000 seconds. B. Bar graph showing the average latency to first ID or average length of time without an ID in cycloheximide and GF109203X treated slices.

3.3 Discussion

The current investigation demonstrated standard prepared slices are more excitable in comparison to sucrose prepared slices, probably as a result of a better preserved inhibitory network. Irrespective of preparation method, CBD+CBZ and LTG+GPT were the least effective AED combinations and TGB+CBZ were the most effective. There is little difference between the two brain slice preparations in terms of resistance and efficacy of combination AEDs. This is of particular interest given the preservation of the inhibitory network, one may expect resistance to be lower in sucrose slices, but given the increased latency to first ID, it is likely enhanced LTP processes in these slices bring levels of resistance to similar standard as seen in standard prepared slices. LTP processes were shown to play a potential role in the resistance toward AEDs.

Previous studies exploring the effects of AEDs on IDs induced *in vitro*, have demonstrated a unifying sensitivity to a large range of AEDs (Armand et al., 1998b; Drier & Heinemann, 1990; Zhang et al., 1995). This overwhelming deficiency in the ability to differentiate between the effectiveness of AEDs *in vitro*, has promoted the existence of 'me too' drugs, which are capable to treating seizures for some patients, but do not improve prognosis for those patients with DRE. Furthermore, by not being able to identify better drugs for sufferers of DRE, the possibility of understanding the mechanisms underlying this severe form of epilepsy remains to be elucidated.

In our laboratory comparisons between standard and modified slice preparation protocols have illustrated improvements to motor cortex and hippocampal slice viability when prepared with modified sucrose aCSF. Additionally, we have demonstrated oscillatory activity stability is significantly improved with modified methods of brain slice preparations, allowing accurate characterisation of pharmacological effects *in vitro* (Modebadze, 2014; Prokic, 2012). It is important to question how improved brain slice preparations effects investigations of AED discovery and efficacy, and whether improved preparations can enrich the ability to differentiate between effective and non-effective AEDs.

3.3.1 The excitability of slices from different brain slice preparations

The current study demonstrated the presence of enhanced excitability in standard prepared brain slices in comparison to sucrose prepared slices. This was evident as a higher percentage of standard slices displayed IDs with shorter latencies to first ID, in comparison to sucrose slices. Additionally, analysis of ID parameters in 0$[Mg]^{2+}$ aCSF supported these findings by showing sucrose prepared slices were the least excitable.

Previous research by Kuenzi et al. (2000) compared the effects of slices prepared from standard and sucrose based aCSF on hippocampal synaptic plasticity, and demonstrated the amount of induced LTP was significantly reduced in sucrose prepared slices. LTP induction

critically depends on the depolarisation of pyramidal cell in the hippocampus, but stimulus-response relationships suggested the sucrose preparation did not affect excitatory synaptic transmission. Moreover, this LTP deficit in sucrose slices was reversed by blocking $GABA_A$ receptor activity with picrotoxin, thus suggesting sucrose aCSF better preserves inhibitory transmission.

Based on these findings, we collaborated with colleagues at Newcastle University to conduct PV interneuron staining in both standard and sucrose prepared slices, before and after ID activity. This investigation led to the finding that PV interneurons are better preserved in sucrose slices in comparison to standard slices. Additionally, it was found that the presence of IDs reduced the number of PV interneurons in both types of slices. These findings suggest the reason for enhanced excitability in standard prepared slices is due to poor preservation of the inhibitory network.

Interneurons have profound effects on the excitability of slices, and abnormalities in the GABAergic circuitry of animals and humans has been proposed to underlie the hyper-excitability seen in epilepsy (de Lanerolle et al., 1989; Kumar & Buckmaster, 2006; Peterson & Ribak, 1989). Moreover, levels of inhibition *in vitro* and *in vivo* are not consistent (Buckmaster & Schwartzkroin, 1995). Whilst such differences can to some extent can be accounted for by the loss of inhibitory axon collaterals during the slicing procedure, it is important for *in vitro* slice preparation methods to be optimised to reduce variance between *in vitro* and *in vivo* environments, in order to investigate AED effects as accurately as possible.

3.3.2 The efficacy of different AED combinations in different brain slice preparations

Comparative investigations into the resistance of the two brain slice preparations, to several combinations of AEDs, showed there were minimal differences between standard and sucrose slices in terms of percentage of slices that were resistant to two AEDs.

Investigations of resistance to specific AED combinations in the different preparations were assessed using two definitions of resistance. Analysis of resistance according to the first definition (continued presence of IDs following application of two AEDs) highlighted standard slices show greater resistance to CBD+CBZ in comparison to sucrose slices. The second definition (less than 50 % reduction in seizure frequency following the application of two AEDs) did not highlight any differences between the two brain slice preparations in response to different combination AEDs.

The majority of combination AEDs here act in mechanistically similar ways, either by acting at Na^+ or Ca^{2+} channels, or both (see section 1.5). The exceptions being TGB acts to enhance GABA transmission (Dalby & Nielsen, 1997; Jackson et al., 1999; Thompson & Gahwiler, 1992), and the mechanisms of CBD remain ambiguous but it has been suggested

to affect: the equilibrative nucleoside transporter, the orphan G-protein-couple receptor GPR55, the $5HT_{1a}$ receptor, the α1 and α3 glycine receptors and the transient receptor potential of Ankyrin type I channel (see Devinsky et al., 2014 for a review).

A higher percentage of sucrose slices displayed greater resistance to TGB+CBZ, in comparison to standard slices. This is surprising, as we have demonstrated sucrose slices better preserve the inhibitory network, therefore we would expect drugs such as TGB which enhance inhibition to work better in such slices. One explanation for these unexpected results may explained by variances in the damage of inhibitory networks in standard prepared slices. Histological investigations of PV interneurons found, some standard prepared slices had a more intact inhibitory network in comparison to other standard prepared slices.

Additionally, it is likely that that the effects of changes to inhibitory networks are not as simple to decipher as expected, and the interplay of changes to excitation also need to be congruently evaluated. Sucrose slices showed longer latencies to first IDs in comparison standard prepared slices, and were more likely to have been exposed to greater NMDA activation which plays an important role in LTP expression. This enhanced exposure to excitation may have resulted in a higher resistance to TGB+CBZ. If this is the case, one may question why is resistance not consistently seen in sucrose slices with other AEDs? As demonstrated TGB+CBZ are particularly effective AEDs, irrespective of slice preparation, in comparison to other AEDs, and it may be this superiority that leads to such results.

The debate over precedence of changes in excitation and inhibition accounting for DRE (Galarreta & Hestrin, 1998; Pavlov et al., 2013; Margineanu & Klitgaard, 2009; Sayin et al., 2003; Woodhall et al., 2005), is a long standing one, which has demonstrated valid points for both sides of the debate. Additionally, other mechanisms of DRE such as inflammation and glia-induced hyperexcitability may also be of importance (Devinsky et al., 2013).

Further in-depth analyses of AED effects on different parameters (e.g. frequency of IDs, IEIs between IDs, duration of IDs and power of IDs) in different brain slice preparation methods, further demonstrated little difference between sucrose and standard prepared slice. Irrespective of different brain slice preparation effects, analysis of these different parameters further showed the CBD+CBZ and LTG+GPT were the least effective AED combinations and TGB+CBZ was the most effective AED combination. Power analyses did not show preparation specific AED effects but did demonstrate the power of IDs in sucrose slices overall was smaller in comparison to standard slice IDs.

The quantitative characterisation of AED effects can be rather complex and many researchers often use frequency of IDs and latency to first ID, as is often done in clinical settings (Brodie et al., 1999b; Buchanan, 1994; de Silva et al., 1996; Sivenius et al., 1994). However, there is a need to consider other parameters (Baker et al., 1991; Yonekawa et al.,

1995) and *in vitro* and *in vivo* techniques offer the ability to extract information on other ID parameters. In this investigation, largely consistent findings between analyses of different parameters have been demonstrated and whilst this is reassuring, it is important to recognise which parameters provide the most insight and predictive power. Regression analyses conducted failed to highlight which out the four parameters considered held the most predictive power, but analyses investigating AED efficacy in different brain slice preparations suggested IEI and duration of IDs would be the most informative.

3.3.3 The role of LTP in the resistance of sucrose perfused slices

Overall there was no significant difference between sucrose and standard prepared slices. Given that the inhibitory network is better preserved in sucrose slices in comparison to standard slices, it is intriguing as to why the level of resistance in sucrose slices persists. Although Knuezi et al (1995) demonstrated LTP is reduced in sucrose slices, here it is evident that latency to first ID is significantly longer. Therefore, one must consider the possibility that this increased length of time exposed to excitatory and LTP inducing agents, results in this increased resistance.

The induction of certain forms of LTP fundamentally relies on the activation of postsynaptic NMDA receptors, as illustrated in figure 3-18. Glutamatergic activation of postsynaptic AMPA receptors allows intracellular entry of Na^+ and K^+ ions and glutamatergic activation of postsynaptic NMDA receptors promotes influx of Ca^{2+} and Na^+ ions once the Mg^{2+} block has been removed. Metabotropic glutamate receptors have also been shown to enhance LTP via activation of protein kinase C (PKC) (McGuinness et al., 1991).

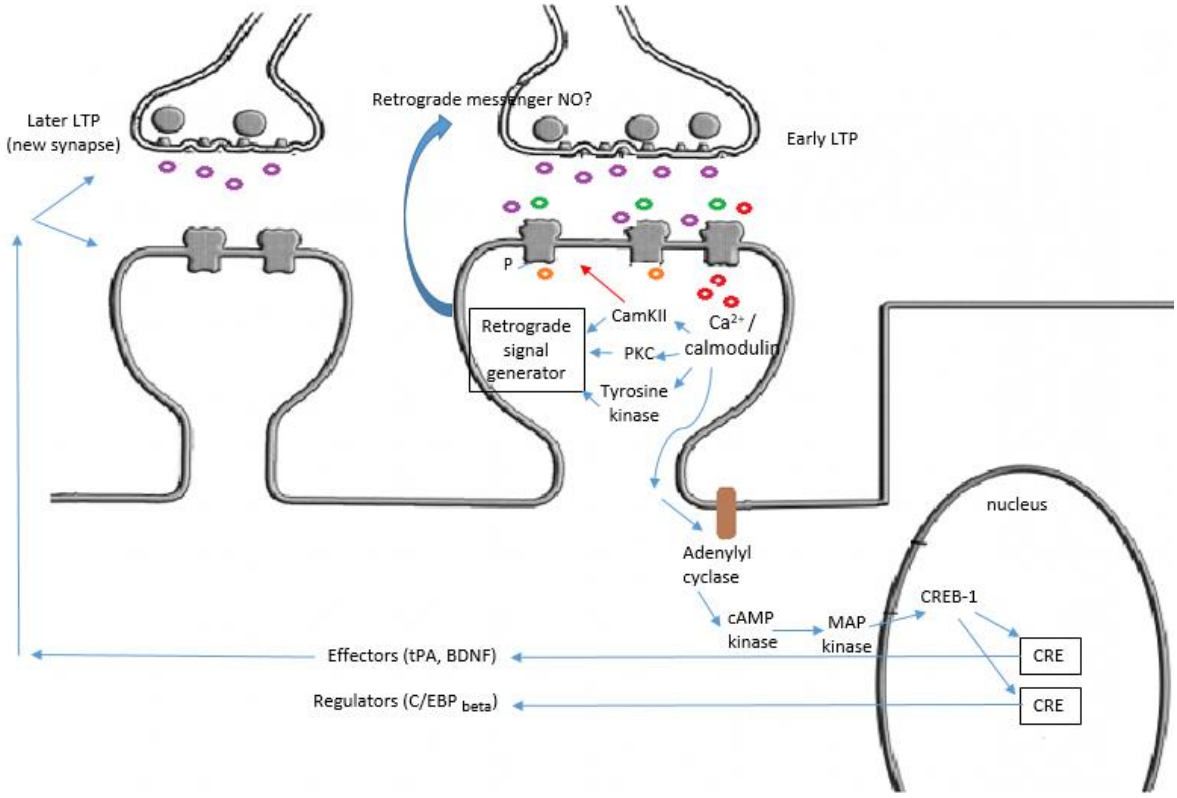

Figure 3- 18. Schematic representation of LTP induced cellular signal transduction pathways. Adapted from Kandel et al. (2012).

The critical rise in intracellular Ca^{2+} as a result of NMDA receptor activation, triggers LTP through a wide range of cellular signal transduction pathways. There are a bewildering number of protein kinases (e.g. PKC, cyclic adenosine 3',5'- monophosphate (cAMP), tyrosine kinase, Src and mitogen-activated protein kinase (MAPK)) involved in LTP (for a review see Lynch, 2004). Despite this number of protein kinases involved in LTP, overwhelming evidence has a suggested an important role of Ca^{2+} activated α-calcium-calmodulin-dependent protein kinase II (CamKII) in initiating LTP effects.

CamKII is one the most abundant proteins in neurons and is expressed pre- and post-synaptically, but expression has been found to be higher in postsynaptic cells where it would be ideally located to respond to changes in calcium concentration (Flink & Meyer, 2002; Lynch, 2004). Activation of CamKII leads to AMPA receptor phosphorylation which ultimately increases AMPA receptor conductance (Barria et al., 1997). CamKII activity continues long after Ca^{2+} signals have returned to baseline, because when CamKII is autophosphorylated at Th^{286} activity is no longer Ca^{2+} dependent (Lisman, 1994). When the kinase is mutated (threonine replaced with alanine) autophosphorylation is prevented and LTP is impaired (Giese et al., 1998). Moreover, CamKII displays pre- and postsynaptic effects (Lynch, 2004; Malenka & Nicoll, 1999). Presynaptically CamKII has been shown to increase phosphorylation of synapsin I and MAP2 (Fukunga et al., 1996). Postsynaptically, CamKII inhibitors have been demonstrated to inhibit LTP (Malenka et al., 1989; Malinow et al., 1989).

To test the hypothesis that sucrose slice AED resistance was due to longer duration of exposure to LTP inducing 0$[Mg]^{2+}$ aCSF, sucrose slices were incubated with the protein synthesis inhibitor, cycloheximide, for 30 minutes prior to 0$[Mg]^{2+}$ aCSF application. No differences between the percentage of cycloheximide treated and sucrose slices that displayed IDs were observed, but there were differences in the latency to first ID. The latency to first ID was considerably longer in cycloheximide treated slices. There were no significant differences between cycloheximide treated and sucrose slices, in terms of IEIs between IDs and duration of IDs in 0$[Mg]^{2+}$ aCSF. These findings suggest that LTP is likely play a role in bootstrapping the generation of IDs, as latency to first ID significantly increases when LTP is blocked, and IDs do not occur in the presence of GF109203X. Complimentary to these findings, chronic pilocarpine epilepsy studies have demonstrated NMDA receptor inhibition with MK-801 prevented the development of epilepsy (Rice & DeLorenzo, 1998). In order for these conclusions to be firmly presented, further investigation showing the links between NMDA receptors and LTP must be made.

There were also no significant differences in terms of percentage of slices that showed resistance to two out of three AED combinations. However, significantly more sucrose slices showed resistance to the LTG+GPT combination in comparison to cycloheximide treated slices. Analysis of AED effects on IEIs between IDs in cycloheximide treated and sucrose slices, further supported the finding of heightened resistance of sucrose slice to the LTG+GPT combination. As cycloheximide treated slices were especially sensitive, to the 'weakest' AEDs, LTG+GPT, these findings further support the notion that LTP is crucial in the establishment of IDs and in the generation of drug resistance.

Overall, cycloheximide treated slices consistently show a much lower resistance to combination AEDs (no more than 20 %), in comparison to non-cycloheximide treated slices of different preparations. These findings coupled with the fact that ID initiation is substantially blocked by the PKC inhibitor, GF109203X, suggests LTP is likely to be involved in seizure initiation and resistance.

3.4 Conclusion

The current study investigated the excitability of standard and sucrose prepared slices. It has previously been demonstrated that differences remain between these two preparations in terms of slice viability, oscillatory stability and induction of LTP. The investigation concluded standard prepared slices are much more excitable than sucrose prepared slices, and immunohistochemistry follow-up investigations suggested this enhanced excitability may due to a poorly preserved inhibitory network in standard slices in comparison to sucrose slices.

As many studies using standard prepared slices in acute models have demonstrated sensitivity to a wide range of AEDs, we further investigated differences between the two

slices preparations to see if differentiations in AEDs could be better identified in sucrose slices, and potentially serve as a better model for assessing drugs targeting DRE. The analysis of several ID parameters, led to the conclusion that there is little difference between sucrose and standard slices in terms of resistance to AEDs. This could possibly be due to extended latency to first ID in sucrose slices, as longer exposure to excitation and LTP inducing 0$[Mg]^{2+}$ aCSF induces more damage in sucrose slices and therefore brings the level of resistance to AEDs seen in these slices to the same as standard slices. Investigation with the protein synthesis blocker, cycloheximide, provided evidence to support this hypothesis as cycloheximide treated slices showed a longer latency to first ID and were less resistant to combination AEDs in comparison to sucrose slices. Additionally, initiation of IDs was blocked in slices treated with the PKC inhibitor, GF109203X. These findings demonstrate the likely role of LTP in ID generation and AED resistance.

Finally, many researchers have highlighted the need to consider assessing AED resistance using different parameters. In this study we concluded IEIs between IDs and duration of IDs held the most predictive power in determining differences between different slices preparations. Even though there were no overtly obvious differences in resistance to AEDs in standard and sucrose slices, sucrose slices evidently better preserve the neuronal networks, which lead to more accurate findings, which has important applications for studies of chronic epilepsy.

Chapter 4 The effects of antiepileptic drugs in layer II of medial entorhinal cortex during epileptogenesis compared to resected epileptic human tissue

4.1 Introduction

4.1.1 Antiepileptic drug effects in acute and chronic models of epilepsy

The discovery of AEDs originally occurred by serendipity, and later the need for preclinical testing of potential AEDs became apparent, hence the common use of pentylenetrazole (PTZ) and maximal electric shock (MES) screening models. The MES model induces seizures by corneal or transauricular electrical stimulation, whereas the PTZ model induces seizures by systematically administering PTZ. Many AEDs focus on restoring, to some extent, the excitation-inhibition balance (Klitgaard, 2005). However, these acute models suffer from several limitations. For example, the MES model has been criticised for being particularly sensitive to drugs which work, at least in part, by blocking Na^+ channels (Meldrum, 1997). Additionally, these models yield false negative results as the MES model is not sensitive to drugs such as LEV and TGB which primarily act through Na^+ independent mechanism (Loscher & Schmidt, 1994). The PTZ model has also been criticised for similar reasons. For example, because PTZ acts as a GABA antagonist, this model suggests VGB and TGB are effective anticonvulsants, but conversely have been shown to be ineffective and even pro-convulsive in some epileptic human patients (Loscher, 2002; Meldrum, 1997).

In a similar fashion to these acute *in vivo* models of seizures, *in vitro* models of seizures (e.g. 0$[Mg]^{2+}$, 4-AP) have also have often found sensitivity to a wide range of AEDs (e.g. CBZ, VPA, PHT, BZDs and barbiturates) (Drier & Heinemann, 1990; Zhang et al., 1995), which promote the development of 'me too' drugs and do not offer insight into the mechanisms of drug resistance or effective treatment as described in section 3.1.

Acute models have led to development of a wide range of AEDs, but these drugs often interfere with normal physiology and therefore result in poor efficacy and safety profiles. Moreover, these models ignore the complex underlying pathophysiology of epilepsy, therefore the prognosis for drug responsive epileptic patients is poor as AEDs do not alter the progression or underlying pathology of epilepsy (Shinnar & Berg, 1996). Furthermore, prognosis for DRE sufferers is even more unsatisfactory, as available AEDs fail to exert antiepileptic effects, and a lack of understanding over the mechanisms that lead to resistance makes overcoming such circumstances with better treatments unlikely. It has been suggested that once a better understanding of the mechanisms underlying drug resistance in epilepsy has been developed, more effective drugs that halt, or even reverse, the process of epileptogenesis can be developed (Loscher, 2002). Several AEDs have been tested in clinical trials for the potential to alter epileptogenesis, and have failed to do so (Temkin, 2001; Temkin et al., 1990), therefore the use of chronic animal models of epilepsy is even more relevant in order to identify truly AEDs.

Chronic models of epilepsy have transformed the development and testing of AEDs by improving treatment outcome predictions and providing better insight into epileptogenesis and DRE mechanisms. One AED that clearly illustrated this point is the identification of LEV as an AED. LEV does not show antiepileptic potential in models with normal animals, but shows a potent antiepileptic effect in chronic epileptic models (Klitgaard, 2001; Klitgaard et al., 1998; Loscher & Honack, 1993). Moreover, LEV has mechanistic actions that are rather different from classic AEDs which generally target Na^{+}, Ca^{2+} and GABA transmission. Specifically, LEV has been shown to inhibit high-voltage activate Ca^{2+} currents (Niespodziany et al., 2001) and suppress inhibitory effects of allosteric modulators of GABA and glycine currents, including zinc and β-carbolines (Rigo et al., 2002). LEV has also been shown to interact with the synaptic vesicle protein 2A (SVA2A) (Bajjalieh et al., 1994; Lynch et al., 2004). SV2A modulates presynaptic neurotransmission by regulating exocytosis of neurotransmitter vesicles. In support, mice lacking SVA2A develop a strong seizure phenotype and usually die within 3 weeks of birth (Lynch et al., 2004).

Another example demonstrating the benefits of chronic models of epilepsy includes the prediction made by the kindling model of epilepsy suggesting clinical failure of NMDA antagonists as an AED, which was suggested by the MES model to be effective (Loscher, 1998; Loscher & Honack, 1991). Additionally, the AEDs diazepam and PHT, have shown opposite effects of superiority as AEDs in acute and chronic models of kindling, with PHT being more effective in the acute model and diazepam being more effective in the chronic model (Pinel, 1983). Although this study has been criticised as acute and chronic models were induced on the same animals, therefore varying levels of tolerance may have been developed, thus confounding the results (Loscher, 2002). Drugs that are effective in the amygdala kindled model have been suggested to be useful in treating complex partial seizures in humans (Loscher et al., 1986; McNamara, 1984), however the mechanism through which certain AEDs aid control of certain types of seizures is less clear.

An acute, *in vivo* study using pilocarpine further demonstrated superiority over classical MES screening models. Following pre-treatment with AEDs pilocarpine was administered and the protective effects were measured by incidence of seizures. Turski (1987) demonstrated, clonazepam (CLO), PHB, VPA and trimethadione (TMD) effectively prevented the incidence of seizure following pilocarpine administration, suggesting the potentiation of GABA was the superimposing mechanism providing protection. However, a similar study has suggested these drugs are not effective at halting pilocarpine induced effects (Morrisett et al., 1987). These contrasting results may have occurred as a result in different treatment protocols (concentration of AEDs and pilocarpine) and time post pilocarpine treatment animals were observed for. For example, Morrisett et al (1987) showed VPA pre-treatment resulted in a longer latency to SE but is unable to stop SE from occurring.

Turski (1987) further showed the AEDs, DPH and CBZ had no effect on preventing the incidence of seizures. Similar findings have been found in the kainate model, *in vitro* (Clifford et al., 1982) and *in vivo* (Fuller & Olney, 1981; Kleinrok et al., 1980). In contrast, MES and PTZ models suggests these drugs are effective (Czuczwar et al., 1982; Turski, 1987). Moreover, ESM and acetazolamide (ACT) significantly exacerbated seizure incidence and brain damage. Other acute pilocarpine studies have demonstrated certain combinations of AEDs (e.g. diazepam and PHB/PHT) work well in reducing pilocarpine induced effects (Lemos & Cavalheiro, 1995).

Studies investigating AED effects in the latent period of chronic KA and pilocarpine models have shown protective effects, in terms of reduced morphological effects and incidence of SRS, of VPA (Bolanos et al., 1998), GPT(Cilio et al., 2001) and LEV (Margineanu et al., 2008) but not VGB (Pitkanen et al., 1999). In contrast there a relatively few studies which investigated AED effects in the chronic phase of pilocarpine and kainate models. This is surprising as some have recognised different mechanisms underlying epileptogenesis and maintenance of SRS, therefore drugs which target prevention of epileptogenesis and drugs which target prevention of SRS may be different (Leite et al., 2002; Silver et al., 1991; Shinnar and Berg, 1996; Loscher, 1998).

One *in vivo* study conducted by Klein et al. (2015) investigating AED effects in chronic epilepsy in the KA model found that six AEDs suppressed secondary generalised convulsive seizures, but focal non convulsive seizures were resistant to CBZ and PHT but not VPA, LEV, PHB and diazepam. These findings support a similar study conducted by Riban et al. (2002). This study went on to investigate resistance of focal seizures in responders and non-responders, and found non-responders were likely to be resistant to more than one AED. From these findings it was suggested this mouse model of KA induced epilepsy is resistant to AEDs which modulate VGSCs, in a similar fashion to the 6-Hz mouse model of difficult to treat partial seizures (Barton et al., 2001; Bankstahl et al., 2013), but was sensitive to drugs with GABAergic mechanisms.

Whilst the study by Klein et al. (2015) suggests the KA model of chronic epilepsy was useful in the testing of DRE, it suffers from several limitations. Firstly, resistance was seen only in non-convulsive focal seizures, not in secondarily convulsive seizures that are often seen in patients of DRE to a devastating degree. Secondly, this studied identified resistance in terms of response to singly administered AEDs, whereas the definition of DRE clearly identifies a failure to respond to two or more AEDs (Berg et al., 2001; Cowan 2002; Kwan & Brodie, 2006). Finally, the presence of high frequency electrographic seizures may be regarded as a strength for assessing AEDs, but it could be questioned whether such a severe seizure state is accurately imitating the human condition and variances are likely to result in differences of AED response too. As with many *in vivo* studies, in this study there also lies the possibility

that differences between responders and non-responders could stem from subtle differences associated with injection site of KA.

Overall, it is evident that because chronic models of epilepsy are more prototypical of underlying pathophysiology of epilepsy in comparison to acute models, they are consequently better equipped to make accurate predictions and test the efficacy of AEDs. Having said this, relatively few studies have explored how response to AEDs change over the course of epileptogenesis or directly addressed whether the nature of epileptogenesis is continuous or not. Moreover, while several different studies have explored AED efficacy at different stages of the chronic KA model of epilepsy, relatively few have been carried out on the pilocarpine model. Additionally, studies investigating AED effects during different stages of epileptogenesis often suffer from limitations such as employing high severity models and classifying resistance based on the response to individual AEDs rather than combinations of AEDs. For these outlined reasons it is the primary aim the current study to investigate the efficacy of combinations of AEDs during epileptogenesis of the refined Li-pilocarpine model, in the MEC.

4.1.2 Antiepileptic drug effects in human patients

One of the fundamental aims of research with chronic animal models of TLE, is to identify mechanisms and drugs which effectively prevent epilepsy in humans. However, it has been demonstrated that there are marked differences that exist between different chronic models of epilepsy in terms of underlying pathophysiology and response to different AEDs. Whilst the amygdala-kindling model has been suggested to be useful for assessing DRE (Loscher, 1997), current knowledge makes it difficult to identify which model is the most appropriate for studying epilepsy. However, as epilepsy generally has multi-factorial aetiologies many models are likely to be relevant and results from different models should all be considered to avoid false negative or positive predictions (Loscher, 2002).

Treiman (1987) assessed the findings of 20 randomised, double-blind, controlled clinical trials assessing AED effects in severely afflicted medically intractable patients. It was found that there were no significant differences in the efficacy of AEDs tested, but all worked significantly better than placebo, and combinations of AEDs worked better than administration of single AEDs in some studies.

Other clinical studies have attempted to use AEDs early on to prevent epileptogenesis, in a similar fashion to some acute animal models tested *in vivo*. Typically in these trials, at risk individuals are identified and treatment is started 12 hours-1 month post injury. Participants are monitored for 6 months to 3 years for seizure occurrence, and some include a period of monitoring after the drug has been stopped, this is when the antiepileptogenesis effect can be examined.

These studies have generally shown PHT (Temkin et al., 1990; Young et al., 1983), PHB (Manaka, 1992), CBZ (Glotzner et al., 1983), VPA (Temkin et al., 1999), Mg^{2+} (Temkin et al., 2007) cannot reliably prevent or suppress epileptic seizures after traumatic brain injury. The findings contrast *in vivo* investigations on AEDs administered during the latent period which have shown VPA protects against spontaneous seizures (Bolanos et al., 1998).

Besides these negative results, these studies have also been illustrated to have substantial limitations. For example, some studies were not blinded, studies had short periods of observation after the drug was stopped, monitoring of compliance or test drug concentrations were not carried out and EEG monitoring of subclinical seizures was not evaluated. Moreover, as drugs were administered early on it is difficult to differentiate effects of seizure suppression and antiepileptogenesis effects. Most of the drugs tested were older generation AEDs, therefore the need to test second generation AEDs in laboratories and, if successful, in clinical trials needs to be evaluated (Temkin, 2009).

Only a few studies have assessed whether drugs which are ineffective in in the treatment of epileptic patients, are also ineffective epileptic human resected tissue *in vitro*. Whilst it may seem more important to evaluate mechanistic actions showing why AEDs do not work in patients, through *in vitro* investigation, this consistency issue is an important one that needs to be addressed in order understand the limits of *in vitro* experimentation. *In vitro* studies exploring AED effects in human resected epileptic tissue have demonstrated 0$[Mg]^{2+}$ aCSF induced IDs are not very sensitive to CBZ, but are sensitive to VGB (Musshoff et al., 1997; Musshoff et al., 2000). In support, Kohling et al. (1998) illustrated CBZ and PHT fail to suppress epileptiform activity. However, it is important to note epileptiform activity in these studies were more representative of LRDs and IIDs, which have been shown to behave in more resistant ways in comparison to IDs in rodent tissue (Drier & Heineman, 1990; Sokolova et al., 1998; Zhang et al., 1995).

Epilepsy is a disease which functionally arises from excessive neuronal excitation, therefore *in vivo* and *in vitro* methods are the ‘gold standard’ scientific approach for investigating mechanisms of pharmaco-resistance (Jones et al., 2016). Our laboratory is one of a prestigious few that has access to studying resected epileptic tissue *in vitro*. As explained by Jones et al. (2016) *in vitro* work with human tissue involves a fair share of complexities surrounding the collection, preparation and storage of human tissue. Additional complexities surround the ability to induce ictal-like events in this type of tissue. However, there is a pressing need for the investigation of AEDs in human tissue and comparison to animal models of epilepsy, which would provide insights into how to improve animal models and mechanisms of resistance. Based on the issue outlined, it is the secondary aim of this study to compare AED effects in the resected epileptic human tissue and epileptic rodent tissue from refined Li-pilocarpine model.

4.2 Results

4.2.1 Neuronal network excitability and the efficacy of AEDs in MEC layer II during epileptogenesis

4.2.1.1 Excitability of layer II of the MEC during epileptogenesis compared to controls

Sucrose perfused transverse hippocampal-entorhinal slices were prepared after SE induction using the Li-pilocarpine model at four stages of epileptogenesis: 24hrs, 1 week, 5 weeks and 12 weeks+. Slices were also prepared from aged-matched controls. Epileptogenesis was initiated using the refined Li-pilocarpine model 1-3 days post weaning (postnatal day 21 - 24) (see section 2.2.1 for more details).

To induce IDs, a 0$[Mg]^{2+}$ aCSF perfusate was applied to all slices. To investigate differences in excitability a between-subjects ANOVA was conducted, which showed no significant interaction effect between status (SE/control) and age (24 hrs/1 week/5 weeks/12 weeks+), $p > 0.05$ (see figure 4-1A and table 4.1). There was a significant main effect of status F (1, 59) = 6.75, $p < 0.05$. Specifically, post-hoc analysis demonstrated a higher percentage of SE slices (48.60 %) showed IDs in comparison to controls (34.31%), $p < 0.05$. There was no significant main effect of age, $p > 0.05$.

To investigate differences in excitability between epileptic and control slices, in terms of latency to first seizure, a between-subjects ANOVA was conducted. Again, there was no significant interaction between status and age, $p > 0.05$ (see figure 4-1B and table 4.1). There was a main effect of status, F (1, 151) = 103.65, $p < 0.01$. Post-hoc analysis showed latency to first seizure was significantly shorter in SE slices (63.11 ± 3.26 ranked seconds) irrespective of stage of epileptogenesis, in comparison to control slices (128.95 ± 5.60 ranked seconds), $p < 0.01$. This analysis was conducted on ranked data, to avoid inaccuracies as a result of violations of the normality assumption.

Table 4.1. The percentage of slices with IDs and latency to first ID in SE and control slices at different stages of epileptogenesis.

		24hrs	1week	5weeks	12weeks+
Percentage	**control**	30.56	40	47.92	18.75
	SE	53.57	47.73	48.04	45.05
Latency	**control**	4801.50 (± 523.62)	8310.92 (± 1118.78)	8086.40 (± 1033.69)	7173.13 (± 678.05)
	SE	2795.10 (± 250.60)	2505.53 (± 238.81)	2980.50 (± 288.71)	3290.49 (± 231.63)

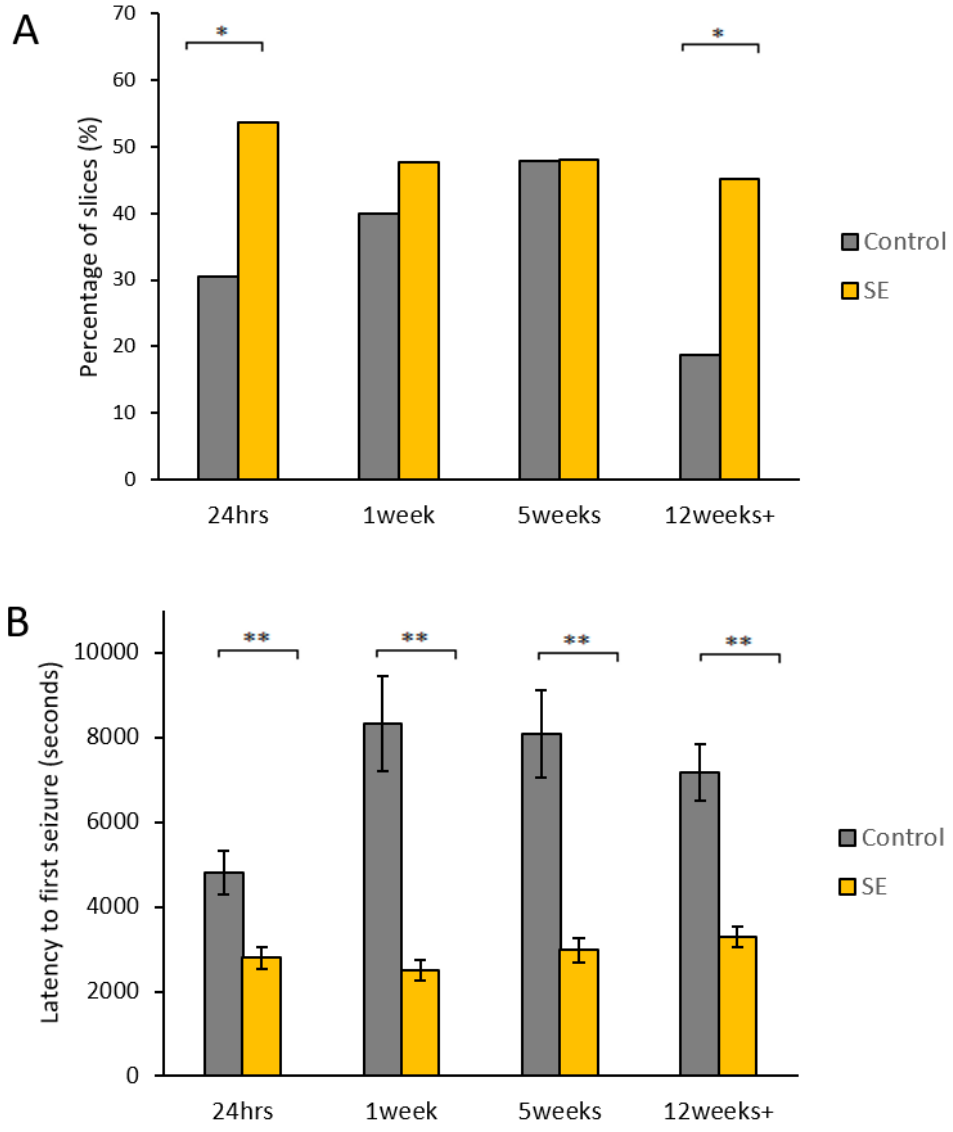

Figure 4-1. The excitability of layer II MEC in control and epileptic slices at different stages of epileptogenesis. A. The percentage of control and epileptic slices which showed IDs. B. Latency to first seizure in control and epileptic slices.

There are clear differences between epileptic and control slices at all stages of epileptogenesis. Once IDs had been initiated, they could reliably last for 2.5 hours at all stages. The next section assesses AED effects in epileptic slices during epileptogenesis. The comparison of AED effects in epileptic and control slices during epileptogenesis was not carried out. Not only was it difficult to induce IDs in control tissue, making the ethics behind using such a large number of animals questionable, but the IEIs between IDs in control tissue was often very long and extremely variable.

To gain further insight into excitability changes, we explored ID parameter changes in 0$[Mg]^{2+}$ aCSF during different stages of epileptogenesis. A one-way between subjects ANOVA showed there were significant differences in the IEIs between IDs at different stages of epileptogenesis, F (3, 111) = 8.78, p < 0.01. Post-hoc analysis showed IEIs at 24hrs post SE

induction (1055.51 ± 133.73 seconds) was significantly greater in comparison to 1 week (465.35 ± 60.84 seconds), 5 weeks (552.48 ± 85.58 seconds) and 12 weeks+ post SE (449.55 ± 68.51 seconds), $p < 0.01$, as illustrated in figure 4-2.

To explore the effects of durations of IDs in 0$[Mg]^{2+}$ aCSF, at different stages of epileptogenesis, a one-way between subjects ANOVA was conducted on raw data. There were significant differences in the durations of IDs at different stages of epileptogenesis, $F(3, 115) = 5.26$, $p < 0.01$. Post-hoc analysis showed the durations of IDs at 5 weeks post SE (81.04 ± 3.72 seconds) were significantly longer in comparison to durations of IDs at 24hrs (63.19 ± 2.90 seconds), 1 week (64.05 ± 2.90 seconds) and 12 weeks+ post SE (66.98 ± 4.41 seconds), $p < 0.05$.

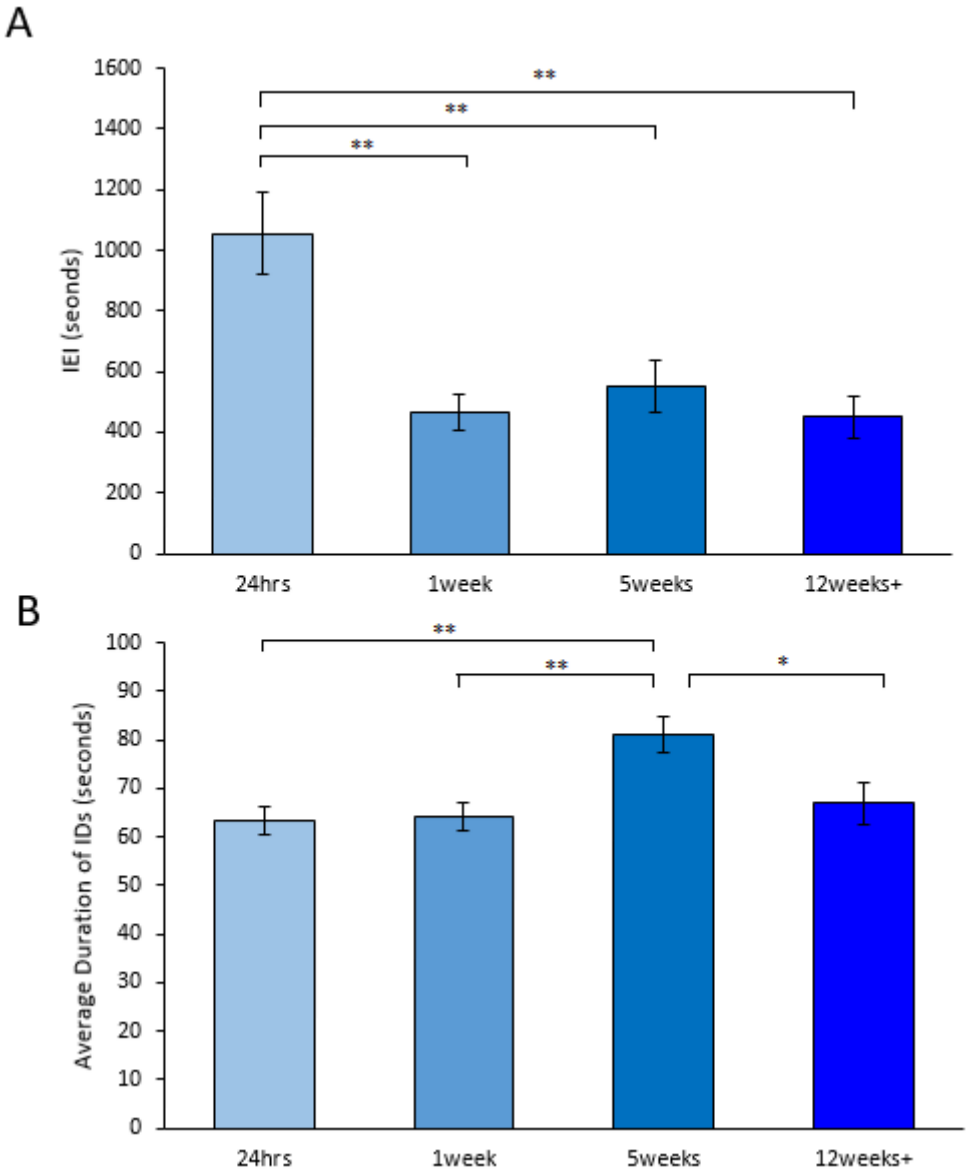

Figure 4-2. ID parameter changes in 0[Mg]$^{2+}$ aCSF during epileptogenesis. A. A bar graph demonstrating the average IEI between IDs at different stages of epileptogenesis. B. Bar graph demonstrating average duration of IDs at different stages of epileptogenesis.

4.2.1.2 The effects of AEDs on ID parameters during epileptogenesis

To provide further insight into changes in efficacy of AEDs during epileptogenesis, a mixed model ANOVA was conducted to investigate changes of IEI with different drug combinations at different stages of epileptogenesis. A box cox transformation was applied to the data to overcome violations of normality (see Appendix 7 for raw data). The box cox transformation is an algorithm applied to non-normally distributed data to identify the ideal transformation to be applied to the data in order to normalise it. A value of lambda (-2 to +2) which minimises standard deviation is calculated. Different transformations are applied depending on the value of lambda, as shown in table 4.2.

Table 4.2. Different data transformations according to the box-cox transformation lambda value

λ	Transformation
-2	$Y^{-2} = 1/Y^2$
-1	$Y^{-1} = 1/Y^1$
-0.5	$Y^{-0.5} = 1/(Sqrt(Y))$
0	log(Y)
0.5	$Y^{0.5} = Sqrt(Y)$
1	$Y^1 = Y$
2	Y^2

There was no significant interaction effect between stage of epileptogenesis and drug combination $p > 0.05$, indicating there was no superiority of AED efficacy, in terms of IEI changes, at different stages of epileptogenesis.

There was a main effect of drugs (i.e. from 0$[Mg]^{2+}$, to first AED and second AED) F (2, 182) = 69.83, $p < 0.01$. Post hoc analysis showed IEIs were significantly shorter in 0$[Mg]^{2+}$ aCSF, compared to first application AEDs and second application of AEDs. Additionally, IEIs of first application of AEDs were significantly shorter than second applications of AEDs, $p < 0.01$, irrespective of specific drug combination and stage of epileptogenesis. As illustrated in figure 4-3 all AEDs had were effective in increasing IEIs between IDs at every stage of epileptogenesis.

There was no significant main effect of stage of epileptogenesis on IEIs, $p > 0.05$, indicating efficacy of AEDs did not change during epileptogenesis in terms of IEIs. There was a significant main effect of drug combination, F (10, 182) = 2.15, $p < 0.05$. Post-hoc analysis showed, irrespective of stage of epileptogenesis, application of the TGB+CBZ combination produced significantly greater increases in IEIs between IDs, in comparison to LTG+GPT and CBD+CBZ, $p < 0.05$, suggesting AEDs of GABAergic mechanisms are more effective in the refined Li-pilocarpine model of epilepsy.

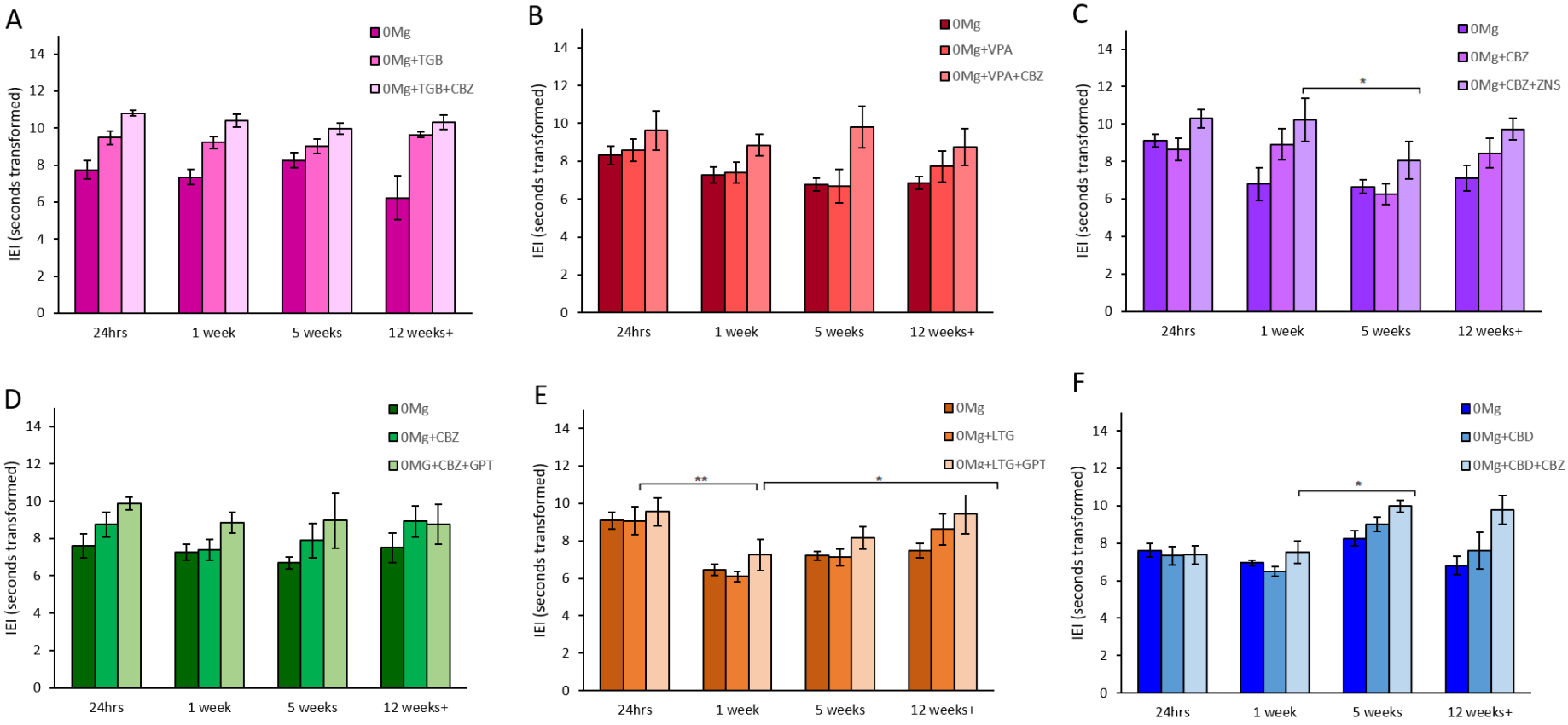

Figure 4-3. The effects of AEDs on IEIs during epileptogenesis. A. The effect of TGB+CBZ on IEIs at different stages of epileptogenesis. B. The effect of VPA+CBZ on IEIs at different stages of epileptogenesis C. The effect of CBZ+ZNS on IEIs at different stages of epileptogenesis D. The effect of CBZ+GPT on IEIs at different stages of epileptogenesis D. The effect of LTG+GPT on IEIs at different stages of epileptogenesis F. The effect of CBD+CBZ on IEIs at different stages of epileptogenesis. There was no superiority of specific AEDs at specific stages of epileptogenesis. Irrespective of stage of epileptogenesis TGB+CBZ had the greatest effect at increasing IEIs.

Exploring AED effects on other parameters, a mixed model ANOVA was conducted to investigate the effects of combination AEDs on the duration of IDs during different stages of epileptogenesis. Although, normality was significantly skewed according to the Shapiro Wilk's test and Kolmogorov-Smirnov test, a box cox transformation did not significantly improve the distribution of data. Additionally, examination of the histograms did not show large variations from normal distribution, therefore the following analysis has been carried out raw data. Moreover, the Box's test of equality of covariance ($p > 0.05$) on raw data indicated, this assumption had not been violated, therefore the analysis was accurate and reliable.

The mixed model ANOVA analysis demonstrated there was no significant interaction, between stage of epileptogenesis and drug combination on the duration of IDs, $p > 0.05$ (see figure 4-4). There was no main effect of stage of epileptogenesis on duration of IDs, $p > 0.05$. There was a significant main effect of drug application, F (2, 52) =3.64, $p < 0.05$. Post hoc analysis illustrated the pooled durations of IDs in 0$[Mg]^{2+}$ aCSF were significantly longer in comparison to the pooled durations of IDs after first and seconds application of AEDs, $p < 0.05$. There were no significant differences in the pooled durations of IDs from first to second application of AEDs, $p > 0.05$. There was a significant main effect of drug combination on the duration of IDs, F (10, 52) = 2.79, $p < 0.01$. Post-hoc analysis however failed to illustrate significant differences between different drug combinations, $p > 0.05$.

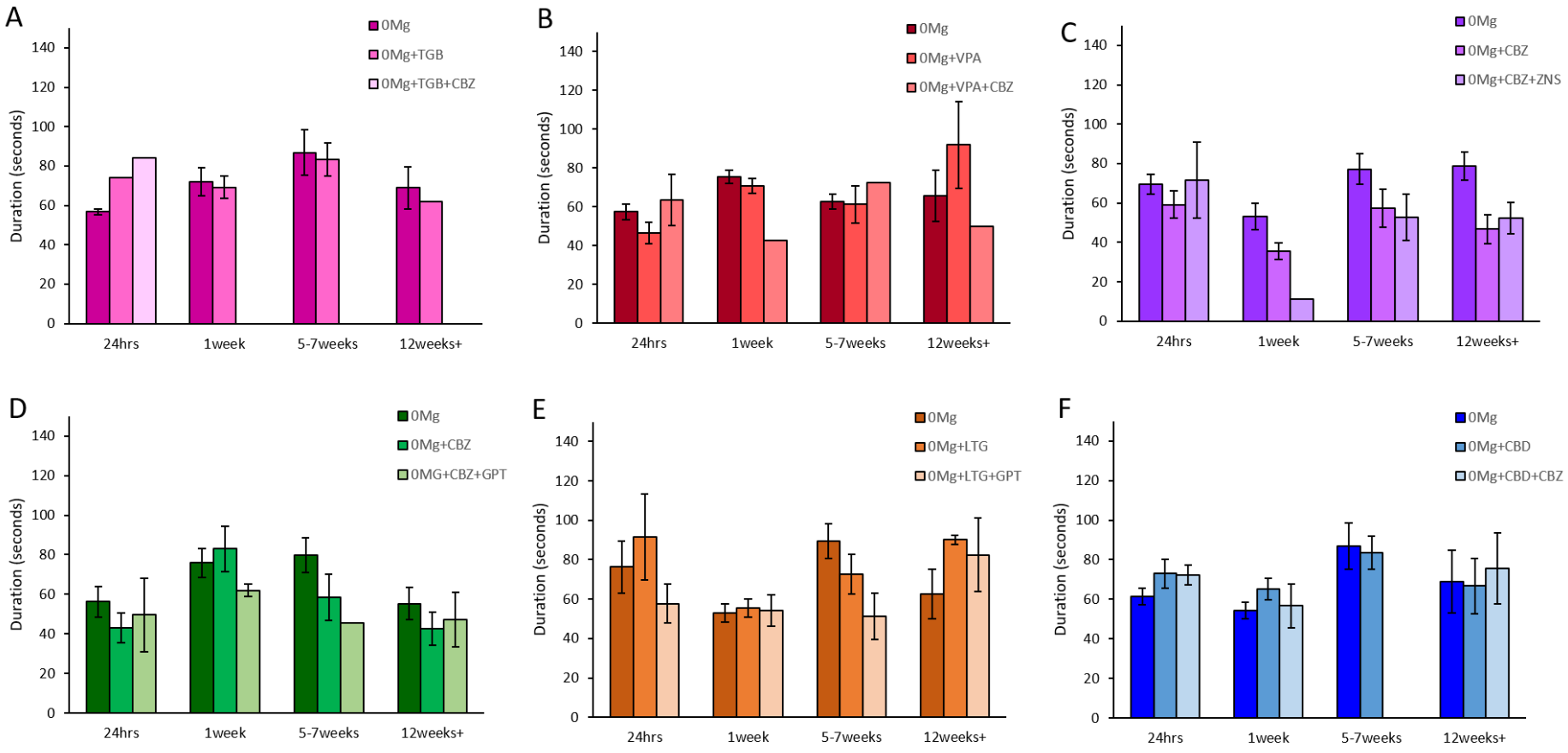

Figure 4-4. The effects of AEDs on the duration of IDs during epileptogenesis. A. The effect of TGB+CBZ on the duration of IDs at different stages of epileptogenesis. B. The effect of VPA+CBZ on the duration IDs at different stages of epileptogenesis C. The effect of CBZ+ZNS on the duration of IDs at different stages of epileptogenesis D. The effect of CBZ+GPT on the duration of IDs at different stages of epileptogenesis D. The effect of LTG+GPT on the duration of IDs at different stages of epileptogenesis F. The effect of CBD+CBZ on the duration of IDs at different stages of epileptogenesis. There was no superior effect of specific AEDs on the duration of IDs at different stages of epileptogenesis.

4.2.1.3 Resistance to combination AEDs in MEC layer II during epileptogenesis

As explained in chapter 3 (section 3.2.4.1) resistance to combination AEDs can be analysed using two definitions of resistance. To test the association between different stages of epileptogenesis (24 hrs, 1 week, 5 weeks and 12 weeks) and pooled resistance to combination AEDs (0 = not resistant, 1 = resistant), a chi-square test of independence was conducted. Figure 4-5A illustrates pooled data showing the percentage of slices that showed resistance to combination AEDs at 24 hrs post SE (33.3 %), 1 week post SE (27.5%), 5 weeks post SE (19.6 %) and 12 weeks+ post SE (19.6 %). There is no significant association between different stages of epileptogenesis and resistance to combination AEDs χ^2 (3, n = 120) = 4.41, $p > 0.05$.

The following analysis investigated if there were differences in resistance to specific AEDs at different stages of epileptogenesis using the first definition of resistance (continued presence of IDs) (see figure 4-5B). A chi square test of independence was conducted for each stage of epileptogenesis. There were no significant association between resistance and different AED combinations at 24 hrs post SE induction, χ^2 (5, n = 30) = 10.25, $p > 0.05$. There was a significant association between resistance and AED combination at 1 week post SE induction, χ^2 (5, n = 31) = 14.71, $p < 0.05$. Post-hoc inspection of adjusted residuals showed 100% of slices responded to TGB+CBZ (adjusted residual = > +/-1.96). There was a significant association between resistance and AED combinations at 5 weeks post SE induction, χ^2 (5, n = 31) =13.92, $p < 0.05$. Post hoc inspection of adjusted residuals showed CBZ+ZNS significantly contributed to this association, as 66.7 % of slices were resistant. LTG+GPT also contributed significantly to this association as 80 % of slices were resistant to these AEDs. CBD+CBZ was also maximally effective at this stage of epileptogenesis, as 100% of slices were susceptible. This finding is consistent with analysis of the ID IEIs. These results suggest CBD+CBZ is most effective during the latter latent period, and may be influential as a tool for preventing epileptogenesis. There was no significant association between resistance and AED combinations during chronic epilepsy (12 weeks+ post SE induction), χ^2 (5, n = 28) = 4.48, $p > 0.05$.

The final analysis investigated if there were differences in resistance to specific AEDs at different stages of epileptogenesis using the second definition of resistance (< 50 % reduction of IDs = resistant) (see figure 4-5C). There was a significant association between resistance and different AED combinations at 24 hours post SE induction χ^2 (5, n = 29) = 12.41, $p < 0.05$. Post hoc inspection of adjusted residuals showed CBD+CBZ significantly contributed to this association as 80 % of slices were resistant. There was a significant association between resistance and different AED combinations at 1 week post SE induction χ^2 (5, n = 30) = 11.67, $p < 0.05$. Post hoc inspection of adjusted residuals failed to show significant contribution, but 80 % of slices showed resistance toward LTG+GPT and CBD+CBZ. Additionally, the majority of slices were responsive (not resistant) towards

VPA+CBZ (80 %) and TGB+CBZ (100 %). There was a significant association between resistance and different AED combinations at 5 weeks post SE induction χ^2 (5, n = 30) = 12.86, $p < 0.05$. Post hoc inspection of adjusted residuals showed LTG+GPT significantly contributed to this association as 80 % of slices were resistant. Also of interest, was the fact that 100% of slices were not resistant towards CBD+CBZ and TGB+CBZ. There was no significant association between resistance and AED combinations during chronic epilepsy (12 weeks+ post SE induction), χ^2 (5, n = 29) = 4.62, $p > 0.05$.

Comparisons of the analyses of resistance to combination AEDs during epileptogenesis suggests, similar results can be produced, although the latter definition (< 50 % reduction of IDs = resistant) is more sensitive to identifying differences in resistance. Both analyses highlight that CBD becomes more effective as epileptogenesis progresses, with optimal response being seen at 5 weeks post SE. Additionally, TGB+CBZ is very effective at all stages of epileptogenesis, whereas LTG+GPT appears to be the least potent combination AEDs.

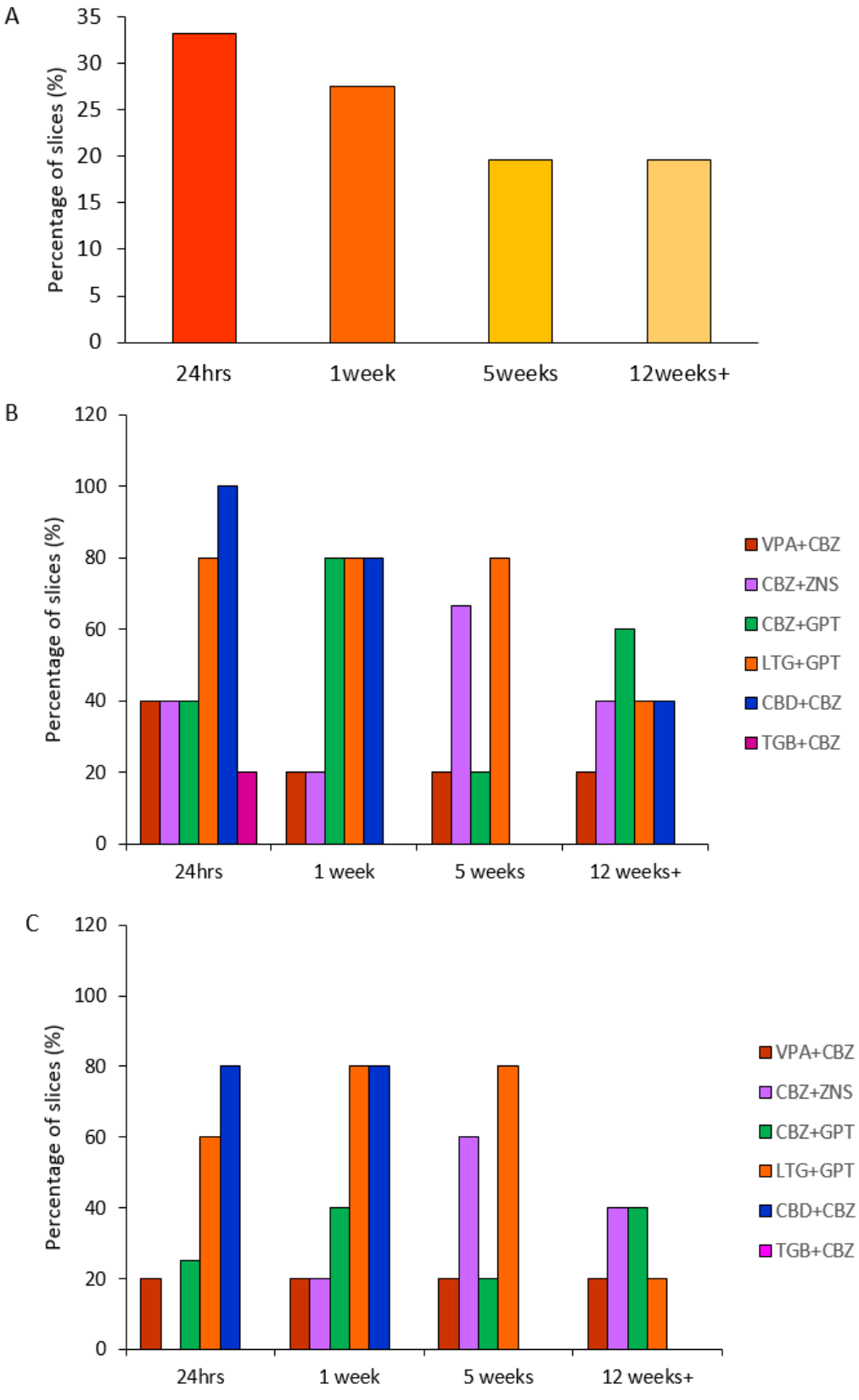

Figure 4-5. Resistance to AEDs during epileptogenesis. A. Pooled data showing the percentage of slices that showed resistance to combination AEDs at 24 hrs post SE, 1 week post SE, 5 weeks post SE and 12 weeks+ post SE. B. Percentage of slices that showed resistance to AED combinations at different stages of epileptogenesis. C. Percentage of slices that showed resistance (<50 % reduction of IDs) to AED combinations at different stages of epileptogenesis.

4.2.2 Comparisons to the Piriform and Entorhinal cortices

4.2.2.1 Differences in the excitability of the PC and EC

Epilepsy is a complex disorder and the EC is not the only site reported to show early dysfunction. As highlighted in section 1.1.1 one of the main cortical inputs to the MEC comes from the piriform cortex (Agster & Burwell, 2009), and also happens to be another site which shows early signs of dysfunction following SE (Gavrilovici et al., 2012; Loscher & Ebert, 1996; Raccine et al., 1988). Similar investigations into the excitability and efficacy of AEDs of the piriform cortex have also been conducted in our laboratory by Jane Pennifold (Pennifold, 2016). A summary of our combined work showing differences in excitability and resistance between the EC and PC can be found in figure 4-6. It is important to acknowledge that these results were obtained from different animals whereby EC slices were prepared using a sucrose based aCSF and PC slices were prepared using a choline based aCSF. Different solutions were utilised to optimise viability of slices for the two different areas *in vitro,* and so loose rather than direct comparisons shall be made between the two brain areas to avoid conclusions being hindered by confounding variables associated with differing homeostatic environments.

To investigate excitability in the PC, in terms of percentage of slices that displayed IDs, a between-subjects ANOVA was conducted. There was no significant interaction between status (control/ SE) and stage of epileptogenesis, therefore excitability did not significantly differ as epileptogenesis progressed, $p > 0.05$ (see figure 4-6). There was a main effect of status F (1, 128) = 4.19, $p < 0.05$. Post-hoc analysis indicated, irrespective of stage of epileptogenesis, more SE slices displayed IDs so were more excitable in comparison to control slices, $p < 0.05$. These PC results are similar to those of EC, whereby significant differences in percentage of slices that displayed IDs were found between SE and control slices, but excitability did not alter between SE and control according to stage of epileptogenesis (see figure 4-1A or 4-6B for comparison). Comparison of figure 4-6A and 4-6B also illustrate the EC is not as excitable as the PC in controls and SE slices.

To investigate excitability in the PC, in terms of latency to first seizure, a between-subjects ANOVA was conducted. There was a significant interaction between status (control/ SE) and stage of epileptogenesis, F (3, 328) = 3.49, $p < 0.05$. Post-hoc analysis showed latency to first ID was significantly shorter in SE slices at 5 weeks post SE, in comparison to controls, $p < 0.01$. Whilst no overall significant interaction between status and stage of epileptogenesis was found in the EC, it was evident there were significant differences between SE and control slices at every stage of epileptogenesis (see figure 4-1B or 4-6D for comparison). Comparison of figure 4-6C and 4-6D show overall the latency to first seizure is greater in EC compared to the PC for control and SE slices, thus showing the EC slices are not as excitable.

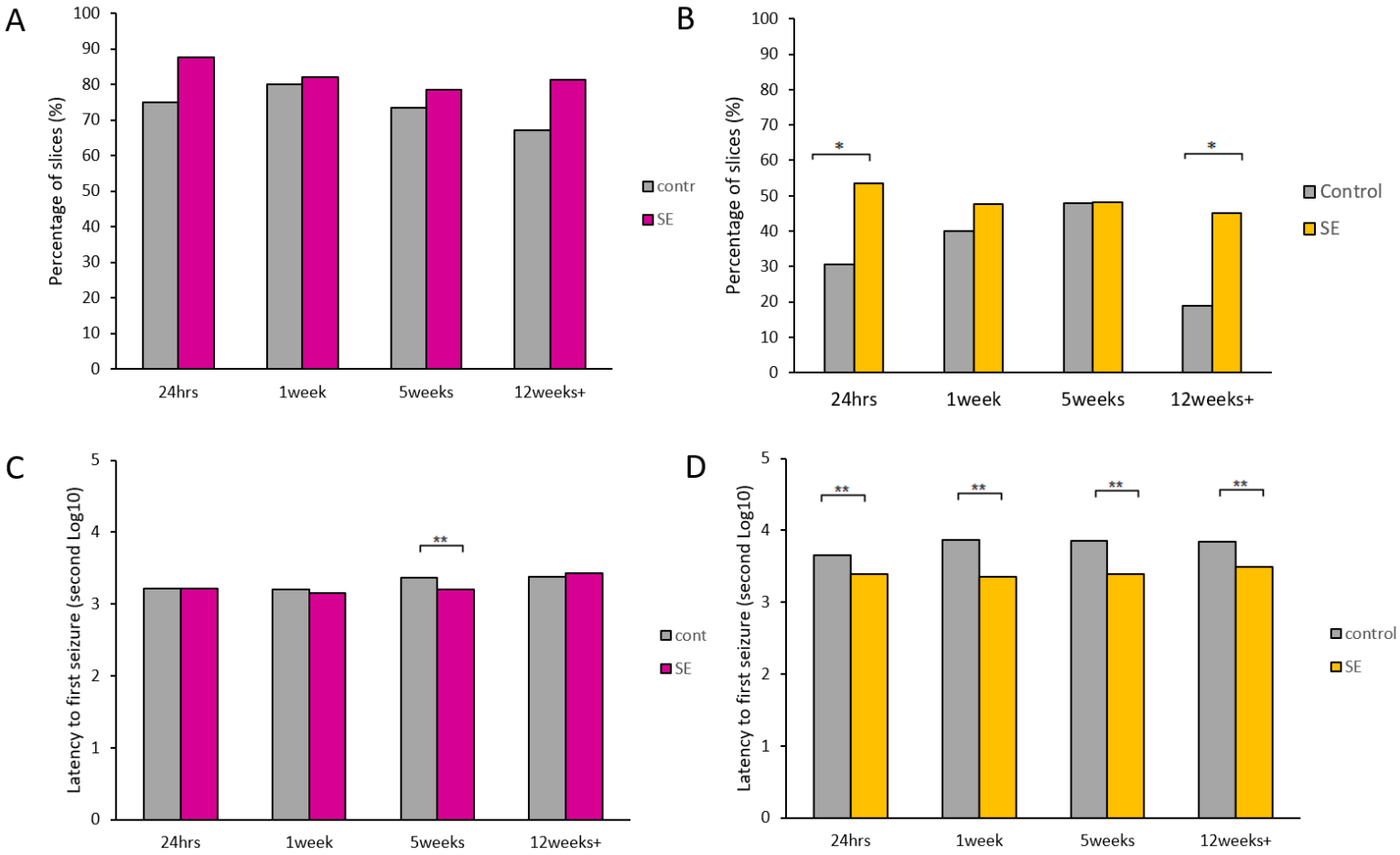

Figure 4-6. The excitability of the PC and EC in control and epileptic animal during epileptogenesis. A. The percentage of control and SE slices that showed IDs in the PC at different stages of epileptogenesis. B. The percentage of control and SE slices that showed IDs in the EC at different stages of epileptogenesis. C. The latency to first seizure in control and SE slices of the PC at different stages of epileptogenesis. D. The latency to first seizure in control and SE slices in the EC during different stages of epileptogenesis.

4.2.2.2 Resistance to combination AEDs during epileptogenesis in the EC and PC

To test the association between different stages of epileptogenesis (24 hrs, 1 week, 5 weeks and 12 weeks) in PC SE slices and pooled resistance (continuation of IDs following the application of 2 AEDs) to combination AEDs (0 = not resistant, 1 = resistant), a chi-square test of independence was conducted. There was a significant association between different stages of epileptogenesis and resistance to combination AEDs χ^2 (3, n = 191) = 33.65, $p <$ 0.01. Post-hoc inspection of the adjusted residuals showed 24 hr SE slices significantly contributed to the association having the most slices with resistance (40.4 %). SE slices at 5 weeks (15.7 %) and 12 weeks+ (13.5 %) post SE induction also significantly contributed to the association. Whilst differences in pooled resistance can be seen the PC SE slices this difference is not evident in the EC. Further comparison of figure 4-7A and 4-7B also shows pooled resistance in both the PC and EC decreases as epileptogenesis progresses but more so in the PC than EC.

A chi square test of independence was conducted for each stage of epileptogenesis to assess if there were differences in resistance to specific AEDs in PC slices. From the four chi-square tests, there was a significant association between resistance and AED combinations at 5 weeks post SE, χ^2 (5, n = 48) = 22.59, $p < 0.01$. Post-hoc inspection of the adjusted residuals showed VPA+CBZ, LTG+GPT and TGB+CBZ significantly contributed to the association. PC slices at 5 weeks post SE showed the greatest resistance to TGB+CBZ (50 %) and no resistance toward VPA+CBZ and LTG+GPT. Interestingly in comparison the EC showed greater resistance to LGT+GPT and CBZ+ZNS at 5 weeks. Additionally, differences in resistance to specific AED combinations can also be seen at 1 week post SE in EC slices. Further comparison of figure 4-7C and 4-7D, highlights that resistance dips in both the EC and PC at 5 weeks post SE.

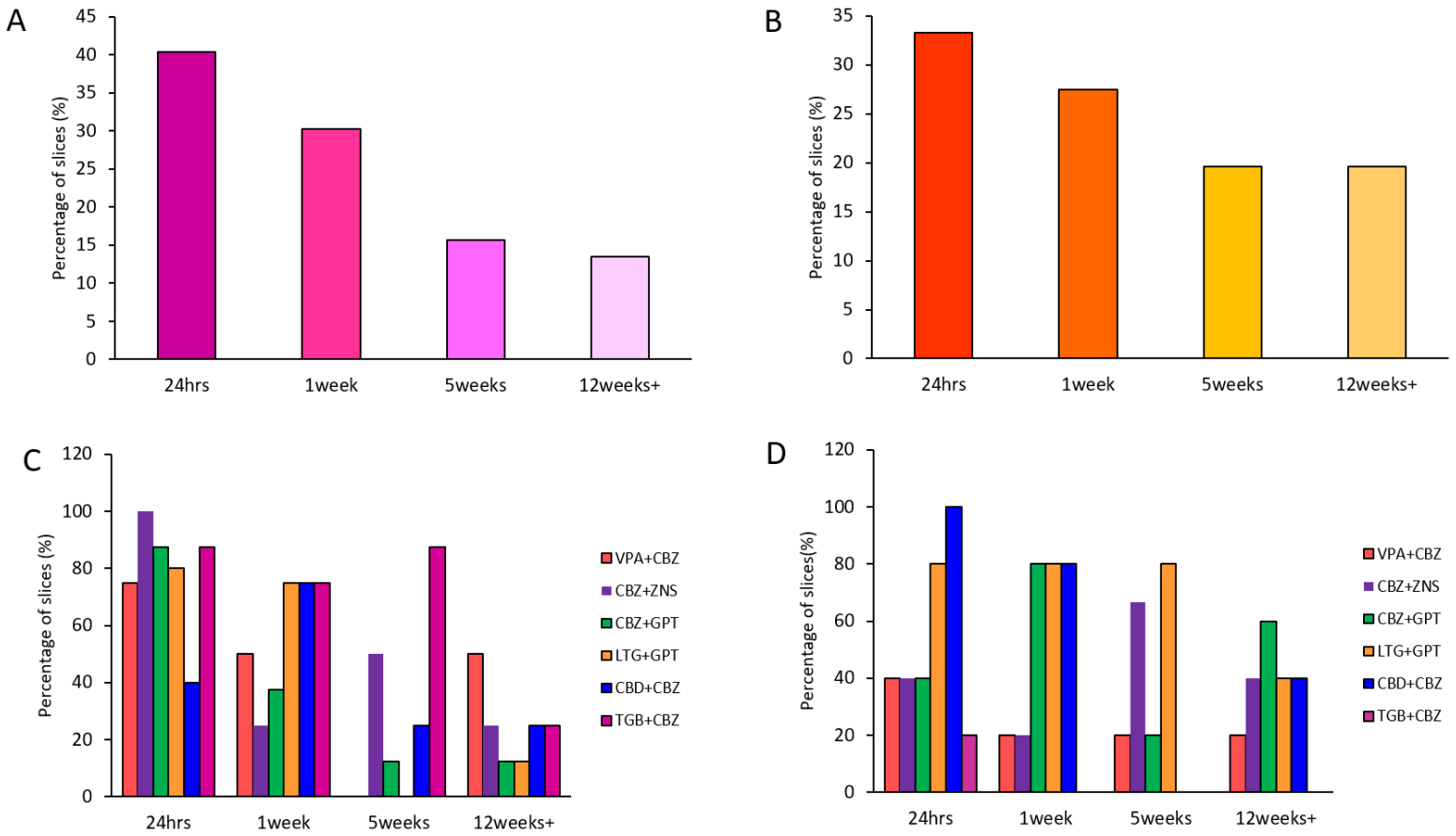

Figure 4-7. Resistance to combination AEDs during epileptogenesis in the piriform and entorhinal cortex. A. Pooled resistance to combination AEDs in the PC at different stages of epileptogenesis. B. Pooled resistance to combination AEDs in the EC at different stages of epileptogenesis. C. Resistance to specific combination AEDs in the PC at different stages of epileptogenesis. D. Resistance to specific combination AEDs in the EC at different stages of epileptogenesis.

4.2.3 Effects of combination AEDs in resected human tissue

Results presented thus far have illustrated slices in which the epileptogenesis process has been initiated are overall more excitable in comparison to control slices. Whilst AED combinations do not show variable efficacy at different stage of epileptogenesis, TGB+CBZ is the most effective AED combination in the EC overall, whilst the converse relationship is shown in the PC. Similarly, LTG+GPT is the least potent AED combination in the EC, but the more effective in the PC. CBD+CBZ has shown greater potency with development of epilepsy in both the EC and PC.

The secondary aim of this study was to compare AED effects in the resected epileptic human tissue and epileptic rodent tissue from refined Li-pilocarpine model, in the hope to better understand mechanisms of resistance and improve models of epilepsy by identifying similarities and differences in response to AEDs. Table 4.3 summarises the types of resected human tissue used in these investigations (n slices: temporal lobe = 9, frontal lobe = 3, motor cortex = 1).

Table 4.3. Summary of human resected tissue used in investigation comparing the efficacy of combination AEDs in human and animal models of temporal lobe epilepsy.

Patient	Human tissue with induced IDs
1	Motor cortex
2	Temporal gyrus and amygdala
3	Sensory motor cortex
4	Medial temporal gyrus and amygdala
5	Inferior frontal gyrus
6	Left parietal gyrus
7	Sensory motor cortex
8	Temporal lobe
9	Medial temporal gyrus

4.2.3.1 Excitability of resected human tissue

To investigate differences in excitability between epileptic rodent and human tissue, in terms of percentage of slices that showed IDs, a Mann-Whitney-U test was conducted. There were significant differences between epileptic rodent and human tissue, $U = 20.50$, $z = -2.09$, $p < 0.05$. A higher percentage of epileptic human tissue slices (76.78 ± 11.06 %) displayed IDs in comparison to chronically epileptic rodent tissue (45.05 ± 3.35 %), as illustrated in figure 4-8C.

There were significant differences between rodent and human tissue, in terms of latency to first seizure, $U = 19.00$, $z = -3.58$, $p < 0.01$. The latency to first ID was significantly longer in the epileptic rodent EC (3290.45 ± 235.28 seconds) in comparison to human TLE tissue (1283.88 ± 365.12 seconds), as illustrated in figure 4-8D.

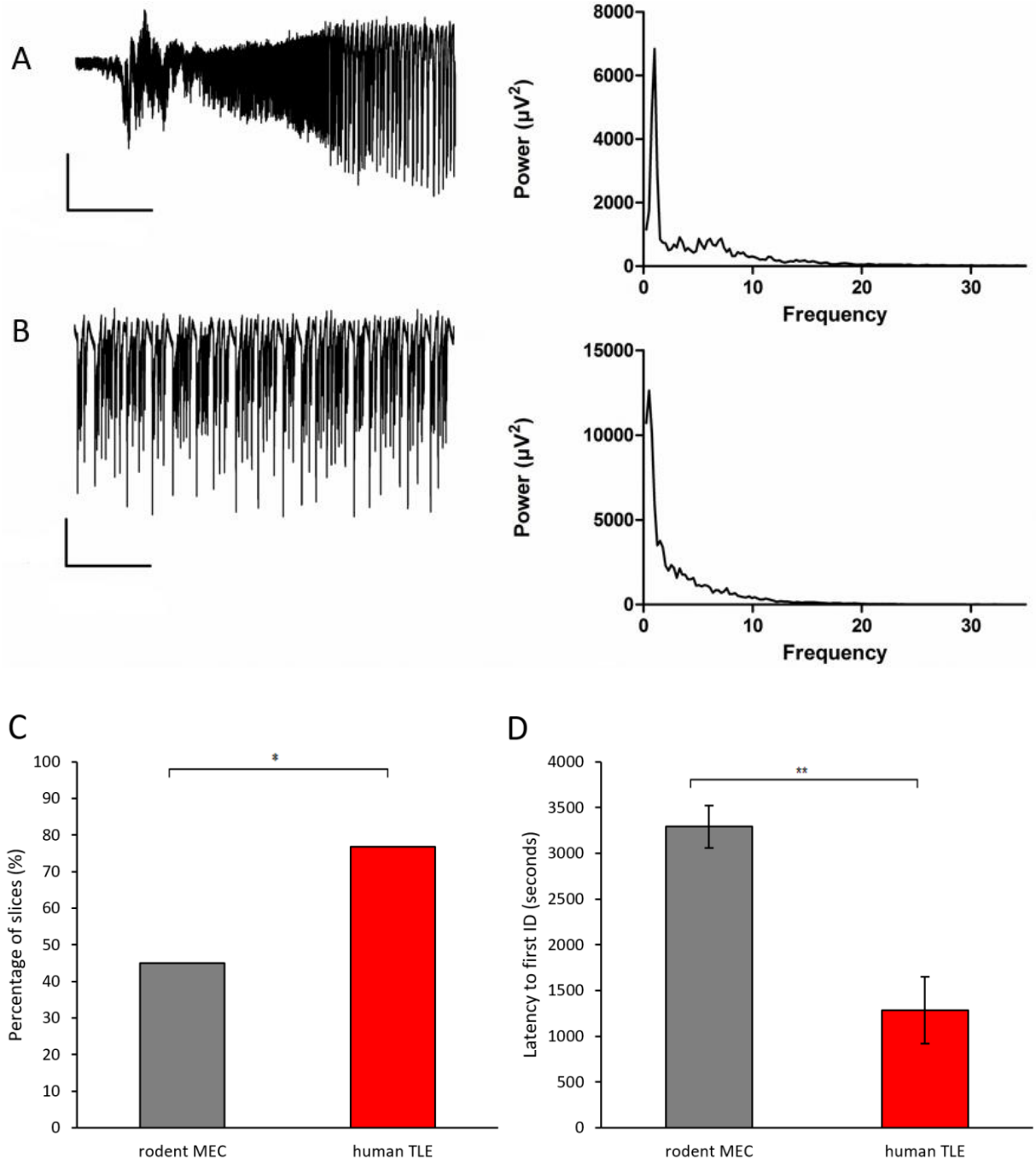

Figure 4-8. The excitability of epileptic rodent and human tissue. A. Evoked ID in human epileptic temporal neocortex. B. Evoked IIDs in human epileptic temporal neocortex C. The percentage of epileptic rodent and human tissue which displayed IDs. D. The latency to first ID in epileptic rodent and human tissue.

To further explore differences in excitability, differences between rodent and human tissue were compared in terms of IEIs between IDs in 0$[Mg]^{2+}$ aCSF for rodent tissue and in 0$[Mg]^{2+}$ and high K^+ or KA (300-400 nM) aCSF for human tissue. There were significant differences between the IEIs of rodent and human tissue in ID inducing mediums, U = 52.00, z = -2.11, p

< 0.05. The IEIs between IDs was significantly longer in human tissue (918.47 ± 223.59 seconds) in comparison to rodent tissue (449.55 ± 223.59 seconds).

Further analysis on ID parameters, included exploring differences of the durations of IDs in ID inducing mediums. As illustrated in figure 4-9, there were no significant differences in terms of the duration of IDs in rodent and human tissue, $U = 82.50$, $z = -1.12$, $p > 0.05$.

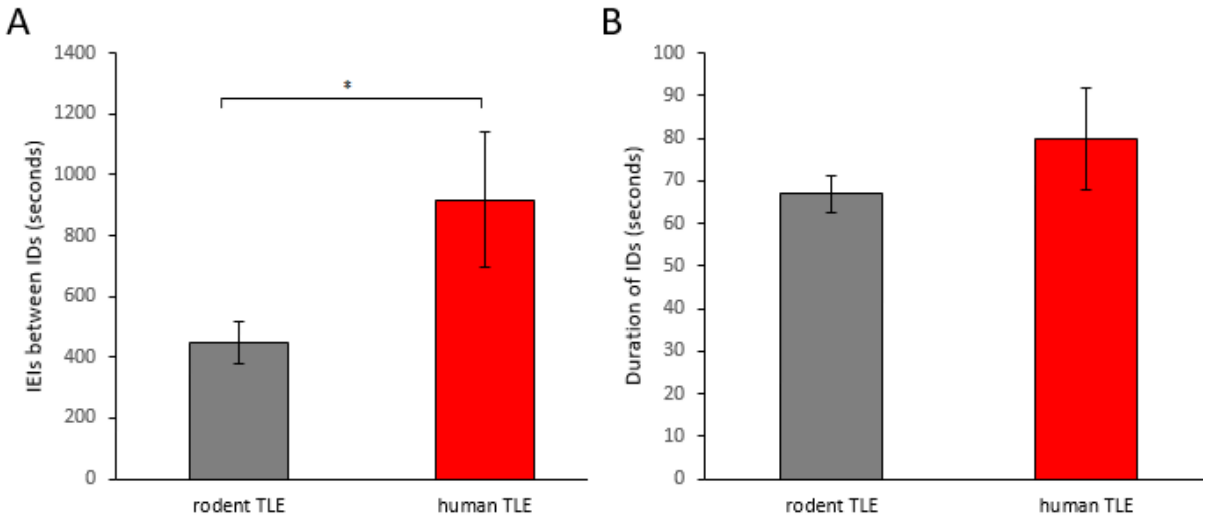

Figure 4-9. Characteristic of ID parameters in epileptic rodent and human tissue. A. Average IEIs between IDs in epileptic rodent (449.55 ± 223.59 seconds) and human tissue in ID inducing mediums. B. Average durations of ID in epileptic rodent and human tissue in ID inducing mediums.

4.2.3.2 Efficacy of AEDs in resected human tissue

Due to the limited sample size on which different AEDs were tested in resected human tissue, meaningful statistical analysis could not be conducted. Nevertheless, the effects of AEDs on the IEIs between IDs are illustrated in figure 4-10. In comparison to epileptic rodent tissue, which did show some degree of resistance to AEDs, human resection of temporal lobe areas (e.g. amygdala, medial temporal gyrus) were all sensitive to all combination AEDs, *in vitro*. There was evidence of some resistance to VPA and CBD alone, but these experiments would need to be repeated to confirm this inference.

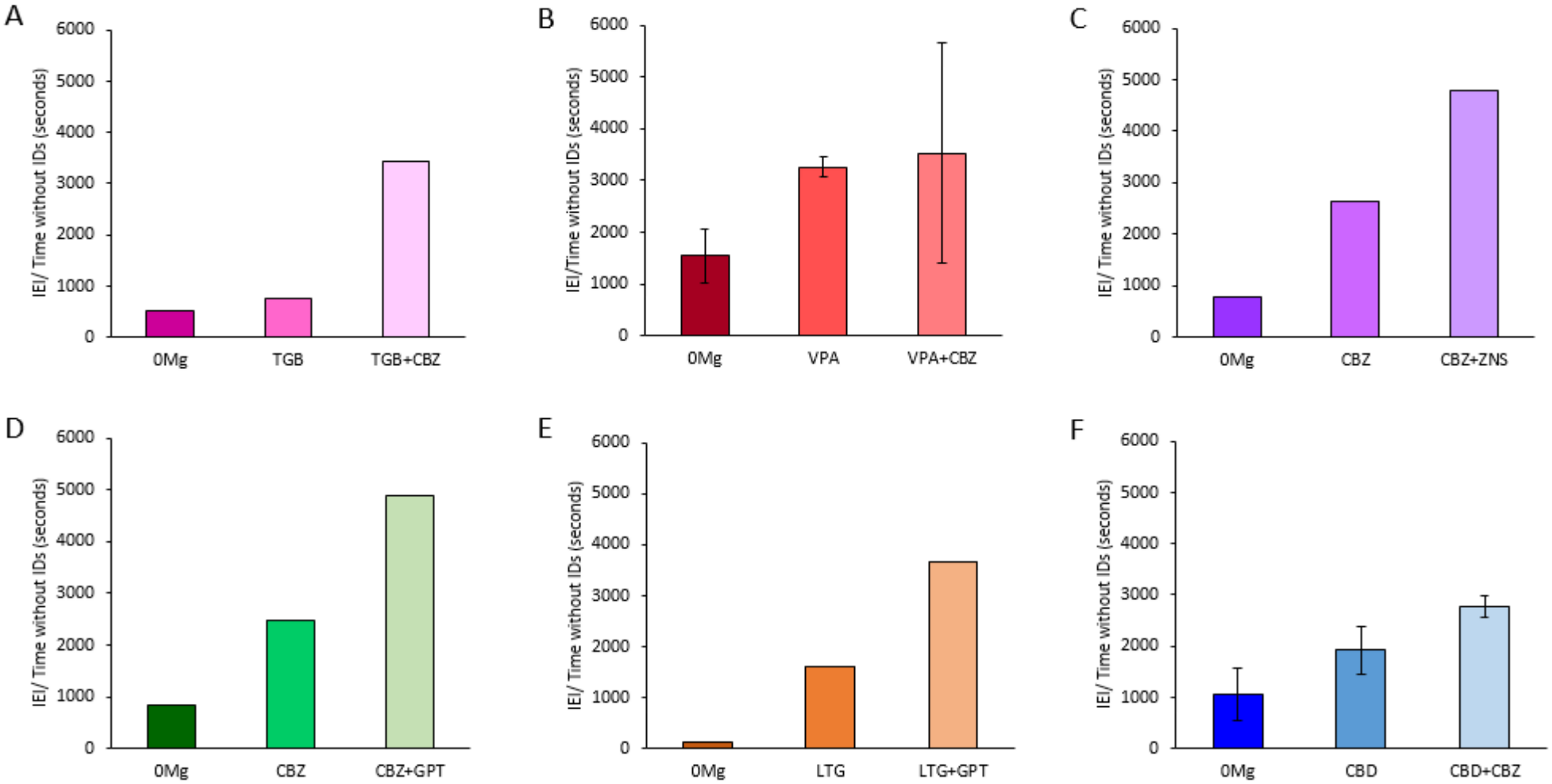

Figure 4-10. The effects of AEDs on the IEIs between IDs in resected epileptic human temporal lobe tissue. A. The effect of TGB+CBZ on IEIs on resected human TLE tissue. B. The effect of VPA+CBZ on IEIs on resected human TLE tissue C. The effect of CBZ+ZNS on IEIs resected human TLE tissue D. The effect of CBZ+GPT on IEIs resected human TLE tissue E. The effect of LTG+GPT on IEIs resected human TLE tissue F. The effect of CBD+CBZ on IEIs resected human TLE tissue.

4.2.3.3 Excitability of different brain regions from human resected tissue

This section explores differences in excitability in human epileptic tissue resected from three different brains areas: the temporal lobe, the frontal cortex and the motor cortex. Although data from all three is presented, differences in excitability will only be statistically analysed between the temporal lobe and frontal cortex, as the sample size of motor cortex slices was limited.

As illustrated in figure 4-11A, epileptic resected tissue from the temporal lobe region of the human brain had the greatest excitability as 76.79 % of slices displayed IDs, followed by motor cortex slices and frontal lobe slices. To compare differences in latency to first ID in human resections from different brain areas a Kruskal-Wallis analysis was carried out. As illustrated in figure 4-11B, there were no significant differences in latency to first ID, in resected tissue from the temporal lobe, frontal cortex and motor cortex, $p > 0.05$.

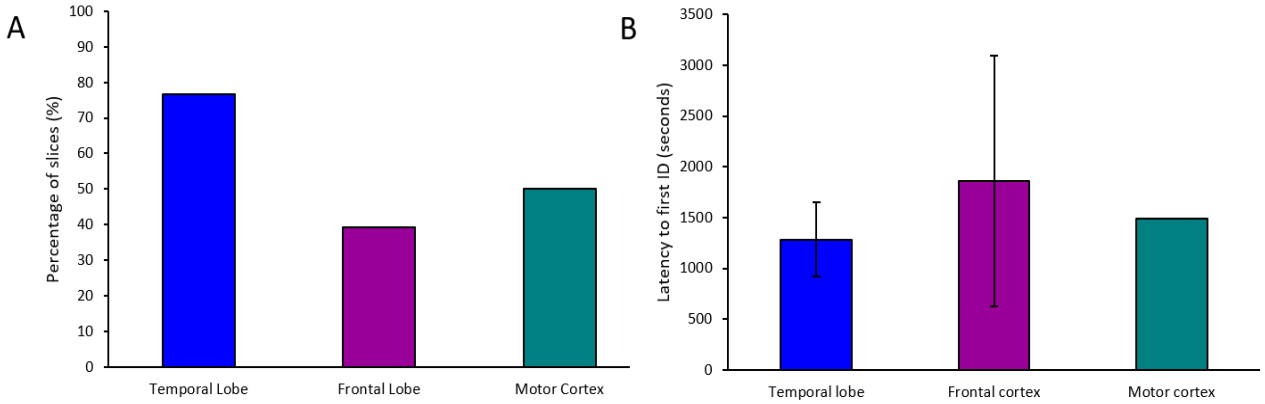

Figure 4-11. The excitability of human epileptic tissue from three different brain regions. A. Percentage of slices which display IDs from temporal lobe, frontal cortex and motor cortex brain regions. B. Latency to first ID in human epileptic tissue from temporal lobe, frontal cortex and motor cortex brain regions.

In terms of ID parameters, a Mann-Whitney-U test showed there were no significant differences in the IEIs between IDs in tissue resected from temporal lobe and frontal cortex areas, $U = 8.00$, $z = -0.82$, $p > 0.05$. Additionally, there were no significant differences between the durations of IDs from epileptic human tissue resected from the temporal lobe and frontal cortex, $U = 11.00$, $z = -0.20$, $p > 0.05$ (see figure 4-12).

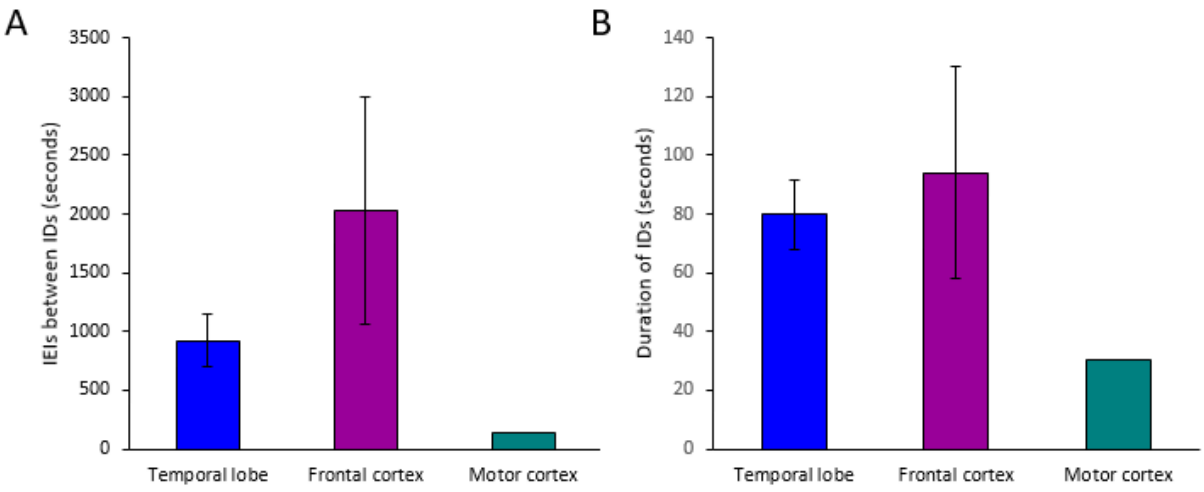

Figure 4-12. Characteristics of ID parameters in 3 different brain regions of the human epileptic brain. A. The IEIs between IDs in the epileptic resected temporal lobe, frontal lobe and motor cortex regions. B. The duration of IDs in epileptic resected temporal, frontal lobe and motor cortex regions.

4.3 Discussion

The current study investigated changes in excitability and the efficacy of combination AEDs during epileptogenesis in an animal model of epilepsy and in resected tissue from human epileptic patients. Slices prepared from animals who had undergone the refine Li-pilocarpine model of epilepsy were significantly more excitable in comparison to controls. Findings suggest immediate network changes. All combination AEDs were effective in reducing IDs at every stage of epileptogenesis, but TGB+CBZ were the most effective and LTG+GPT were the least effective overall. Resistance to AEDs was between 20-33% across all stages of epileptogenesis, and resistance to CBD+CBZ decreased as epileptogenesis progressed. Excitability in resected human tissue showed tissue from temporal lobe regions were more excitable in comparison to the motor cortex and frontal lobe regions. However, resected tissue required more excitation in order to induce IDs in comparison to epileptic rodent tissue. Preliminary investigations of AED efficacy illustrated all combination AEDs were effective in reducing IDs, with some resistance toward VPA and CBD. Overall, it is evident epilepsy induces subtle changes in network dynamics that increase excitability in the temporal lobe and other brain regions in rodent models and resected human tissue.

Acute *in vivo* (e.g. MES and PTZ) and *in vitro* (e.g. 0$[Mg]^{2+}$, 4- AP,) studies provided a great initial step in the screening of AEDs for epilepsy treatment. However, these studies suffer from several limitations. As a result chronic models of epilepsy have been employed and have transformed the development and testing of AEDs by improving treatment outcome predictions and providing better insight into epileptogenesis and mechanisms of DRE. Chronic models have identified AEDs with unique mechanisms such as LEV (Bajjalieh et al., 1994; Lynch et al., 2004). Chronic models have further enabled exploration of the protective

effects of AEDs at different stages of epileptogenesis (Bolanos et al., 1998; Margineanu et al., 2008; Morrisett et al., 1987; Turski, 1987).

Whilst several different studies have explored AED efficacy at different stages of the chronic KA model of epilepsy, relatively few have been carried out on the pilocarpine model. Additionally, studies investigating AED effects during different stages of epileptogenesis often suffer from limitations such as employing high severity models and classifying resistance based on the response to individual AEDs rather than combinations of AEDs. For these outlined reasons it was the primary aim the current study to investigate the efficacy of combinations of AEDs during epileptogenesis of the refined Li-pilocarpine model, in layer II of the MEC.

One of the fundamental aims of research with chronic animal models of TLE, is to identify mechanisms and drugs which effectively prevent epilepsy in humans. However, it has been demonstrated that there are marked differences that exist between different chronic models of epilepsy in terms of underlying pathophysiology and response to different AEDs. For example, clinical studies have shown VPA cannot prevent or suppress epileptic seizures after traumatic brain injury (Temkin et al., 1999), but *in vivo* investigations on AEDs administered during the latent period have shown VPA protects against spontaneous seizures (Bolanos et al., 1998).

Evidently there is a need to also investigate AEDs in human tissue in comparison to animal models of epilepsy, to provide insights into how to improve animal models and better understand mechanisms of resistance. Therefore the secondary aim of this study was to compare excitability and AED effects in the resected epileptic human tissue to epileptic rodent tissue from refined Li-pilocarpine model.

4.3.1 Excitability and efficacy of AEDs in layer II MEC during epileptogenesis

The current study demonstrated enhanced excitability of slices that had undergone the refined Li-Pilocarpine model of epilepsy. Irrespective of the stage of epileptogenesis, significantly more SE slices displayed IDs in 0$[Mg]^{2+}$ aCSF, with shorter latencies to first ID in comparison to their control counterparts. Further investigation of ID parameter characteristics during epileptogenesis, showed the IEI's between IDs was the longest slices 24 hours post SE induction, and therefore these slices were not as excitable in comparison to slices from later stages of epileptogenesis. These results suggest there is an active process underlying the suppression of ictogenesis at this early stage of epileptogenesis. Elevation in inhibition has been suggested to be a compensatory response of brain in attempt to decrease seizure propensity (Fritschy et al., 1999). Moreover, other ID parameters such as duration are largely unaltered at different stages of epileptogenesis suggesting compensation mechanisms are specific to ID triggering mechanisms rather than the nature of the intrinsic discharge itself.

In terms of evaluating whether epileptogenesis is a continuous process or not, these findings show support for the step-function hypotheses of epileptogenesis. The step-function hypothesis suggests the mechanisms responsible for seizure generation are mature at the time of the first seizure, after the latent period. On the other hand, the continuous-function hypothesis suggests the mechanisms responsible for seizure generation are not complete at the time of the first seizure, and seizure frequency increases as epileptogenesis progresses (Dudek & Staley, 2012). Evidently, fundamental network changes occur imminently after from the Li-Pilocarpine model of epilepsy has been carried out. These network changes increase the excitability of networks and promotes the generation of IDs, thus supporting the step-function hypothesis. However, this step difference appears after SE induction rather than after the latent period.

Investigations of the efficacy of AEDs during epileptogenesis illustrated that all combinations of AEDs significantly increased the intervals between IDs, and there was no superiority of certain AED combinations at different stages of epileptogenesis. Irrespective of stage of epileptogenesis, TGB+CBZ increased IEI's between IDs significantly more than LTG+GPT and CBD+CBZ. Combination AEDs also reduced duration of IDs overall. These findings suggest AEDs with GABAergic mechanisms are more effective in this refined Li-Pilocarpine model of epilepsy.

In support of these findings, many experimental and clinical studies have demonstrated GABA has an important role in the mechanism and treatment of epilepsy (Trieman, 2001). For example, GABAergic abnormalities have been observed in genetic (Roberts et al., 1985) and acquired models of epilepsy (Esclapez & Trottier, 1989; Houser et al., 1986). GABA agonists have been shown to suppress seizures (Vicini et al., 1987; Rogers et al., 1994), and conversely GABA antagonists have been shown to induce seizures (Jones & Lambert, 1990a, b). Moreover, in human TLE patients, $GABA_A$ receptors have been shown to be reduced (McDonald et al., 1991).

Others have suggested that this excitation-inhibition imbalance occurs as a result of the disconnection of inhibitory and excitatory neurons, as proposed by dormant interneuron hypothesis (Sloviter, 1987). In support, some studies which have found that inhibition is not completely impaired by chronic epilepsy, but more likely to be overshadowed by abnormal enhanced excitation (Mody & Heinmann, 1987; Tolner et al., 2007). On the contrary, Kumar & Buckmaster (2006) demonstrated chronic epileptic adult rats show a significant loss of GABAergic neurons in the EC and gephyrin-positive puncta in EC layers I and II. Additionally, excitatory synaptic drive to nearby surviving interneurons appeared to be intact, suggesting hyper-excitability is not due to dormancy but an actual loss of inhibitory input.

It is clear that disrupting the equilibrium between excitation and inhibition in the brain is likely to induce seizures, however, epilepsy is much more mechanistically complex. This is evident

as AED discovery thus far has typically targeted reducing excitation or enhancing inhibition, which has offered control over seizures for some but not all patients. Additionally, enhancing GABAergic inhibition can be pro-epileptic in some circumstances too (e.g. absence seizures) (Loscher, 2002). Abnormal enhancement of GABAergic inhibition has been observed in a number of studies (Klassen et al., 2006; Naylor et al., 2005; Zhan & Nadler, 2009). Whilst this elevation in inhibition has been suggested to be a compensatory response of brain in attempt to decrease seizure propensity (Fritschy et al., 1999) others have reported abnormally enhanced inhibition, paradoxically, promotes seizures. For example, Cope et al. (2009) showed in a genetic model of absence epilepsy, GAT-1 deficiency lead to enhanced levels of $GABA_A$ inhibition.

Whilst enhancements in inhibition have been linked to the initiation of seizures, on the other side of the paradox, a lack of excitation leading to hyopfunctional networks has also been suggested to promote seizures (Duncan et al., 2008; Turrigiano, 2012). One mechanism through which this has been suggested to occur is through homeostatic synaptic scaling (Turrigiano et al., 1998 Turrigiano et al., 2008). Neuronal networks must maintain stability in the face of plastic challenges such as changes in the cell size or synapse number or strength, that alter excitability and stability. Synaptic scaling is a form of homeostatic plasticity, whereby perturbing network activity generates compensatory changes in synaptic strength to maintain balance. For example, neurons can detect changes in their own firing rates through calcium-dependent sensors that regulate receptor trafficking to increase or decrease accumulation of glutamate receptors at synaptic sites through a range of signalling pathways (Turrigiano, 2012). According to this research a lack of excitation in hypofunctional networks, could generate seizures themselves through synaptic scaling whereby a shift in the activity set point occurs.

Moreover, it important to consider the interactions of excitation and inhibition. For example, severe myoclonic epilepsy is characterised by mutations of VGSCs which impair sodium current and action potential firing in hippocampal inhibitory interneurons without detectable changes in pyramidal neurons. As a result, AEDs such as PHT and CBZ which block sodium channels, act in pro-epileptic ways (Oakley et al 2011; Rogawski & Loscher, 2004). Additionally, numerous studies have demonstrated the ability of glutamate in modulating inhibition (Belan & Kostyuk, 2001) which can have enhancing and inhibiting effects depending on the type of receptors (NMDA, KAR and mGluRs), sensitivity and location (pre- or postsynaptic) of receptors.

Overall, the emphasis of the excitation-inhibition imbalance in epilepsy is of limited value, as this over simplistic view, of a clearly multi-factorial disease, ignores the mechanisms that render an epileptic brain resistance to AEDs (Margineanu & Klitgaard, 2009). Conversely, however, assessing the myriad of genetic, developmental and acquired factors that influence

the interplay between synaptic reorganisations leading to excitation-inhibition derangement, it is important to generate a cohesive understanding by asking crucial question to ensure development of knowledge and not just information (Sloviter, 2004).

The measurement of resistance to AEDs in clinical studies as well as physiological *in vivo/ in vitro* investigations have often been flawed. The current study explored how resistance to different combination AEDs changed during epileptogenesis according to two measurements of resistance. Findings based on the first definition (continued presence of IDs following application of two AEDs) of resistance showed there were no stage dependent effects and resistance was demonstrated in 20 - 33% of slices through epileptogenesis. Resistance was interestingly the highest in the least excitable slices at 24 hours post SE induction. Thereafter, resistance decreased during the latent period before beginning to slightly rise in chronic epilepsy. A more definitive increase in resistance may have been observed in chronic epilepsy, had more time been left between confirmation of epileptic status through the PSBB task and experimentation. As shown in chapter 3, LTP plays a significant role in the resistance to AEDs in acute models, as sucrose slices had longer latencies to first ID and therefore longer exposure to the excitatory, LTP inducing 0$[Mg]^{2+}$ aCSF. The reinforced network thereafter demonstrated slightly more resistance to AEDs in comparison to standard prepared slices. Complimentary to these findings, chronic pilocarpine epilepsy studies have demonstrated NMDA receptor inhibition with MK-801 prevented the development of epilepsy (Rice & DeLorenzo, 1998). Future investigation of how the length of time spent in the chronic epileptic state without treatment influences resistance to AEDs will determine the role of LTP in chronic epilepsy.

There were very few differences between the two analyses of resistance for specific AED combinations at different stages of epileptogenesis. Overall, some level of resistance was maintained for most drug combinations. Both analyses demonstrated TGB+CBZ was highly effective at abolishing IDs at all stages of epileptogenesis in comparison to other drug combinations. This finding correlate with other analyses of ID parameters. Specifically, TGB+CBZ was shown to increase IEIs between IDs at every stage of epileptogenesis. According to the second definition of resistance (< 50% reduction in ID frequency) the CBD+CBZ combination becomes more effective as epileptogenesis progresses. However, the analyses based on the first definition of resistance suggests the CBD+CBZ combination is maximally effective at 5 weeks post SE, suggesting it may be influential as a tool for preventing epileptogenesis. This definition of resistance allows us to pinpoint with greater accuracy when CBD+CBZ would be most effective, hence allowed the differentiation between therapies that target disease prevention and disease modification (Sloviter & Bumanglag, 2013).

4.3.2 Excitability and resistance to AEDs in the PC and EC

Connections between the PC and EC are well characterised (Agster & Burwell, 2009). The PC is a three layered cortical structure and has been shown to be an easily excitable, seizure-prone region (Hoffman & Haberly, 1991; Loscher & Ebert, 1996; Raccine et al., 1988). Similar investigations into the excitability of the PC and efficacy of AEDs during epileptogenesis, carried out by Pennifold (2016), allowed the comparison of the PC and EC areas in this refined Li-Pilocarpine model of epilepsy and in controls. These investigations suggested the PC is highly excitable in normal and pathological conditions, in comparison to the EC.

It is important to acknowledge that these results were obtained from different animals whereby EC slices were prepared using a sucrose based aCSF and PC slices were prepared using a choline based aCSF. Different solutions were utilised to optimise viability of slices for the two different areas *in vitro*. However, experimental observations of hippocampal slices prepared using choline based aCSF showed a tendency for increased spontaneous epileptiform activity, hence these results could be an effect of the different solutions used. On the other hand, Hamidi et al. (2014) demonstrated when the connectivity between the PC and EC is preserved the duration of IDs in the two areas is reduced. This view that interconnectivity hampers ictogenesis is supported by evidence from Barbarosie and Avoli (1997) who found IDs could not be recorded in the EC when connected to the hippocampus. In contrast, the studies carried out on the EC here were done in combined EC-hippocampal slices, whereby IDs could still be induced in the EC. Nevertheless, it would be of interest to further investigate how excitability changes during epileptogenesis when the PC and EC areas are connected.

Investigation of PC drug resistance showed resistance was greatest at 24hrs post SE and more likely to show resistance in comparison to the EC at this stage. There were no further differences in resistance at other stages of epileptogenesis, but resistance did appear to be the lowest at 5 weeks post SE.

It is of particular interest that resistance to combination AEDs was lower 5 weeks post SE in PC and EC slices, as this time point coincides with the final developmental stage of adolescence whereby the immature brain is transformed into adult form. The mechanism responsible for this transition is synaptic pruning (Selemon, 2013) and reorganisation of connections have been shown to be activity dependent (Changeux & Danchin, 1976; Shatz, 1990). As a result of these developmental processes the balance of excitation and inhibition are altered in favour of inhibition (Bourgeois & Rakic, 1993; Rakic et al., 1986) as excitatory synapses are selectively degenerated but inhibitory synapses are spared. Additionally, D2 dopamine receptors on interneurons undergo maturation, leading stimulation to result in excitation of interneurons, thus promoting inhibition (Tseng & O'Donnell, 2007). These

developmental processes in which inhibition is enhanced may explain why resistance to AEDs may be reduced at 5 weeks post SE. Moreover, perturbations in this developmental refinement such as the presence of seizures or underlying excitability lead to the survival of too many excitatory synapses during synaptic pruning resulting in a chronically unstable and excitable network. In support of this view, Zhou et al. (2009) demonstrated the LGI1 gene regulates glutamatergic pruning pre-and post-synaptically, and in mice expressing a mutated

PC slices at 5 weeks post SE showed the greatest resistance to TGB+CBZ and no resistance toward VPA+CBZ and LTG+GPT. Interestingly in comparison the EC showed greater resistance to LGT+GPT and CBZ+ZNS at 5 weeks post SE. Moreover, CBD+CBZ and TGB+CBZ are particularly effective combinations of AEDs in both areas of the brain. TGB enhances GABA transmission by blocking reuptake of GABA into neurons and glia (Trieman, 2001). The mechanisms of CBD remain ambiguous but it has been suggested to affect: the equilibrative nucleoside transporter, the orphan G-protein-couple receptor GPR55, the 5HT1a receptor, the α1 and α3 glycine receptors and the transient receptor potential of Ankyrin type I channel (see Devinsky et al., 2014 for a review). Further investigation, into network changes that promote the efficacy of these drugs would provide better insight into the mechanisms of epileptogenesis and DRE.

4.3.3 Excitability and efficacy of AEDs in resected human tissue

4.3.3.1 Excitability in resected temporal lobe tissue

Investigations of the excitability of epileptic resected human tissue and rodent tissue of the temporal lobe regions, illustrated significantly more human tissue slices showed IDs in comparison to rodent tissue. Additionally, human tissue slices had shorter latencies to first ID. However, it is important to acknowledge that the human tissue we received was highly variable from patient to patient and even slice to slice. In some slices IDs were easily induced with just a 0$[Mg]^{2+}$ aCSF perfusate, in most other slices this would not be enough drive to induce IDs in the network and harsher solutions such as 0$[Mg]^{2+}$/high K^{+} aCSF and/or 200- 400 nM kainate would be required in order to induce IDs. Therefore, these comparisons suggest human tissue is much more excitable than epileptic rodent tissue, but these findings should be treated with caution given the complexities surrounding the induction of IDs in resected human tissue.

Comparisons of ID parameter characteristics showed human tissue showed longer IEIs between IDs in comparison to rodent tissue. There were no differences in the two types of tissue with regard to the durations of IDs. These findings taken together with the fact most human tissue slices required harsher inducing ID inducing solutions, suggest *in vitro* this tissue is not as excitable as chronic epileptic rodent tissue.

As reviewed by Köhling and Avoli (2006), many studies of resected human tissue require pro-convulsant agents such as GABA antagonists and 4-AP and/or alterations to the aCSF concentrations of Na^+, K^+ and Mg^{2+}, in order to observe epileptiform-like activity *in vitro*. On the other hand, recent observations of spontaneous pathological activity have been reported (Cunningham et al., 2012; Huberfield et al., 2007; Roopun et al., 2010). It has been suggested increases in the reporting of spontaneous activity could be a result of improved slice preparation methods, such as the replacement of sodium chloride with sucrose or choline and the addition of several antioxidants (Jones et al., 2016). These improved slicing methods were adopted within our laboratory, and whilst spontaneous IIDs and HFOs were regularly present, spontaneous IDs very rarely occur. Other factors that may have contributed to this lack of spontaneous ictal activity include: variations in surgical technique, variations in the brain area the sample is taken from and time taken to transport and slice the sample. Having said this, spontaneous ictal events are only very occasionally evident in chronic epileptic rodent tissue in our recordings. It is important to acknowledge that *in vitro* preparation represent a reduced system of the intact brain, therefore it is not unusual characteristic to require electrical or chemical stimulation in order to induce IDs.

Comparisons of 'control' and epileptic human tissue have shown no epileptiform activity can be induced in control tissue, but can be observed in epileptic tissue (Jones et al., 2016). These findings are representative of the excitability differences in EC control and epileptic tissue described here. The key point to take away from this study, however, is that human TLE tissue required more excitation than chronic epileptic rodent tissue. Further exploration of the functional implications of morphological changes may provide insights as to why more excitation was required.

Earlier studies investigating the functional implications of morphological changes demonstrated cell dispersion and recurrent sprouting lead to circuitry changes which promote seizure activity (Houser, 1990). Additionally, using organotypic cultured slices, Hocke et al. (2007) conducted histological examinations and showed hippocampal cell loss associated with TLE.

Investigations of the electrophysiological properties of neurons in human epilepsy have shown parameters of intrinsic neuronal excitability appear to be rather normal. For example, action potential discharges, voltage-gated Na^+, K^+ and Ca^{2+} currents appear to be remarkably similar to those found in rodent tissue (Beck et al., 1996; Isokawa et al., 1991; Strowbridge et al., 1992).

Whilst intrinsic cellular properties appear rather 'normal', synaptic mechanisms have been shown to be more abnormal. For example, NMDA-dependent activity has been shown to be considerably prolonged, and was correlated to a loss of spine density (Isokawa & Levesque, 1991; Isokawa et al., 1997). Additionally, Mathern et al. (1997) demonstrated increased

NMDAR2 hybridization in the DG and increased AMPA GluR3 mRNA densities in the hippocampus of epileptic patients. The number of $GABA_A$ receptors is been shown to be reduced in hippocampal resected tissue (McDonald et al., 1991; Johnson et al., 1992; Olsen et al., 1992; Wolf et al., 1994), but this is most likely a secondary change associated with cell loss. Paradoxically, however, others have found mechanisms of synchronous activity relies on $GABA_A$ receptor activation in Taylor's type focal cortical dysplastic (FCD) tissue (D'Antuono et al., 2004). FCD is characterised by disruption of cortical lamination and large balloon cells. The ability of GABA to cause extracellular increase in K^+ and the decreased ability of $GABA_B$ agonist, baclofen, to control release of GABA from interneurons, was suggested to be the mechanisms through which GABA mediated synchronisation occurred.

Investigating the functional implications of synaptic abnormalities on plasticity, Wilson et al. (1998) showed short-term plasticity in disturbed in sclerotic epileptic tissue in comparison non-sclerotic tissue. Specifically, paired-pulse depression was increased in the performant path and decreased in associational pathways. This increased inhibition in the performant pathway, maybe a compensatory mechanism aimed at protecting against seizures, whereas reduced inhibition in associated areas may support seizure initiation. Furthermore, *in vitro* investigations have demonstrated, LTP induced by high-frequency stimulation or forskolin (increases intracellular cAMP) is dramatically reduced in sclerotic tissue, in comparison to non-sclerotic tissue from TLE patients (Beck et al., 2000). The level of damage and reorganisation within epileptic tissue increases variability in findings. In support, it has been shown epileptiform activity can easily be induced in sclerotic tissue (10mM K^+) in comparison to non-sclerotic tissue (12mM K^+) (Gabriel et al., 2004).

Many KA and pilocarpine models of epilepsy have been criticised on the basis that they produce severe widespread brain damage that does not accurately reproduce that human pathology (Sloviter & Bumanglag, 2013). One of the main advantages to the refined Li-Pilocarpine model of epilepsy used within our laboratory is the reduction in severity. The model induces subtle alterations in network dynamics, without significant loss of neuronal network function (Modebadze et al., 2016). The results presented in chapter 3 of different slice preparations and previous epileptic human tissue studies have shown epileptiform activity is more difficult to induce in tissue that is less damaged. Despite some discrepancy between the induction in epileptiform activity between rodent and human tissue here, which could partially be attributed to tissue removal procedures, it is clear the refined Li-pilocarpine model of epilepsy can provide fruitful insights into the mechanisms of epilepsy.

4.3.3.2 AED efficacy in temporal lobe resected tissue

All six combination AEDs were demonstrated to increase IEIs in resected temporal lobe human tissue. There appeared to be some resistance to VPA and CBD alone, but results would need to be replicated with larger samples in order to draw firm conclusions from these

findings. These preliminary findings suggest AEDs that are potentially ineffective in treating TLE in patients, maybe effective *in vitro*.

In contrast, others have shown resistance to CBZ and PHT (Kohling et al., 1998; Musshoff et al., 1997), however these studies explored AED effects on IID and LRD type epileptiform activity, which is typically shorter in duration in comparison to IDs and not representative of behavioural convulsions observed in patients. The current study assessed the effects of AEDs on longer duration IDs.

As highlighted, the *in vitro* brain slice preparation represents a reduced system. As a result of severed connections represented in this preparation the mechanisms of drug resistant present in patients, may not be replicable *in vitro*. On the other hand, these results are preliminary and as some resistance was shown toward VPA and CBD results from larger samples may reveal different insights.

4.3.3.3 Excitability in resected tissue from different brain regions

Investigations of the excitability of epileptic human tissue of different brain areas, showed more temporal lobe slices showed IDs in comparison to motor cortex and frontal lobe slices. There were no differences in the latency to first ID. These results suggest of the three epileptic regions examined the temporal lobe is the most excitable. In terms of ID parameters IEIs between IDs and the duration of IDs were the shortest in the motor cortex, followed by the temporal lobe and finally the frontal lobe slices.

These findings are supported by statistics which show temporal lobe epilepsy is the most common form (41% of cases) (Curia et al., 2014). Additionally, studies have shown the intrinsic cellular characteristics of temporal lobe areas, give rise to their participation in epileptogenesis (Jones & Heinemann, 1988). It is likely that these characteristics are similar in resected human tissue too.

4.3.4 Conclusion

The current study investigated changes in excitability and the efficacy of combination AEDs during epileptogenesis in an animal model of epilepsy and in resected tissue from human epileptic patients. Irrespective of stage of epileptogenesis slices prepared from animals who had undergone the refine Li-pilocarpine model of epilepsy were significantly more excitable in comparison to controls. Findings demonstrate immediate network changes. All combination AEDs were effective in reducing IDs at every stage of epileptogenesis, but TGB+CBZ were the most effective and LTG+GPT were the least effective overall. These findings suggest AEDs of GABAergic mechanisms are particularly effective. However, the focus on targeting inhibition or excitation in AED therapy should be avoided as it has not been fruitful thus far. Alternative mechanisms are considered in chapter 5. Resistance to AEDs was between 20-33% across all stages of epileptogenesis. There was some evidence of variation to specific

combination of AEDs at different stages. For example, CBD+CBZ was shown to be particularly effective as epileptogenesis progressed, with the optimal efficacy at 5 weeks post SE, suggesting a therapeutic role in the prevention of epileptogenesis perhaps.

Comparisons of the PC and EC areas demonstrated that the PC in control and epileptic animals was more excitable than the EC. Both regions showed a dip in resistance during the latent period (5 weeks post SE) of epileptogenesis, when developmental synaptic pruning processes and maturation of receptors are likely to enhance AED effects. Both the PC and EC responded well to CBD+CBZ and TGB+CBZ in chronic epilepsy.

Investigations of excitability in resected human tissue showed tissue from temporal lobe regions were more excitable in comparison to the motor cortex and frontal lobe regions. However, resected tissue required more excitation in order to induce IDs in comparison to epileptic rodent tissue. This discrepancy could be attributed to the likelihood that damage within human tissue is likely to be subtle (Gabriel et al., 2004). One of the main criticisms of many kainate and pilocarpine models is that they are often severe and do not imitate human pathology, however the refined Li-pilocarpine model used within our laboratory has been demonstrated to show subtle alterations in network dynamics, without significant loss of neuronal network function (Modebadze et al., 2016). Preliminary investigations of AED efficacy illustrated all combination AEDs were effective in reducing IDs, with some resistance toward VPA and CBD.

Overall, it is evident epilepsy induces subtle changes in network dynamics that increase excitability in the temporal lobe and other brain regions in rodent models and resected human tissue. Although, AED efficacy on the whole has shown to be effective *in vitro* preparations of rodent and human tissue, it is important to remember this preparation is representative of a reduced system. Nevertheless, subtle changes in AED efficacy during epileptogenesis can be seen, the mechanisms for which can be further explored.

Chapter 5 Mechanisms underlying NMDA induced network alterations during epileptogenesis in layer II of the MEC.

5.1 Introduction

The typical time course of epileptogenesis is characterised by initial neuronal insult followed by a seizure free latent period and finally the presence of spontaneous recurring seizures (SRS) (Dudek & Staley, 2012; Sloviter & Bumanglag, 2013). The latent period has been regarded as the pre-epileptic state during which a cascade of molecular and structural mechanisms are activated, leading to the promotion of SRS.

Studies exploring epileptogenic mechanisms have typically focused on the imbalance between excitation and inhibition leading to the disruption of the brain's equilibrium and resulting in the epileptic seizures. This approach has been illustrated to be of limited value in understanding seizures and DRE (Margineanu & Klitgaard, 2009).

Alternatively, the drug-transporter (Loscher & Potschka, 2002; Sisodiya, 2003) and drug-target hypotheses (Remy et al., 2003; Loup et al., 2000) suggest alterations to drug-transporter proteins which transport drugs across the BBB and alterations to drug targets themselves may result in resistance to AEDs. Whilst these maybe somewhat relevant explanations, they do not account for all possibilities of drug resistance (Baltes et al., 2007), therefore other possibilities, such as the involvement of inflammatory processes, functional glia and altered intracellular communication should be also explored (Margineanu & Klitgaard, 2009).

5.1.1 Excitatory modulation of inhibition

Despite inhibitory neurons comprising of just 10-25% of the neuronal networks (Markram et al., 2004), they play a critical role in the modification of neuronal activity. Inhibitory neurons have been shown to be important in the development of cortical circuitry (Allendoerfer & Shatz, 1994), and in the plasticity of excitatory synapses (McBain & Fisahn, 2001). Several neurotransmitters, such as dopamine (Zhou & Hablitz, 1999b), serotonin (Zhou & Hablitz, 1999a), GABA (Jarolimek & Misgeld, 1997) and glutamate (Satake et al., 2000) have been suggested to modulate presynaptic GABA release from interneurons.

Glutamate receptors have been well documented to be involved in plasticity associated with LTP and epilepsy, therefore understanding the mechanisms behind excitatory modulation of inhibition can also provide insights into how LTP and epilepsy can be manipulated (Belan & Kostyuk, 2002). Numerous studies have demonstrated the ability of glutamate in modulating inhibition (see Belan & Kostyuk, 2002 for a review).

A study by Xue et al. (2011) explored how NMDA receptor activation influences GABAergic transmission. It was initially demonstrated NMDA application elicits an inward current and phasic synaptic activity in CA1 pyramidal cells. There were two types of NMDA-induced

responses one with characterised by small amplitude changes (common type I) and another of larger amplitude changes (less common type II). To assess whether NMDA increases EPSCs or IPSCs the $GABA_A$ receptor antagonist, SR-95531, was applied and abolished NMDA-induced increases in phasic synaptic activity, thus confirming NMDA receptor activation increases the frequency of IPSCs. Variations to the duration of NMDA application demonstrated a biphasic increase in amplitude, suggesting the presence of two components to NMDA induced responses. The frequency of IPSCs induced by NMDA peaked at a duration of 3 seconds.

Further investigations included exploring whether changes to the frequency and amplitude were attributed to an increase in presynaptic GABAergic interneurons firing or postsynaptic pyramidal $GABA_A$ receptor sensitivity. Puff application of TTX abolished NMDA-induced synaptic activities in pyramidal neurons, suggesting presynaptic GABAergic interneurons increase firing with NMDA application. Additionally, recording from GABAergic interneurons indicated NMDA application activates NMDA receptors located at the soma/dendrites of interneurons. Induced activity comprised of either an intense discharge of action potentials during the rising phase of depolarisations or in spontaneously firing interneurons, action potentials initially increased and then ceased when the depolarising action reached a plateau (see top traces in figure 5-1A and B). These two differential responses in interneurons to NMDA application possibly explains initial differences in type I and type II NMDA induced amplitude changes in pyramidal neurons, respectively. Different interneuron responses occurred as a result of differential expression of voltage-gated calcium channels (VGCCs), which mediate GABA release. Interneurons with P/Q type VGCCs were involved in type I responses, whereas interneurons with N-type VGCCs were involved in type II responses.

Intracellular pyramidal cell loading of the NMDA antagonist, MK-801 demonstrated postsynaptic NMDA receptors are also involved in NMDA induced IPSCs. Finally, the possibility that postsynaptic activation leads to recruitment of NO as a retrograde messenger was tested, using the NO scavenger, PTIO. PTIO reversibly inhibited NMDA induced IPSCs. These findings indicate a mechanisms of feedback inhibition, to prevent over-excitation.

Overall, these findings suggest NMDA receptor activation enhances $GABA_A$ inhibition between hippocampal interneurons and pyramidal cells, through two distinct mechanisms. Firstly, through activation of NMDA receptors in presynaptic interneurons and secondly through postsynaptic pyramidal NMDA receptor activation. This latter mechanism recruits retrograde messenger NO pathways to enhance GABA release from interneurons. As depicted in figure 5-1, activation of postsynaptic NMDA receptors leads to an influx of Ca^{2+} which phosphorylates protein kinase C (PKC) to activate NO synthase. NO then diffuses to presynaptic interneuron sites, where it is absorbed by the haem group of guanylate cyclase.

Guanylate cyclase reacts with GTP to produce cGMP which increases transmitter release and decreases transmitter reuptake.

NO is produced by a group of isoenzymes, the nitric oxide synthases (NOS), which converts L-arginine to citrulline. The three isoenzymes are: endothelial (eNOS), inducible (iNOS) and neuronal (nNOS). For activation eNOS and nNOS require the formation of Ca^{2+} calmodulin complexes, whereas iNOS is synthesised following inflammatory stimulation and is commonly expressed in astrocytes and microglia. Agents such as heme, tetrahydrobiopterin, Flavin adenosine dinucleotide, Flavin mononucleotide and reduced nicotinamide-adenine dinucleotide phosphate are required for the catalytic activity of all NOS isoenzymes. As mentioned one way in which NO mediates cell signalling is through activation of cGMP, but another way involves post-translational modifications including nitrotyrosination and S-nitrosylation (Banach et al., 2011).

The role of NO in modulating neurotransmitter release at excitatory synapses is well documented (see Garthwaite, 2008 for review). For example, during activation of hippocampal pyramidal cell muscarinic receptors retrograde signalling by NO, controls cannabinoid receptor (CB_1R) dependent depolarisation-induced suppression of inhibition (Makara et al., 2007). On the other hand, others have shown inhibitory enhancing effects of NO signalling pathways in other brain regions (Nugent et al., 2009; Yang & Cox, 2007). Additionally, NO has also been implicated in LTP, as stimulation of glutamatergic afferents activates Ca^{2+} influx through NMDA receptors and promotes NO production which acts as a retrograde messenger to enhance glutamate release (Haley et al., 1992).

NMDA receptors have been extensively documented to play a central role in LTP at glutamatergic synapses, but the role of NMDA receptors in the LTP of inhibitory circuits has only more recently been elucidated. Expressions of NMDA receptor subunits varies according to types of interneurons (e.g. PV interneurons, somatostatin interneurons, etc.) (Moreau & Kullmann, 2013). Additionally, at presynaptic locations NMDA molecular mechanisms (e.g. BDNF, NO, glutamate) of GABAergic synaptic plasticity also vary (Castillo et al., 2011). These different variations contribute to the effective routing of activity in healthy inhibitory circuits.

The NMDA receptor mediated influence over inhibition is very complex and in a similar fashion of mGluRs and KARs, has different models of action dependent on locations and sensitivity NMDA receptor subunits on different types on interneurons (Castillo et al., 2011; Moreau & Kullmann, 2013; Tang et al., 2006) and pre- and post-synaptic locations of NMDA receptors (Xue et al., 2011). To add another layer of complexity, the intracellular signalling pathways NMDA receptors activate, e.g. NO, also have numerous effects.

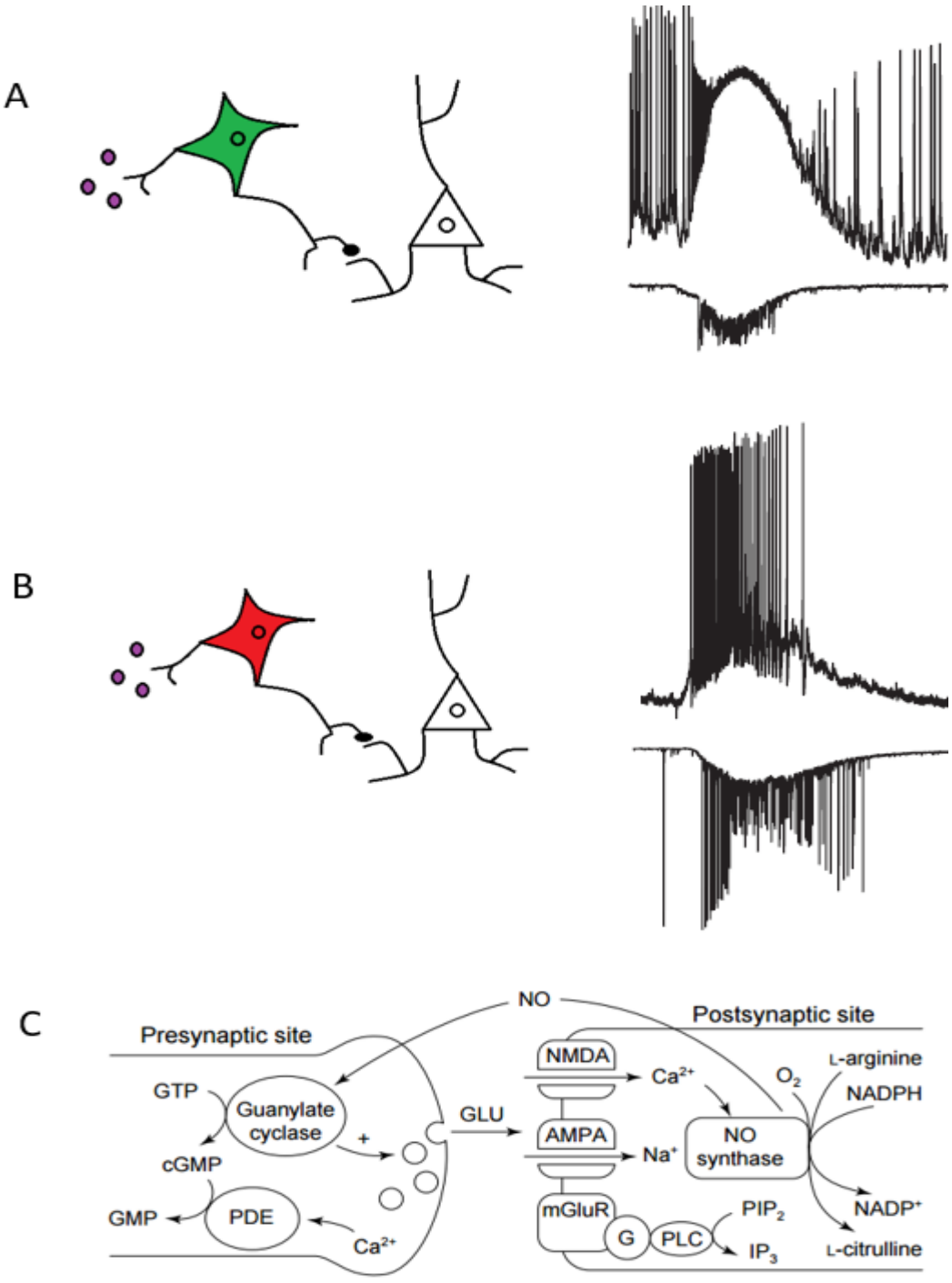

Figure 5-1. A representation of NMDA receptor activation enhancing GABAergic transmission onto hippocampal pyramidal cells through pre- and postsynaptic mechanisms (adapted from Xue et al., 2011). A. Presynaptic activation of NMDA receptors on interneurons with higher expression P/Q type VCCs (top trace), leads to a NMDA induced type I response (smaller amplitude) of sIPSCs in pyramidal cells (bottom trace). B. Presynaptic activation of NMDA receptors on interneurons with higher expression of N- type VCCs (top trace), leads to a NMDA induced type II response (larger amplitude) of sIPSCs in pyramidal cells (bottom trace). C. Postsynaptic activation of NMDA receptors on pyramidal cells, enhances GABA release from presynaptic terminals of interneurons through recruitment of retrograde messenger NO.C. Schematic representation of the retrograde messenger action of NO (adapted from Hölscher, 1997).

5.1.2 Changes in NMDA receptors during epileptogenesis

The involvement of NMDA receptors is critical in LTP and as evidenced in section 5.1.2 also plays a significant role in the intricate control of neuronal excitation by modulating inhibition through a variety of mechanisms. Interestingly, pathological LTP mechanisms have been suggested as an explanation of epileptogenesis and DRE.

In contrast to AMPA and KA subtypes of glutamate receptors, NMDA receptors are also permeable to Ca^{2+} ions in addition to Na^{+} and K^{+} ions. Furthermore, NMDA receptors have slower kinetics which is attributed to a higher affinity for glutamate (Conti & Weinberg, 1999). Under normal physiological conditions brief controlled elevations in Ca^{2+} transmitted via NMDA receptors, trigger LTP through a wide range of signal transduction pathways (e.g. PKC, cyclic adenosine 3',5'- monophosphate (cAMP), tyrosine kinase, Src and mitogen-activated protein kinase (MAPK) (for a review see Lynch, 2004). These plasticity induced structural and electrophysiological changes are fundamental to learning and memory (Malenka & Nicoll, 1999; Gnegy, 2000; West et al., 2001; Tzounopoulos & Stackman, 2003).

The Ca^{2+} hypothesis of epileptogenesis proposes the neuronal effects of Ca^{2+} lie on a continuum. One end of the spectrum is characterised by brief increases in Ca^{2+} associated with physiological effects of learning and memory. The middle ground is characterised by a reversible but prolonged and sub-lethal elevation in intracellular Ca^{2+} that trigger pathological plasticity of epileptogenesis. Finally, the extreme end of the spectrum is characterised by irreversible elevation in Ca^{2+} and neuronal death (DeLorenzo et al., 2005).

In terms of Ca^{2+} effects during epileptogenesis, it has been postulated that during initial CNS insult, sub-lethal increases in Ca^{2+} that do not cause cell death are experienced. During the latent period persistent elevations in Ca^{2+} initiate pathological LTP. Finally during chronic epilepsy persistent elevations in Ca^{2+} maintains SRS (DeLorenzo et al., 1998; Rice & DeLorenzo, 1998; Pal et al., 2000, 2001; Sun et al., 2002, 2004).

Pathological plasticity induces a wide range of changes including a reduction in GABA transmission proposed to be a result of $GABA_A$ receptor internalisation (Blair et al., 2004) and an upregulation in NMDA receptor function and/ or expression at postsynaptic sites (Avanzini & Franceschetti, 2003; Dalby & Mody, 2001; Morimoto et al., 2004). Additionally, presynaptic NMDA receptor alterations have been demonstrated in chronic epilepsy. Presynaptic regulation of glutamate release is powerfully controlled by heteroreceptors and autoreceptors. Heteroreceptors regulate the syntheses and/or release of mediators other than their own ligand and are effected by $GABA_B$, adenosine and muscarinic receptors. Autoreceptors are sensitive to the neurotransmitters released by the neurons in which it resides and is effected by mGluRs, KARs and NMDA receptors. Yang et al. (2006)

demonstrated in the layer V of the EC there is a developmental decrease in NR2B autoreceptor function, however in chronically epileptic animals this is reversed as sEPSCs were sensitive to the NR2B antagonist, Ro 25-6981.These results suggest enhanced NR2B activity which may occur through upregulation or redistribution of receptors. This enhanced NR2B activity represents a recapitulation of earlier development. Similarly, others have suggested epileptic network resemble earlier development (Cohen et al., 2003).

Additionally, others have also reported evidence for enhanced NR2B expression in human and animal models of epilepsy (DeFazio & Hablitz, 2000; Mathern et al., 1999; Ying et al., 2004). For example, epileptiform discharges from patients with cortical dysplasia have been shown to be suppressed with the NR2B antagonist, ifenprodil (Moddel et al., 2005). In the freeze-lesion induced animal model, ifenprodil also increases the threshold for evoking discharges (DeFazio & Hablitz, 2000).

Under normal physiological conditions the NMDA receptor subunit NR2A, is three times more likely to open in response to glutamate in comparison to NR2B. On the other hand, when NR2B receptors do open they stay open for twice as long in comparison to NR2A. Whilst NR2A is insensitive to different glutamate concentrations, NR2B responds in a graded fashion in response to different glutamate concentrations. The activity of NR2A is also insensitive to location of the synapse, whereas NR2B receptors located closest to release site were opened three times as often as NR2B receptors located further away. As mentioned NR2B receptors normally get displaced by NR2A receptors during development and this coincides with a reduction in spontaneous activity, but Santucci and Raghavachari (2008) also suggested NR2B receptors become preferentially located extrasynaptically, where higher concentrations of glutamate are required for them to open. The possible functions of this extrasynaptic NR2B receptor locations was suggested to detect events that cause large amounts of glutamate to be released or detect glutamate release by astrocytes which may play a role in synchronisation. Finally, both NR2B and NR2A receptors could both drive CamKII autophosphorylation required for LTP but NR2B was more reliably involved. Given that research by Yang et al (2006) and others have shown an upregulation in NR2B expression in epilepsy coupled with the variable electrophysiological profile and involvement in LTP it seems likely that the altered NR2A to NR2B ratio can play an influential role in the maintenance of seizures.

Dravid et al (2007) investigated the potencies of structurally distinct NMDA channel blockers at recombinant NMDA receptors (NR1+NR2A/NR2B/NR2C/NR2D), the results from which suggest MK801 would serve a good probe to investigate the changes of NMDA subunit receptor changes and the influence on the generation and maintenance of seizures in epilepsy. Dravid et al. (2007) specifically showed that inhibitory potency varied with stereochemistry of channel blockers and NMDA receptor subunit composition. At pH 7.6

MK801 inhibitory potency was similar for all four NR2 subunits, but potency of (-) MK801 was more variable. At NR2A subunits (+) MK801 was 23 times more potent than (-) MK801. For NR2B, NR2C and NR2D subunits (+) MK801 was 3.6, 1.6 and 4.4 times, respectively, more potent than (-) MK801 at pH 7.6. Additionally, the potency of (+) MK801 and (-) MK801 is greater at NR2A when pH is reduced, but this shift in potency is not seen at NR2B subunits. Evidently Dravid et al. (2007) have demonstrated the robust nature of MK801 isomers on NR2 subunits, which could be useful to investigate the changes of NMDA subunit receptor changes and the influence on the generation and maintenance of seizures in epilepsy.

Moreover, other studies have already begun to show the usefulness of MK801 as a tool in investigating NMDA receptor changes in epileptiform activity and behaviour, but the mechanisms underlying such changes remain to be elucidated. Moderate doses of the NMDA receptor antagonist MK801 have been shown to attenuate seizure activity (Avoli et al., 1996). On the other hand, low doses of NMDA receptor antagonist, MK-801, (0.1- 0.4 mg/kg) has been widely reported to increase locomotion (Deutsche et al., 1997; Wu et al., 2005; Wegnener et al., 2011) and epileptic activity (Stafstrom et al., 1997; Starr & Starr, 1993).

Whilst much research has focused using low dose MK801 to model schizophrenia (Tang et al., 2006), relatively few studies have focused on the mechanisms behind the biphasic MK801 effects on seizure activity. The mechanisms through which MK-801 worsens seizure activity has been suggested to be due disinhibitory effects in which MK-801 selectively inhibits NMDA receptors on interneurons (Stafstrom et al., 1997). Others have highlighted in normal tissue there appears to be substantial differences in the sensitivity to MK-801 which may correlate to anatomical localisations of different NMDA receptor subtypes and account for paradoxical effects of low doses of MK-801 (Tang et al., 2006). As NMDA receptors are altered in epileptogenesis, it can be hypothesised as the NMDA drive in epileptic networks change during epileptogenesis further differences in MK801 sensitivity maybe evident between control and epileptic tissue at different time points.

Moreover, an alternative explanation of low dose MK801 seizure potentiating effects have been suggested to occur as a result of interdependence between D1 and NMDA receptors in the control of behaviour (Starr & Starr, 1993). Five subtypes of dopamine receptors (D1-D5) have been commonly described, all of which are G-protein-coupled transmembrane receptors. D1 receptors are the most abundant are found in the striatum, limbic areas, thalamus and hypothalamus. D2 receptors are the next most common are mainly found in the pituitary gland. Activation of D1 receptors increases intracellular cAMP, which activates protein kinase A (PKA) leading to phosphorylation of DARPP-32. DARPP-32 inhibits protein phosphatase-1 and calcineurin, thus promoting the action of protein kinases and favouring

protein phosphorylation which is an amplifying mechanism. Generally, D2 receptors oppose the actions of D1 receptors (Girault & Greengard, 2004).

Activation of D1 receptors have been shown to accentuate pilocarpine-induced seizure activity (Burke et al., 1990). Also, in the study of dopamine receptors in Parkinson's disease, MK801 has been shown to potentiate D1 stimulant induced increases in motor activity, but attenuates D2 agonist effects (Goodwin et al., 1992). Starr and Starr (1993), demonstrated both MK801 and the NO synthesis inhibitor, L-NAME, produced convulsant effects in synergy with D1 receptor agonist, SK&F38393. These results suggest that MK801 works in synergy with D1 receptors to activate NO signalling pathways leading to seizure activity.

NO has been reported to significantly increase glutamate release at excitatory synapses (McNaught & Brown, 1998) and conversely decrease glutamate release (Sequeira et al., 1997). Biphasic effects of NO on glutamate release have been suggested to occur as a result in intracellular levels of cGMP (Ferrara and Sardo, 2004). Manipulations of NO have been shown to have pro and anticonvulsant effects. For example, demonstrating anticonvulsant effects, the NO precursor, L-arginine has effectively reduced susceptibility to seizures (Herberg et al., 1995; Prezgalinski et al., 1996), and NOS inhibitors have increased seizure susceptibility (Urbanska et al., 1996).The mechanisms through which this NO anticonvulsant effect occurs has been proposed to include: negative feedback on the NMDA receptor through competitive blockade of the recognition site (Manzoni et al., 1992), functional interaction with the redox modulatory site of NMDA receptors (reduction enhancing and oxidants depressing NMDA activity) (Lei et al., 1992; Sanchez et al., 2000) and NO induced enhancement of GABAergic transmission (Tsuda et al., 1997). Conversely proconvulsant effects of NO are related to changes in cerebral blood flow. For example, where vasodilation has been reduced with NOS inhibitors, seizures have been prevented or delayed (Bittermam & Bitterman, 1998). Similarly, KA inductions of seizure activity have been directly associated with increases in NOS activity (Yasuda et al., 2001).

Overall, numerous investigations have demonstrated glutamate-receptors modulate GABAergic inhibition through a variety of mechanisms, which are complexly dependent and intertwined with spatial-temporal distribution patterns of extracellular glutamate concentrations and differential expressions of glutamate receptors on different types of neurons (Belan & Kostyuk, 2002). Undoubtedly, changes to different glutamate receptors will disrupt the excitability of networks. Whilst the expression of KARs increase as a result of axonal sprouting (Pinheiro & Mulle, 2008), axonal sprouting itself maybe a secondary mechanism. On the other hand, NMDA receptors have been extensively documented to be involved in seizure generation and LTP. Pre- and postsynaptic NMDA receptor activation of interneurons and pyramidal cells play an intricate role in regulating the excitation of neuronal networks. Numerous studies have also suggested NMDA receptors are altered significantly

over the course of epileptogenesis and MK801 could be a useful tool in investigating NMDA receptor changes during epileptogenesis. A prominent mechanism through which NMDA receptors exert control over excitability is via activation of NO signalling pathways which also has pro and anticonvulsant action and has been proposed to play a fundamental role in the development of epileptogenesis. The current study assessed how NMDA drive of inhibition changes during epileptogenesis in comparison to controls. Secondly the role of the NO pathway in mediating NMDA drive of inhibition during epileptogenesis was evaluated.

5.2 Results

5.2.1. NMDA drive of inhibition during epileptogenesis

To explore NMDA drive of inhibition during epileptogenesis, transverse combined hippocampal-entorhinal slices were prepared from rats that had undergone the refined Li-pilocarpine model at four different time points: 24 hours, 1 week, 5 weeks and 12 weeks+ post SE. Slices from age matched controls were also prepared. Slices were exposed to 0$[Mg]^{2+}$ aCSF for 25 minutes, after which a MK801 (+) MK801 maleate (Dizocipline)) dose response (0.001 – 30 μM) experiment was conducted to evaluate which concentrations promote or inhibit IDs in epileptic and control tissue *in vitro*.

As illustrated in Figure 5-2 to 5-6, IDs could readily be evoked in control and epileptic slices at different stages of epileptogenesis with 0.1-0.3 μM MK801 application. Statistical analysis of this data is represented in figure 5-7. Due to both independent and dependent variables being categorical in nature, analyses options were limited and therefore had to be made simpler.

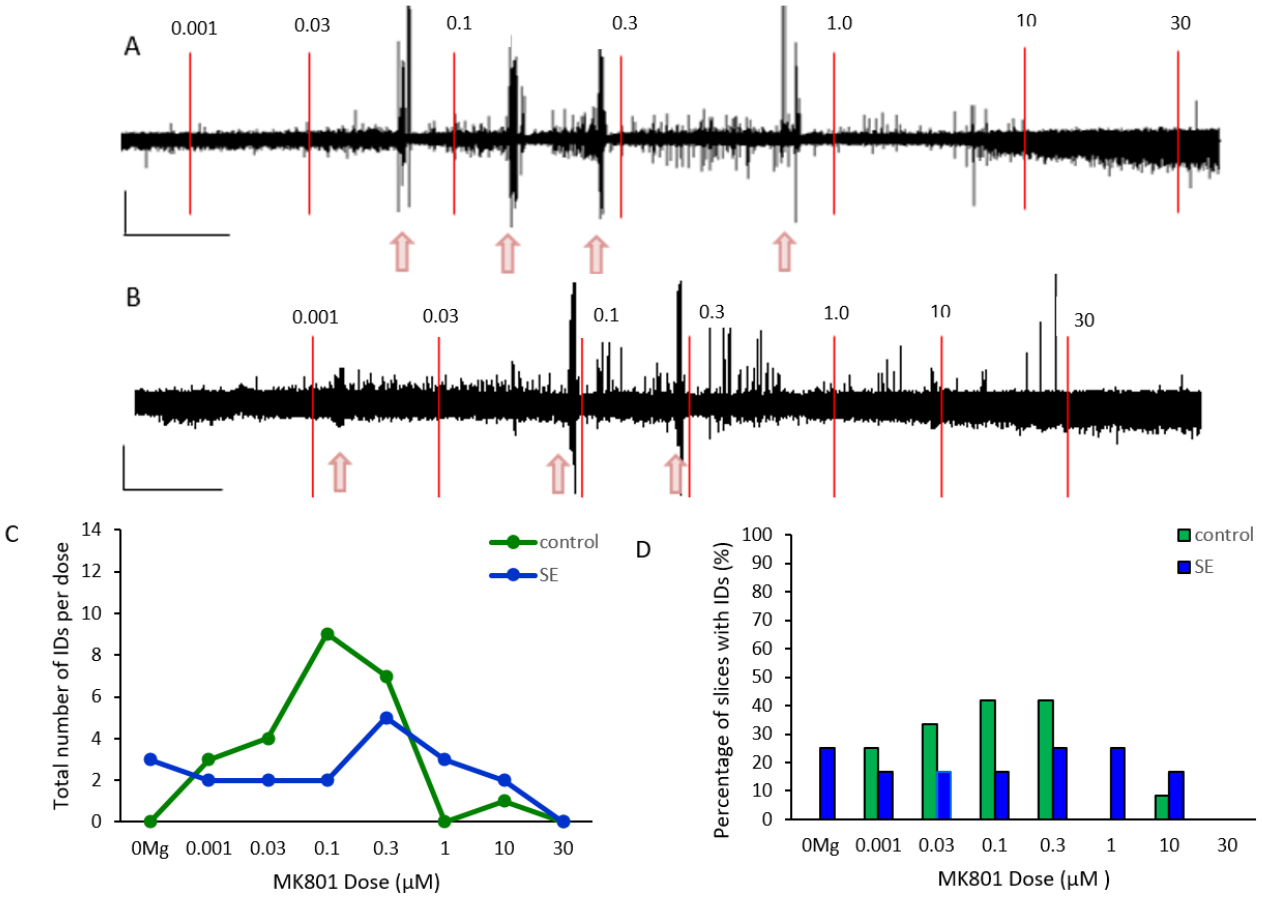

Figure 5-2. Ictal discharges at different MK801 concentrations in control and epileptic slices 24 hours post SE. A. Ictal discharges in a 60 g control rat evoked by different MK801 concentrations (0.001 – 30 µM) Scale: 500 µV x 1000secs. Red lines indicate the point of application. Red arrows indicate IDs. B. Ictal discharges evoked in a 24 hour SE rat by different MK801concentrations. Scale: 100 µV x 1000 secs. C. Comparison of the total number of IDs at different MK801 concentrations in control (n = 12) and 24 hour SE rats (n = 12). D. Comparison of the percentage of slices which showed IDs with different MK801 concentrations in control and 24 hour SE rats.

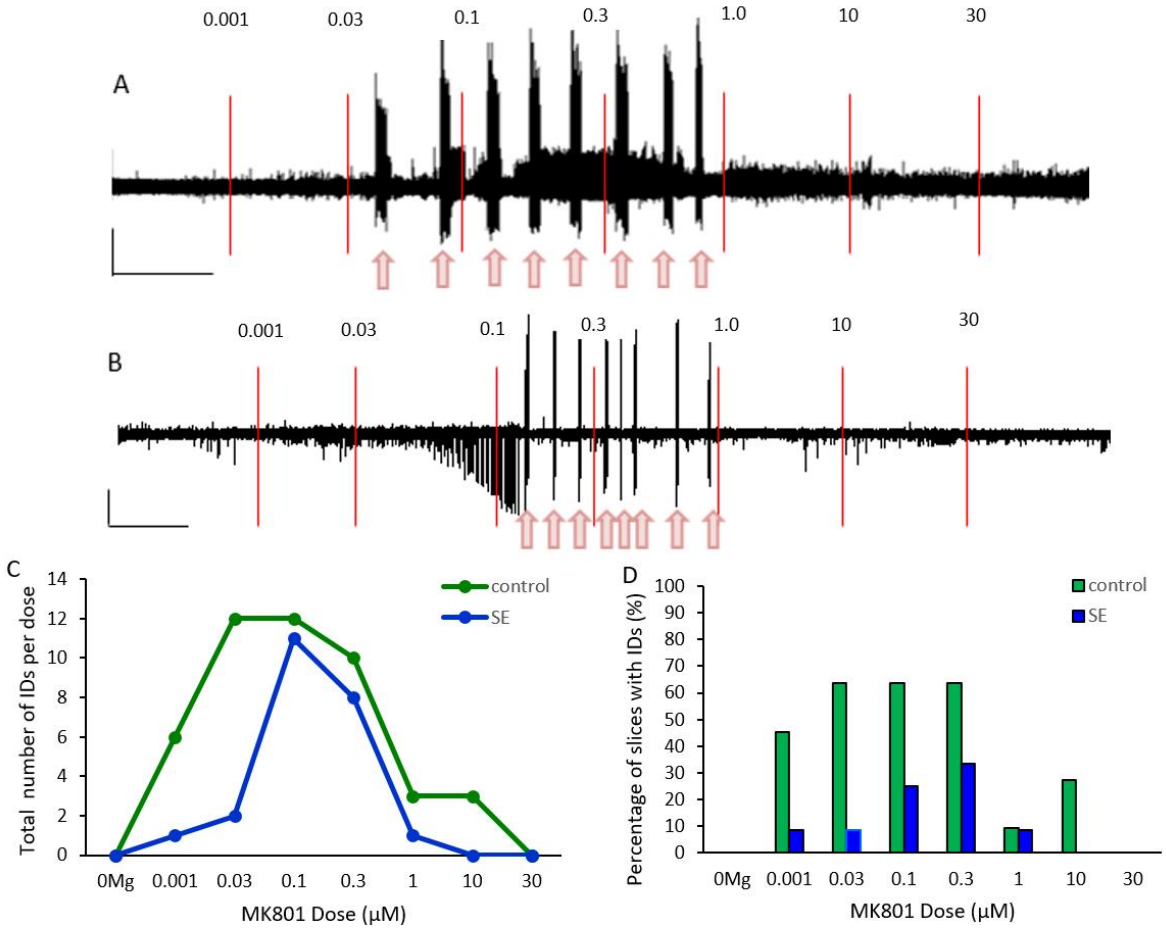

Figure 5-3. Ictal discharges at different MK801 concentrations in control and epileptic slices 1 week post SE. A. Ictal discharges in a 85 g control rat evoked by different MK801 concentrations (0.001 – 30 μM) Scale: 100μV x 1000 secs. Red lines indicate the point of application. Red arrows indicate IDs. B. Ictal discharges evoked in a 1 week SE rat by different MK801 concentrations. Scale: 500 μV x 1000 secs. C. Comparison of the total number of IDs at different MK801 concentrations in control (n = 12) and 1 week SE rats (n = 12). D. Comparison of the percentage of slices which showed IDs with different MK801 concentrations in control and 1 week SE rats.

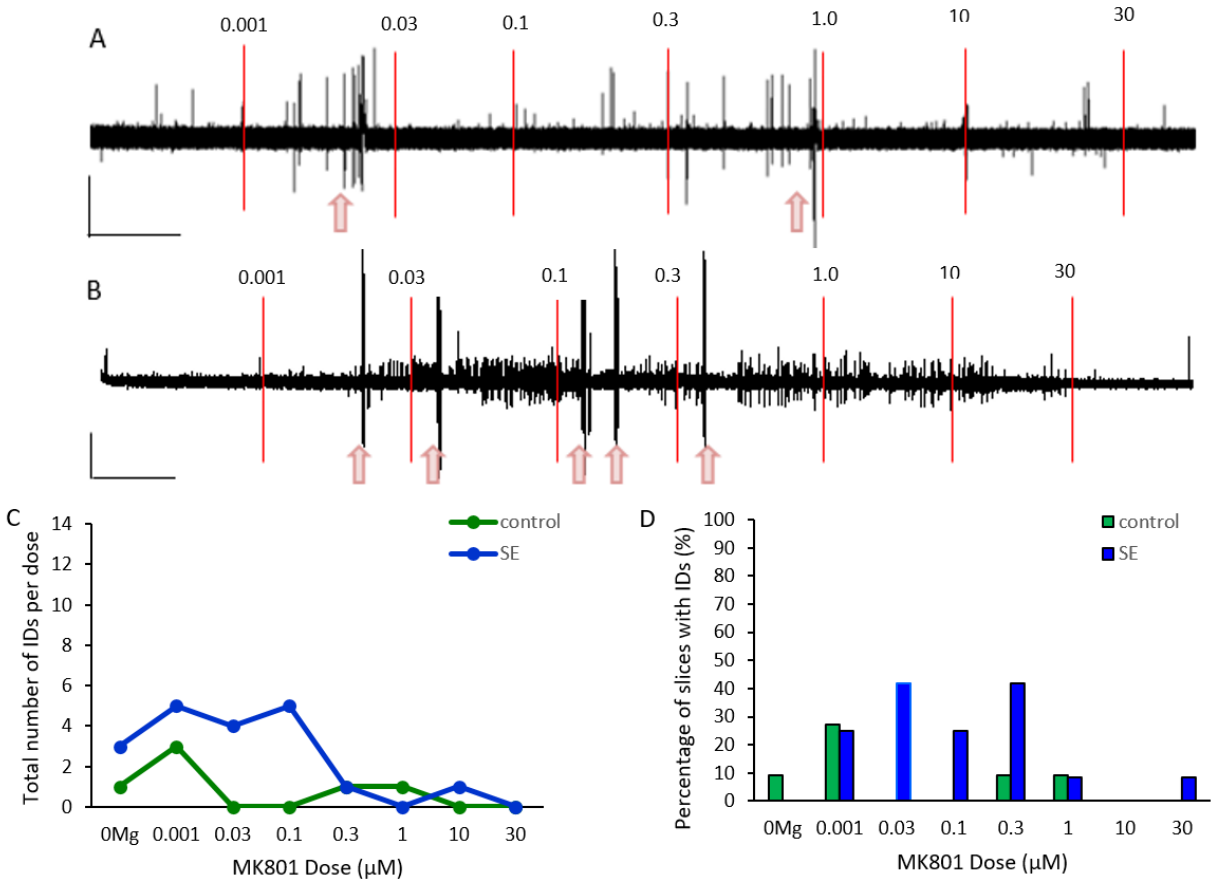

Figure 5-4. Ictal discharges at different MK801 concentrations in control and epileptic slices 5 weeks post SE. A. Ictal discharges in a 290 g control rat evoked by different MK801 concentrations (0.001 - 30 µM) Scale: 200 µV x 1000 secs. Red lines indicate the point of application. Red arrows indicate IDs. B. Ictal discharges evoked in a 5 week SE rat by different MK801 concentrations. Scale: 500 µV x 1000 secs. C. Comparison of the total number of IDs at different MK801 concentrations in control (n = 12) and 5 week SE rats (n = 12). D. Comparison of the percentage of slices which showed IDs with different MK801 concentrations in control and 5 week SE rats.

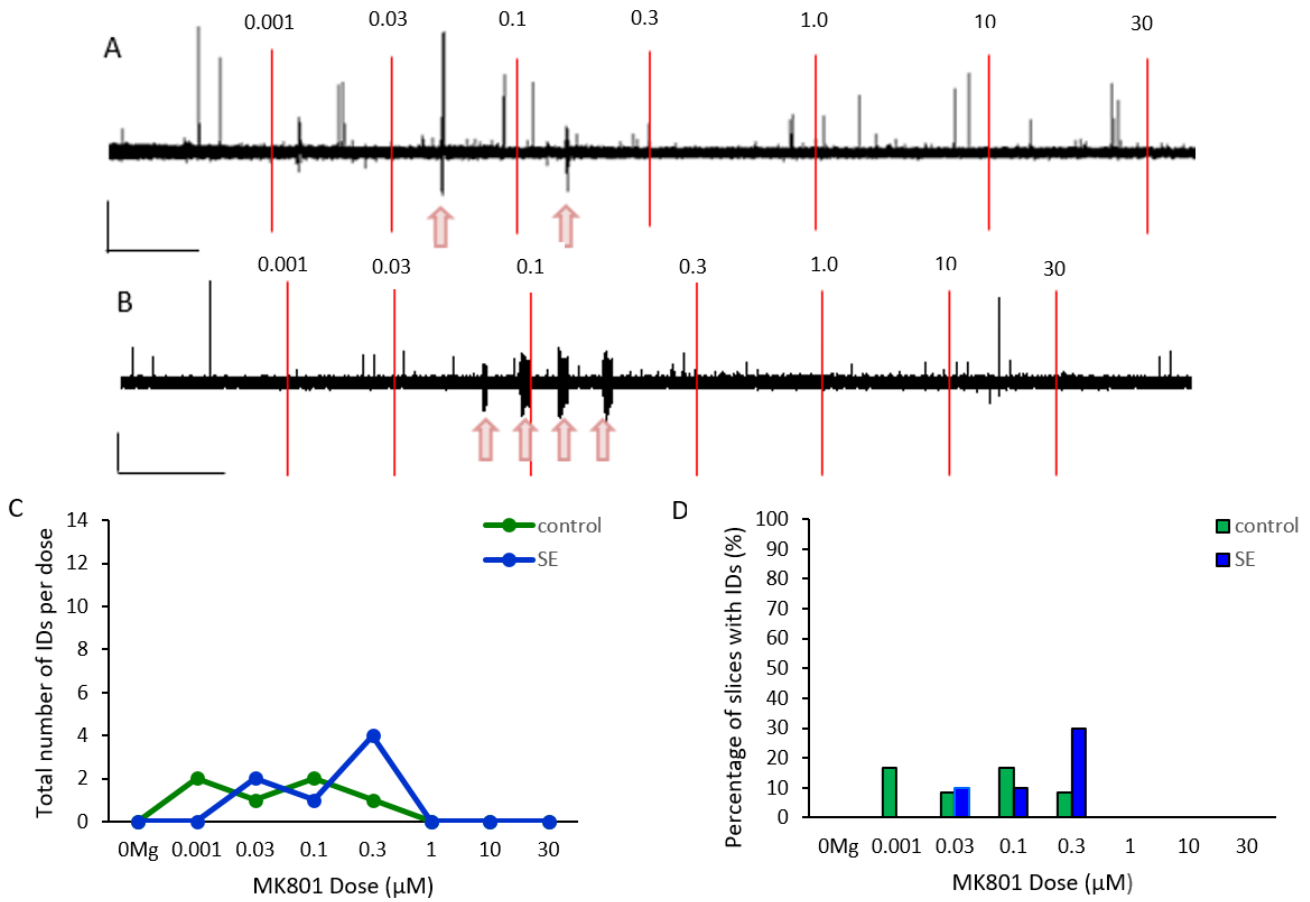

Figure 5-5. Ictal discharges at different MK801 concentrations in control and epileptic slices 12 weeks+ post SE. A. Ictal discharges in a 450 g control rat evoked by different MK801 concentrations (0.001 - 30 µM) Scale: 200 µV x 1000 secs. Red lines indicate the point of application. Red arrows indicate IDs. B. Ictal discharges evoked in a 12 weeks+ SE rat by different MK801 concentrations. Scale: 500 µV x 1000 secs. C. Comparison of the total number of IDs at different MK801 concentrations in control (n = 8) and 12 weeks+ SE rats (n = 12). D. Comparison of the percentage of slices which showed IDs with different MK801 concentrations in control and 12 weeks+ SE rats.

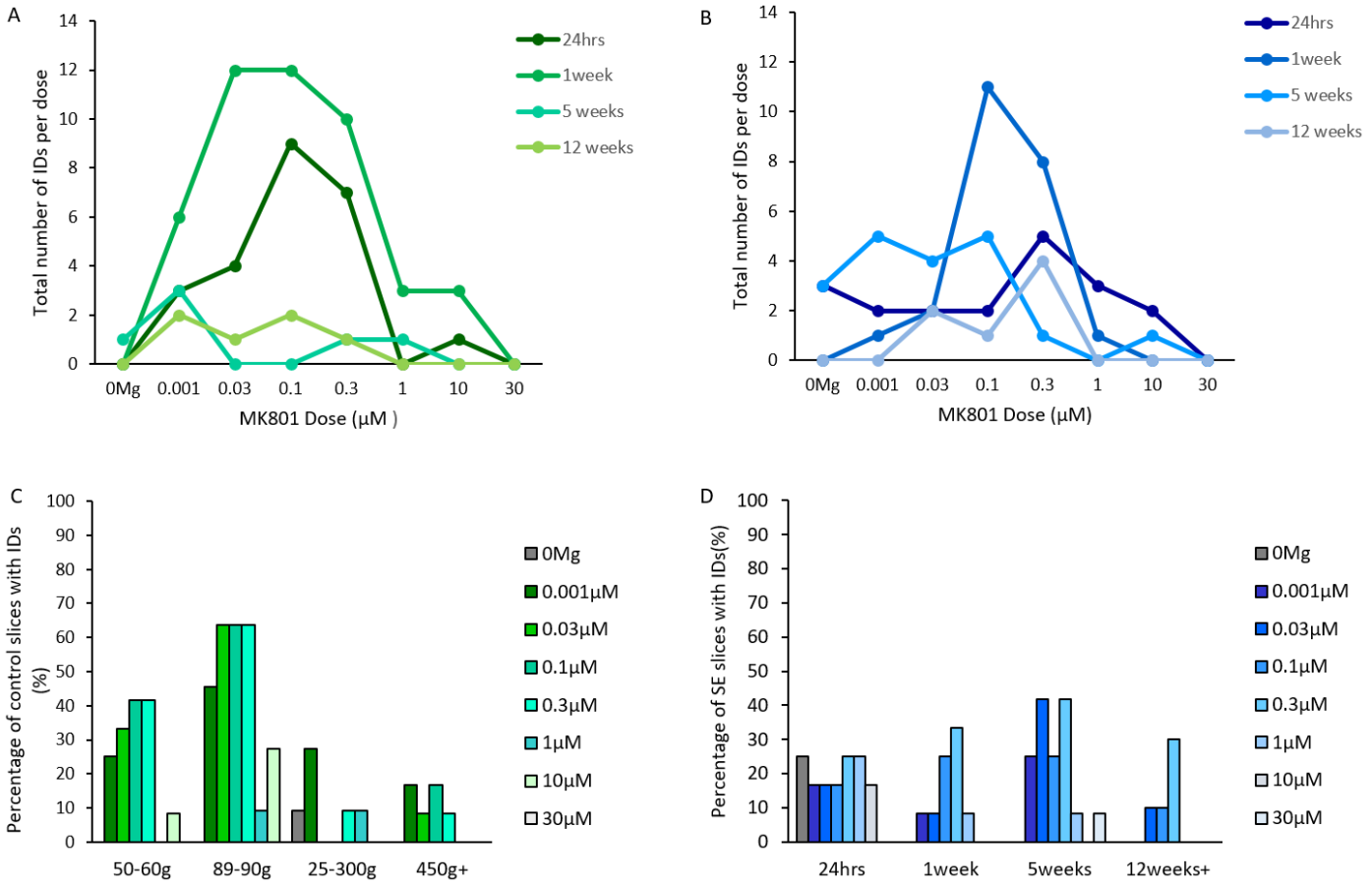

Figure 5-6. Excitability of control and epileptic slices with different MK801 concentrations at different stages of epileptogenesis. A. Total number of IDs at different MK801 concentrations in control slices at different developmental stages of epileptogenesis. B. Total number of IDs at different MK801 concentrations in epileptic slices at different developmental stages of epileptogenesis. C. Developmental changes in the percentage of control slices with IDs at different MK801 concentrations. D. Developmental changes in the percentage of epileptic slices with IDs at different MK801 concentrations.

To statistically assess whether there were differences in the number of slices that showed IDs at different MK801 concentrations, two Friedman's tests conducted on control and epileptic tissues. The results of the Freidman's test of control slices showed, irrespective of age, there were significant differences in the number of control slices that showed IDs at different MK801 concentrations, χ^2 (6, n = 46) = 37.36, $p < 0.01$ (see Figure 5-7A). Control slices showed more IDs at concentrations of 0.1 µM and 0.3 µM MK801. Based on this information, post-hoc planned comparisons were carried out with Wilcoxon signed-rank tests, with a Bonferroni correction applied ($p < 0.01$). As shown in figure 5-7A there were significant differences between the number of control slices that showed IDs at 0.1 µM MK801 and 1 µM ($Z = - 3.21$, $p < 0.01$), 10 µM ($Z = - 3.16$, $p < 0.01$), 30 µM ($Z = - 3.74$, $p < 0.01$) MK801 concentrations. There were also significant differences between, 0.3 µM MK801 and 1 µM ($Z = - 3.00$, $p < 0.01$), 10 µM ($Z = - 2.89$, $p < 0.01$), 30 µM ($Z = - 3.74$, $p < 0.01$) MK801 concentrations. The results show, irrespective of age 0.1- 0.3 µM MK801 were the optimal concentrations for evoking IDs in control tissue.

Similarly, the results of the Freidman's test of SE slices showed, there were significant differences in the number of SE slices that showed IDs at different MK801 concentrations, χ^2 (6, n = 46) = 37.36, $p < 0.01$ (see Figure 5-7B). SE slices showed higher number of IDs at 0.3 µM MK801. Based on this information, post-hoc planned comparisons were carried out with Wilcoxon signed-rank tests with a Bonferroni correction applied ($p < 0.01$). As shown in figure 5-7B there were significant differences between the number of control slices that showed IDs at 0.1 µM MK801 and 30 µM MK801 ($Z = -2.53$, $p < 0.01$). There were also significant differences between, 0.3 µM MK801 and 1µM ($Z = - 2.67$, $p < 0.01$), 10 µM ($Z = - 3.60$, $p < 0.01$), 30 µM ($Z = - 3.74$, $p < 0.01$) MK801 concentrations. The results show, irrespective of age 0.1- 0.3 µM MK801 were the optimal concentrations for evoking IDs in epileptic tissue.

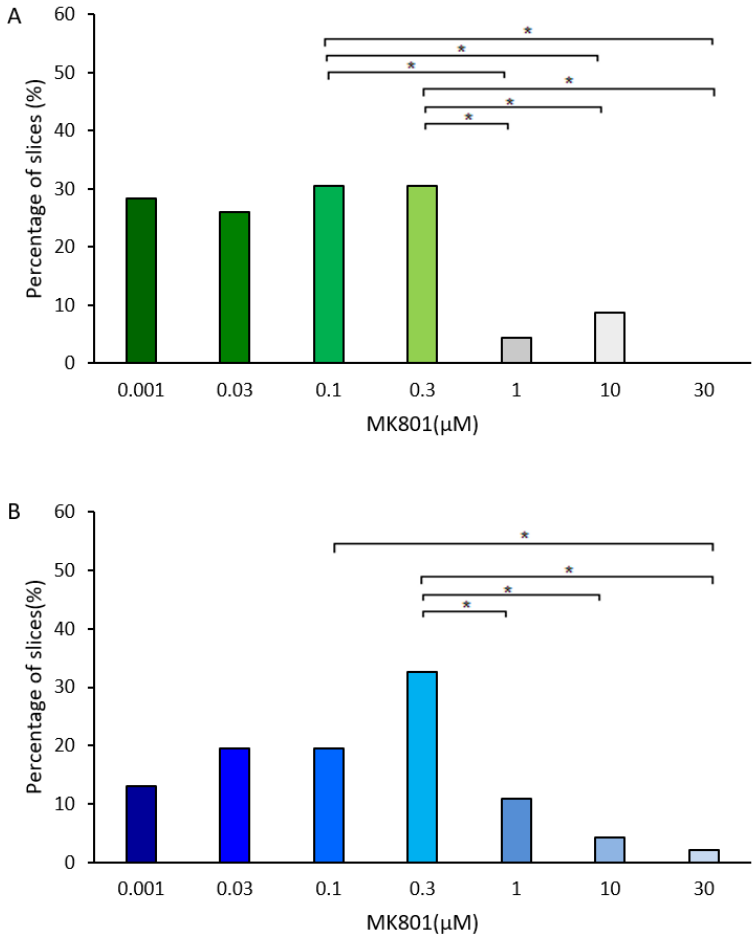

Figure 5-7. Excitability of control and epileptic slices with different MK801 concentrations. A. Percentage of excitable control slices (pooled ages) with different concentrations of MK801. B. Excitability of SE slices (pooled ages) with different concentrations of MK801.

There was a trend for excitability (percentage of slices with IDs and accumulative number of IDs) with 0.1- 0.3 μM MK801 applications to gradually decrease in control animals post 80-90 g. However, this trend was not present in tissue that had undergone the refined Li-pilocarpine model of epilepsy. As illustrated in figure 5-8 and summarised in table 5.1, excitability in SE slices remained high during the later latent period (5 weeks post SE) and during the chronic phases of epileptogenesis (12 week+ post SE).

To investigate if there were significant differences in the percentage of slices with IDs with 100 nM MK801 application in control and epileptic slices at different stages of epileptogenesis, a logistic regression analysis was conducted (see Figure 5-8A). The full model containing predictor variables, status and age was significant, χ^2 (7, N= 91) = 17.05, $p < 0.05$, indicating the model was able to distinguish between the presence and absence of IDs in slices at 100 nM MK801. The model explained between 17.1% (Cox & Snell R square) and 25.2 % (Nagelkerke R square) of the variance, and correctly classified 78 % of cases. Status, age and the status x age interaction effect did not make a unique significant

contribution to the model, $p > 0.05$. These results suggest there were no differences between the excitability of control and epileptic slices during epileptogenesis.

To assess if there were significant differences in the percentage of slices with IDs with 0.3 µM MK801 application in control and epileptic slices at different stages of epileptogenesis, a logistic regression analysis was conducted (see Figure 5-8B). The full model containing predictor variables, status and age was not significant, χ^2 (7, N= 91) = 12.51, $p > 0.05$, indicating the model was not able to distinguish between the presence and absence of IDs in slices at 0.3 µM MK801 (i.e. no better than chance). The model explained between 12.8 % (Cox & Snell R square) and 18.0 % (Nagelkerke R square) of the variance, and correctly classified 71.4 % of cases. Status, age and the status x age interaction effect did not make a unique significant contribution to the model, $p > 0.05$. These results suggest there were no differences between the excitability of control and epileptic slices during epileptogenesis.

These results indicate there were no differences in excitability induced by 0.1- 0.3 µM MK801 applications between control and epileptic slices at different stages of epileptogenesis. Nevertheless, there is a clear trend for excitability to decrease gradually in controls but not in epileptic slices with development (see Figure 5-8A and B).

Table 5.1. Summary of excitability of control and SE slices with 0.1- 0.3 µM MK801

			Percentage of slices with IDs (%)		Total number of IDs	
Status	**Age**	**N**	**0.1µM MK801**	**0.3µM MK801**	**0.1µM MK801**	**0.3µM MK801**
Control	24 hours	12	41.67	41.67	9	7
	1 week	11	63.64	63.64	12	10
	5 weeks	11	0	9.09	0	1
	12 weeks+	12	16.67	8.33	2	1
SE	24 hours	12	16.67	25.00	2	5
	1 week	12	25.00	33.33	11	8
	5 weeks	12	25.00	41.67	5	1
	12 weeks+	10	10.00	30.00	1	4

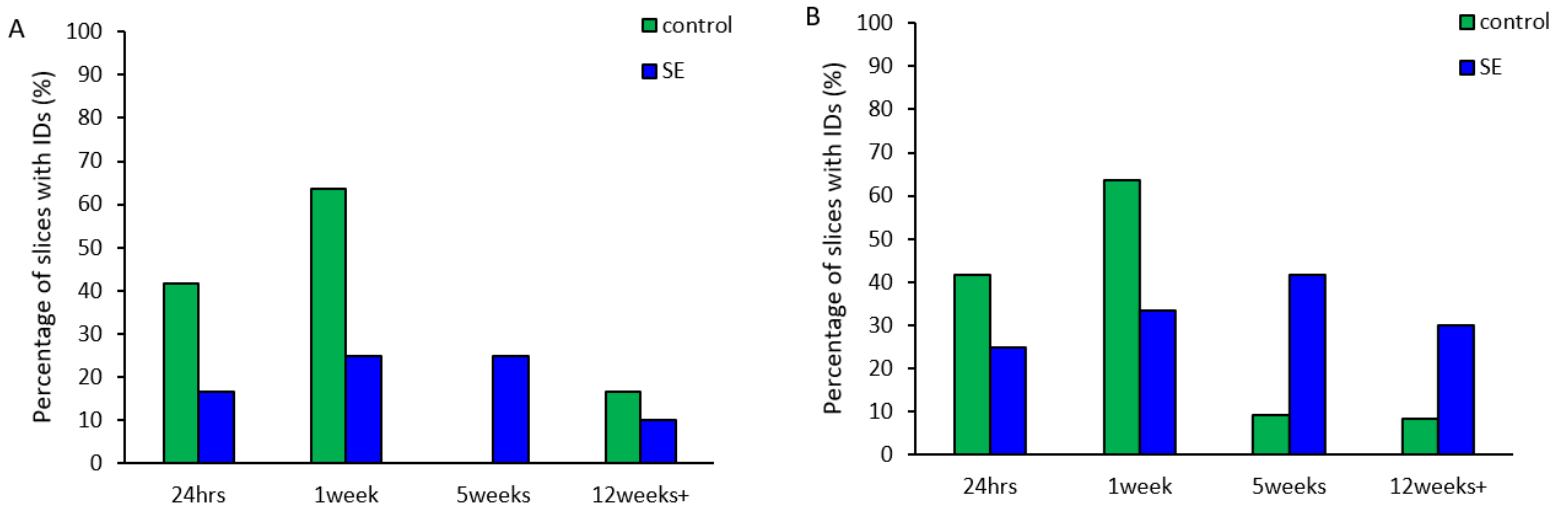

Figure 5-8. Excitability of control and epileptic slices at 0.1 and 0.3μM MK801. A. Percentage of slices with IDs with 0.1 μM MK801 in control and epileptic slices at different stages of epileptogenesis. B. Percentage of slices with IDs with 0.3 μM MK801 in control and epileptic slices at different stages of epileptogenesis.

It could be argued that the presence of IDs present in these experiments, was not due to MK801 application and instead occurred as a result of 0$[Mg]^{2+}$ aCSF. However, it is clearly illustrated in figures 5-2 to 5-5 that IDs occurred prominently during application of 0.1- 0.3 μM MK801. Moreover, examination of the latency to first ID in control and epileptic slices, with and without MK801 application showed large differences, suggesting bursts of ictal activity was due to the application of MK801, as demonstrated in figure 5-9.

A between-subjects ANOVA showed there were significant differences between pooled control slices with and without MK801 application (0.001- 30 μM) in the latency to first seizure F (1, 57) = 26.29, $p < 0.01$ (see Figure 5-9A). Post-hoc analysis showed control slices without MK801 has a longer latency to first ID (7149.88 ± 500.92 seconds) in comparison to control slices with MK801 (3602.76 ± 203.17 seconds). There was no interaction effect between MK801 application and age in control slices, $p > 0.05$ (see Figure 5-9B).

Further ANOVA analysis showed there were significant differences in the latencies to first ID in epileptic slices with and without MK801 application, F (1, 130) = 18.41, $p < 0.01$ (see Figure 5-9A). Post-hoc analysis showed SE slices without MK801 showed shorter latencies to first ID (2890.36 ± 128.31 seconds) in comparison to SE slices with MK801 (4871.90 ± 536.67 seconds). There was no interaction effect between MK801 application and age in epileptic slices, $p > 0.05$ (see Figure 5-9C).

Looking at figure 5-9B and 5-9C, it would appear that MK801 is more pro-convulsant in controls and more anticonvulsant in epileptic slices. To test this hypothesis, a between subjects ANOVA was conducted, but there was no significant interaction between MK801 application, SE status and age F (3, 187) = 2.22, $p = 0.09$.

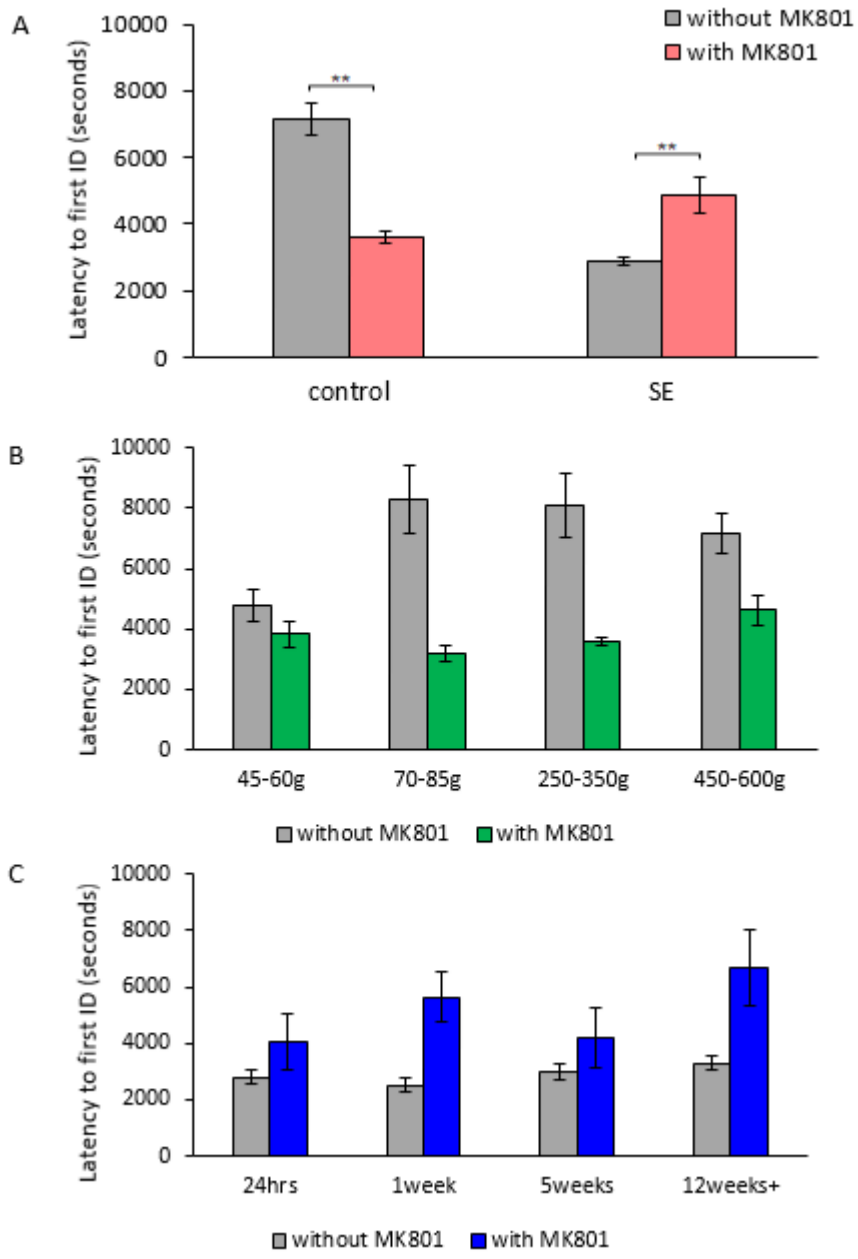

Figure 5-9. Latencies to first IDs in control and epileptic tissue, without and with MK801 application (0.001- 30µM). A. Latency to first ID in control and epileptic tissue with and without MK801 application B. Latency to first ID in control tissue, at different ages, without and with MK801 application. C. Latency to first ID in epileptic tissue, at different ages, without and with MK801 application.

5.2.2. Role of NO pathway in the NMDA drive of inhibition

As demonstrated in section 5.2.1 the NMDA receptor antagonist, MK801, was capable of evoking IDs in control and epileptic tissue. This trend decreased with development in controls but not epileptic tissue. Here, the role of NO mediating MK801 induced IDs is evaluated. Epileptic slices were exposed to 0$[Mg]^{2+}$ aCSF and the nitric oxide synthase inhibitor, 7-nitroinadazole (7-NI) (200 µM), after which MK801 concentrations (0.03 – 10 µM) were applied to evaluate whether IDs still precipitated.

To investigate whether there were significant differences in the percentage of SE slices that showed IDs at 0.1 μM MK801 in 0$[Mg]^{2+}$ aCSF or 0$[Mg]^{2+}$ and 7-NI aCSF, a logistic regression analysis was conducted (see Table 5.2 for summary). The full model containing predictor variables condition (in 0$[Mg]^{2+}$ aCSF or 0$[Mg]^{2+}$ and 7-NI aCSF) and age was not significant χ^2 (7, 92) = 3.31, $p > 0.05$, indicating the model was not able to distinguish between the presence and absence of IDs in slices at 0.1μM MK801 (i.e. no better than chance). The model explained between 3.5 % (Cox & Snell R square) and 5.5 % (Nagelkerke R square) of the variance, and correctly classified 79.3 % of cases. Condition, age and the status x age interaction effect did not make a unique significant contribution to the model, $p > 0.05$. These results suggest application of the NO synthase inhibitor, 7-NI, did not affect the percentage of slices with IDs induced by 0.1 μM MK801 at different stages of epileptogenesis (see figures 5-10 and 5-11).

To investigate whether there were significant differences in the percentage of SE slices that showed IDs at 0.3 μM MK801 in 0$[Mg]^{2+}$ aCSF or 0$[Mg]^{2+}$ and 7-NI aCSF, a logistic regression analysis was conducted. The full model containing predictor variables condition (in 0$[Mg]^{2+}$ aCSF or 0$[Mg]^{2+}$ and 7-NI aCSF) and age was not significant χ^2 (7, 92) = 7.96, $p > 0.05$, indicating the model was not able to distinguish between the presence and absence of IDs in slices at 0.3 μM MK801 (i.e. no better than chance). The model explained between 8.3 % (Cox & Snell R square) and 12.8 % (Nagelkerke R square) of the variance, and correctly classified 78.3% of cases. Condition, age and the status x age interaction effect did not make a unique significant contribution to the model, $p > 0.05$. These results suggest application of the NO synthase inhibitor, 7-NI, did not affect the percentage of slices with IDs induced by 0.3 μM MK801 at different stages of epileptogenesis (see figure 5-10 and 5-11).

Table 5.2. Percentage of SE slices with IDs with and without 7-nitraindazole

			Percentage of slices with IDs (%)				
Condition	**Age**	**N**	**0.03μM MK801**	**0.1μM MK801**	**0.3μM MK801**	**1.0μM MK801**	**10μM MK801**
SE without 7-NI	24 hours	12	16.67	16.67	25.00	25.00	16.67
	1 week	12	8.33	25.00	33.33	8.33	0
	5 weeks	12	41.67	25.00	41.67	8.33	0
	12 weeks+	10	10.00	10.00	30.00	0	0
SE with 7-NI	24 hours	10	20.00	10.00	10.00	0	0
	1 week	12	0	16.67	8.33	0	8.33
	5 weeks	12	33.33	33.33	16.67	0	0
	12 weeks+	12	16.67	25.00	25.00	8.33	16.67

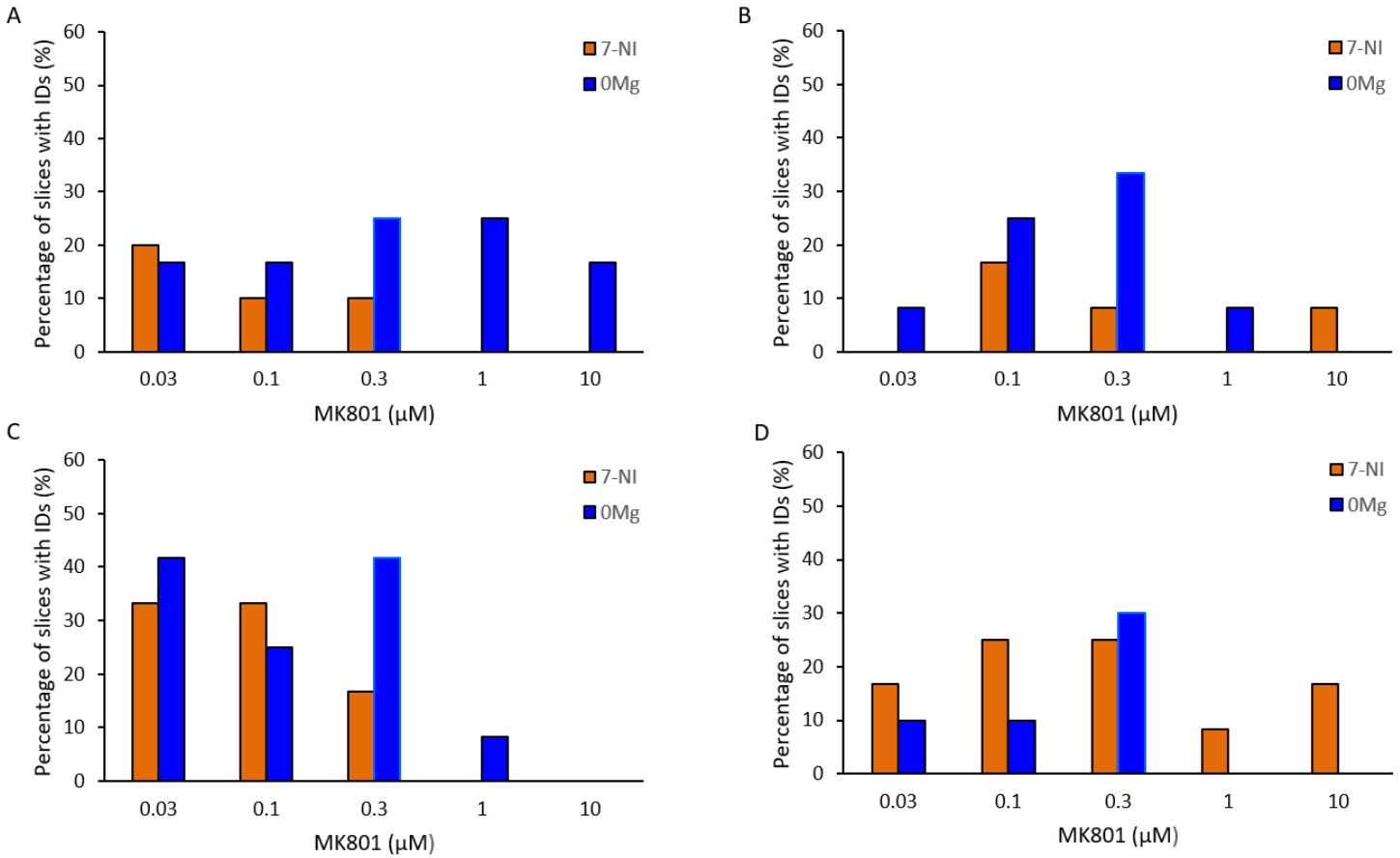

Figure 5-10. Percentage of slices with IDs at different MK801 concentrations in 7-nitraindazole (200 μM) and/or 0[Mg]$^{2+}$ aCSF during epileptogenesis. A. Percentage 24 hours SE slices with IDs at different MK801 concentrations in 7-NI and/or 0[Mg]$^{2+}$. B. Percentage 1 week SE slices with IDs at different MK801 concentrations in 7-NI and/or 0[Mg]$^{2+}$. C. Percentage 5 week SE slices with IDs at different MK801 concentrations in 7-NI and/or 0[Mg]$^{2+}$. D. Percentage 12 weeks+ SE slices with IDs at different MK801 concentrations in 7-NI and/or 0[Mg]$^{2+}$.

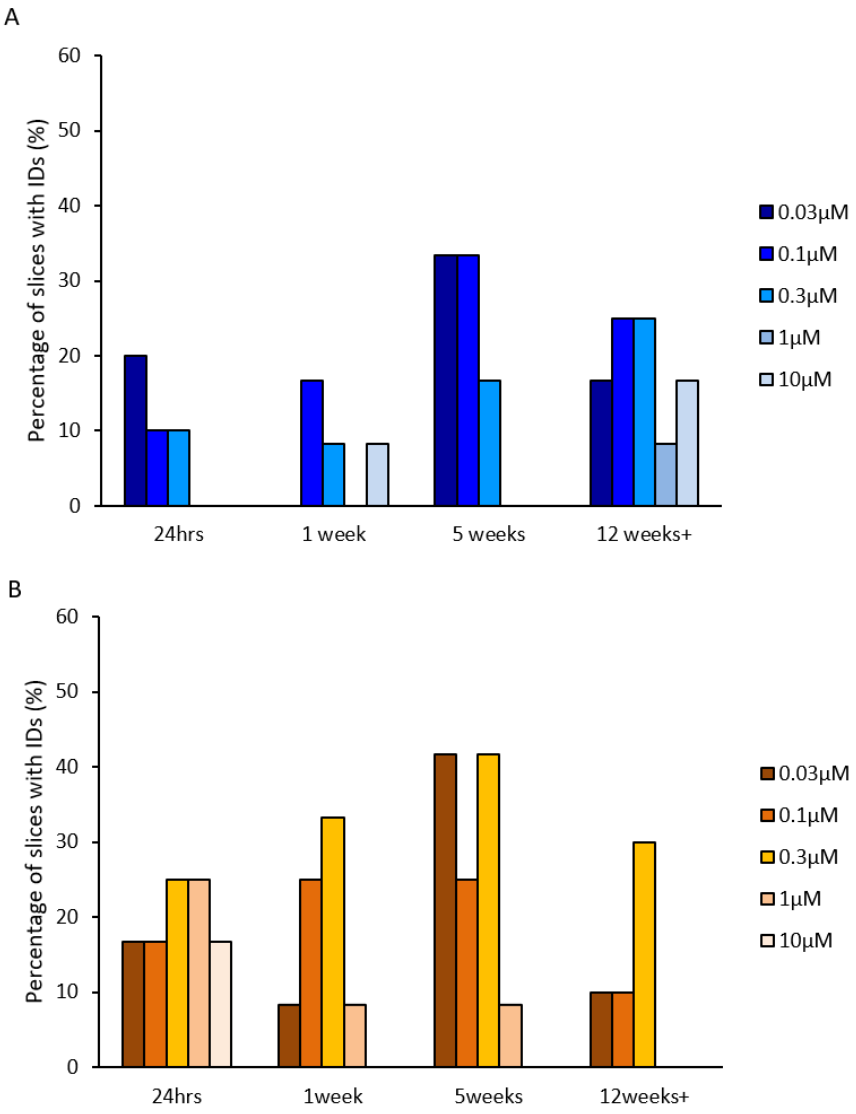

Figure 5-11. The effects of 7-nitraindazole on MK801 ID inducing effects during epileptogenesis. A. Percentage of SE slices with IDs at difference MK801 concentrations, during epileptogenesis. B. Percentage of SE slices with IDs at different MK801 concentrations, during epileptogenesis after 7-NI exposure.

5.2.3. Changes in GABAergic events during epileptogenesis

The findings presented thus far demonstrate that NMDA receptor activity deviates from normal physiological development during epileptogenesis. This is evident through a trend in which low MK801 concentrations (0.1- 0.3 μM) readily induces IDs throughout epileptogenesis, whereas in controls this excitability decreases. As illustrated in section 5.2.2, these ID inducing effects of low MK801 concentrations are not mediated through the NO signalling pathway, as the NO synthase inhibitor, 7-nitraindazole failed to significantly reduce excitability. Here, the developmental changes of GABAergic events during epileptogenesis are explored further.

Bazelot et al. (2010) demonstrated interneurons, but not pyramidal cells, were capable of evoking field potentials at monosynaptic latencies. These inhibitory fields were shown to be GABAergic in nature and originated from postsynaptic sites as they were suppressed by low external Cl^-. Further electrode array investigation revealed events generated at dendritic sites had similar amplitudes to perisomatic events, but occurred less frequently. Based on this study, the alterations to GABAergic activity during epileptogenesis are explored here on a similar neuronal network level.

In a similar fashion to Bazelot et al (2010), figure 5-12 illustrates slow field events are inhibitory in nature. Blocking of excitatory transmission with CNQX (20 μM) and AP5 (50 μM) reduced the frequency of events. Additional application of bicuculline (20 μM) suppressed slow extracellular events. Variable effects of amplitude were evident, but this experiment was only conducted in an 80 g and 250 g control rat for confirmatory purposes.

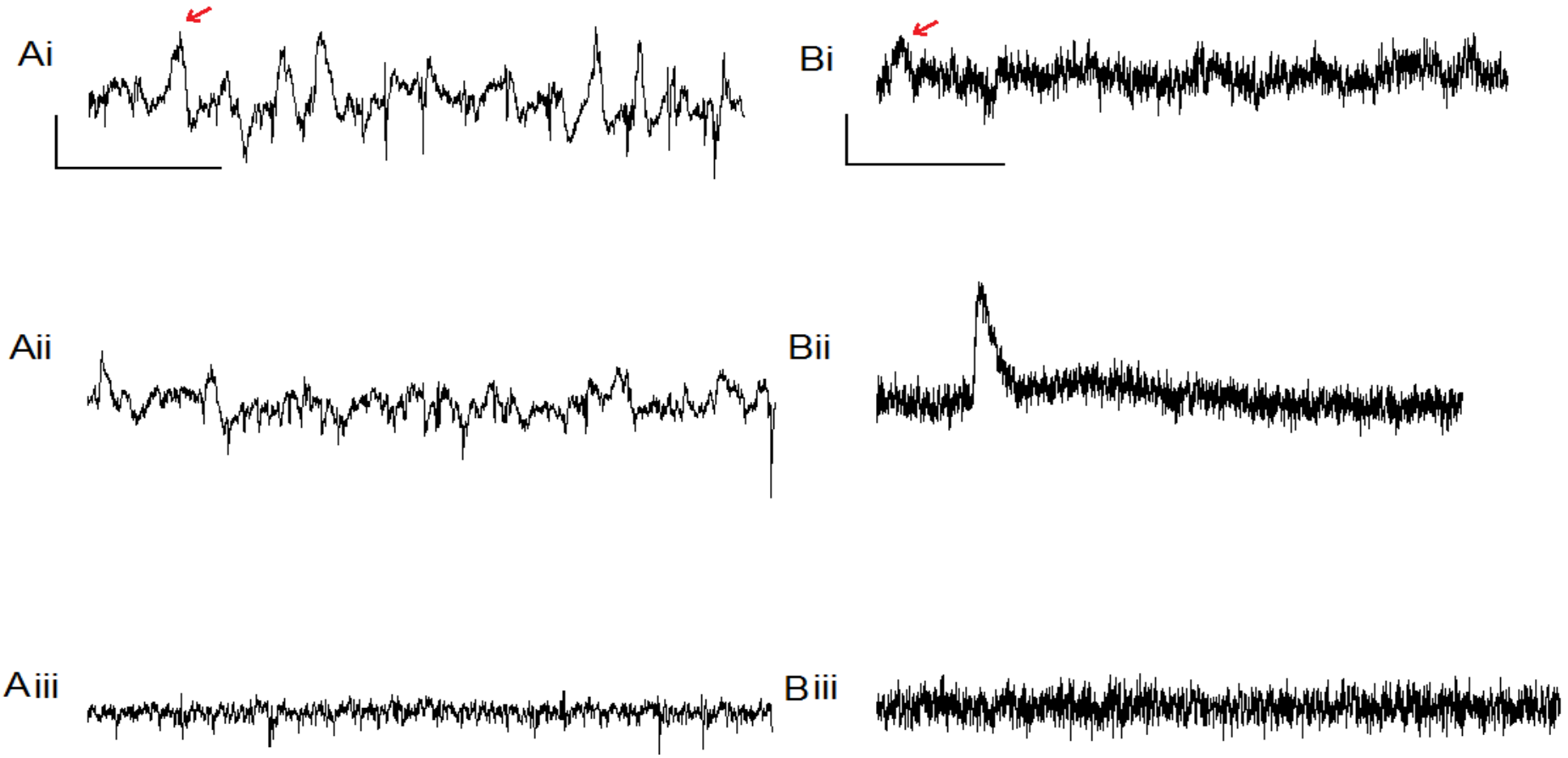

Figure 5-12. Extracellular events mediated by $GABA_A$ receptors in CA3 and layer II MEC. Ai. Extracellular CA3 trace showing spikes and extracellular field IPSPs (arrow) in the presence of KA (50 nM). Scale: 100 μV x 100ms. Aii. Extracellular CA3 trace showing spikes and extracellular field IPSPs in the presence of CNQX (20 μM) and AP5 (50 μM). Note reduction in frequency of field IPSPs. Aiii. Suppression of CA3 field IPSPs in the presence of bicuculline (20 μM). Bi. Extracellular MEC trace showing spikes and extracellular field IPSPs (arrow) in the presence of KA (50 nM). Scale: 50 μV x 100 ms. Bii. Extracellular MEC trace showing spikes and extracellular field IPSPs in the presence of CNQX (20 μM) and AP5 (50 μM). Note reduction in frequency of field IPSPs. Biii. Suppression of MEC field IPSPs in the presence of bicuculline (20 μM).

Results presented in section 5.2.1 demonstrated clear difference in ID inducing effect of low concentration MK801 (0.1- 0.3 μM) in SE and control slices during the latent period. A summary of the effects of MK801 in field IPSPs (IEIs and amplitude) in controls and SE slices at 1 week and 5 weeks post SE induction is presented in Table 5.3.

Table 5.3. The effects of MK801 on field IPSPs in control and SE slices

			IEI (seconds)		Amplitude (μV)	
Status	**Age**	**N**	**0[Mg]$^{2+}$**	**0.3μM MK801**	**0[Mg]$^{2+}$**	**0.3μM MK801**
Control	1 week	6	328.83 (± 126.00)	294.37 (± 72.27)	91.70 (± 4.85)	87.16 (± 2.58)
	5 weeks	4	7032.68 (± 873.31)	4326.66 (± 1238.60)	96.42 (± 12.50)	92.58 (± 3.83)
SE	1 week	6	1062.66 (± 348.77)	1801.33 (± 543.73)	95.08 (± 16.88)	93.83 (± 15.30)
	5 weeks	6	662.14 (± 118.33)	715.87 (± 185.23)	106.11 (± 12.47)	118.67 (± 8.66)

To investigate differences in the inter-event-intervals (IEIs) between field IPSPs in SE and control slices at 1 week post SE a mixed-model ANOVA was conducted on ranked data, due to the low complexity and small sample size used. As illustrated in figure 5-13C, there were significant differences in the IEIs between field IPSPs in control and SE slices at 1 week post SE, F (1, 10) = 13.04, $p < 0.01$. The IEIs between field IPSPs in SE slices was significantly longer in comparison to control slices. These results suggest inhibition is reduced in SE slices during the early latent period. There was no significant effect of MK801 on the IEIs between field IPSPs, $p > 0.05$. There was also no interaction effect between the effect of MK801 and status of animals (control vs SE), $p > 0.05$.

To investigate differences on the amplitude of field IPSPs in SE and control slices at 1 week post SE a mixed-model ANOVA was conducted on ranked data. As illustrated in figure 5-13D, there was no significant differences in the amplitude of field IPSPs in control and SE slices at 1 week post SE, $p > 0.05$. There was also no significant effect of MK801 the amplitude of field IPSPs, $p > 0.05$. Finally, there was no significant interaction effect between effect of MK801 and status of animal (control vs SE), $p > 0.05$.

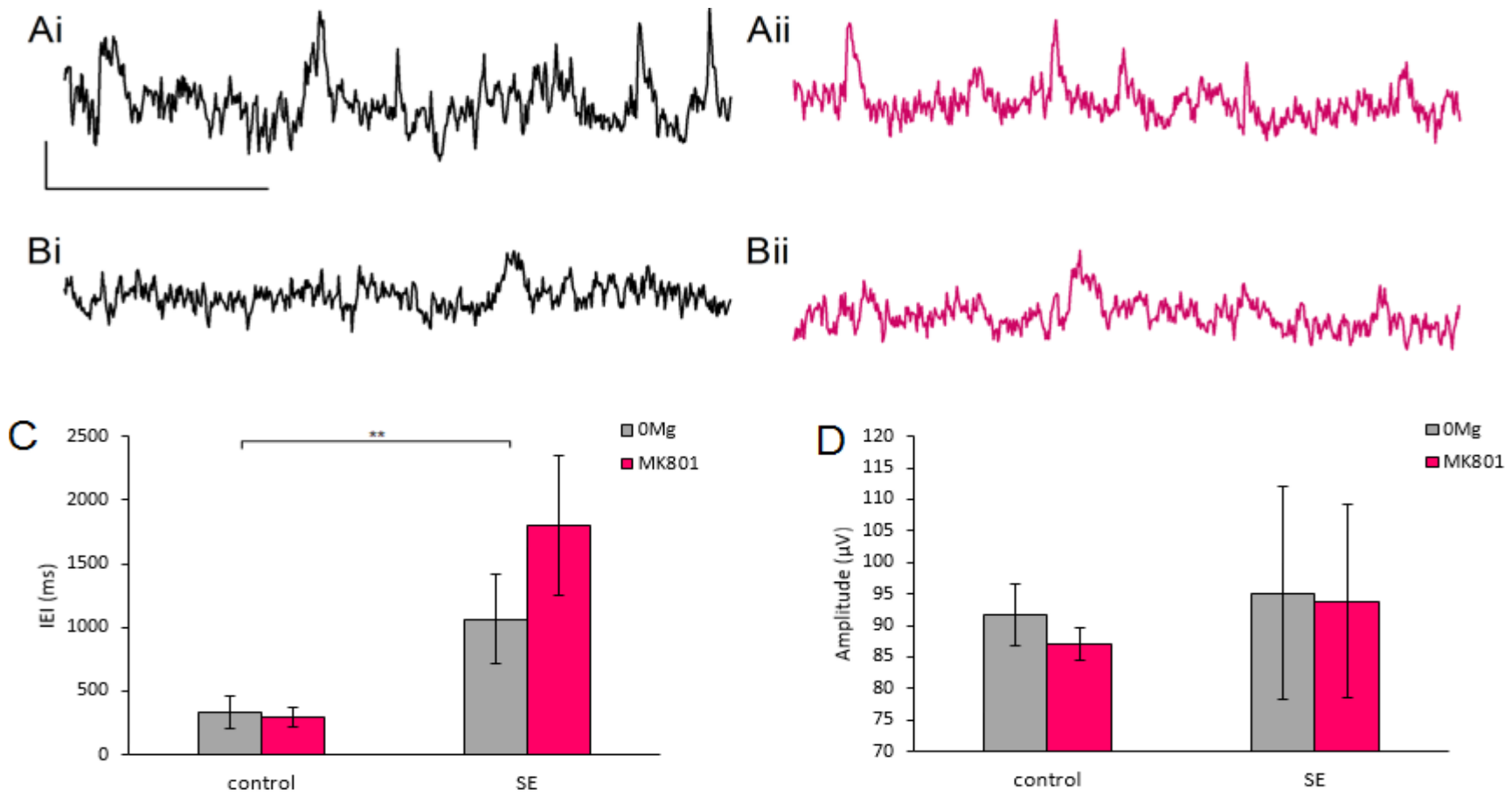

Figure 5-13. The effects of MK801 on field IPSPs in control and SE slices at 1 week post SE. Ai. Field IPSPs in an 80 g control slice in 0[Mg]$^{2+}$ aCSF (Bandpass second order filter 1-300 Hz). Scale: 20 µV x 200 ms. Aii. The effect of MK801 (300 nM) on field IPSPs in 80 g control slice. Bi. Field IPSPs in a 1 week SE slice in 0[Mg]$^{2+}$ aCSF. Bii. The effect of MK801 (300nM) on field IPSPs in a 1 week SE slice. C. The effects of MK801 on the IEIs between field IPSPs in control (0[Mg]$^{2+}$ aCSF: 328.83 ms ± 126.00, MK801: 294.37 ms ± 72.27) and 1 week SE slices(0[Mg]$^{2+}$ aCSF: 1062.66ms ± 348.77, MK801: 1801.33 ms ± 543.73). D. The effects of MK801 on the amplitude of field IPSPs in control (0[Mg]$^{2+}$ aCSF: 91.70 µV ± 4.85, MK801: 87.16 µV ± 2.58) and 1 week SE slices (0[Mg]$^{2+}$ aCSF: 95.08 µV ± 16.88, MK801: 93.08 µV ± 15.31).

To investigate differences in the IEIs between field IPSPs in SE and control slices at 5 weeks post SE a mixed-model ANOVA was conducted on ranked data. As illustrated in figure 5-14C, there were significant differences in the IEIs between field IPSPs in control and SE slices at 5 weeks post SE, F (1, 7) = 16.20, $p < 0.01$. The IEIs between field IPSPs were significantly longer in control slices compared to SE slices at 5 weeks post SE. These results suggest inhibition is enhanced in SE slices during the later latent period (5weeks) and contrast the results in figure 5-12 showing reduced inhibition in SE slices at 1 week post SE induction, and perhaps indicative of a compensatory mechanism. There was no significant effect of MK801 on the IEI's between field IPSPs, $p > 0.05$. There was also no interaction effect between the effect of MK801 and status of animals (control vs SE), $p > 0.05$. Despite these results it is interesting that there is a trend for MK801 to reduce IEIs between field IPSPs in controls but not SE slices at 5 weeks post SE.

To investigate differences on the amplitude of field IPSPs in SE and control slices at 5 weeks post SE a mixed-model ANOVA was conducted on ranked data. There was no significant differences in the amplitude of field IPSPs in control and SE slices at 5 weeks post SE, $p > 0.05$. There was also no significant effect of MK801 the amplitude of field IPSPs, $p > 0.05$. Finally, there was no significant interaction effect between effect of MK801 and status of animal (control vs SE), $p > 0.05$.

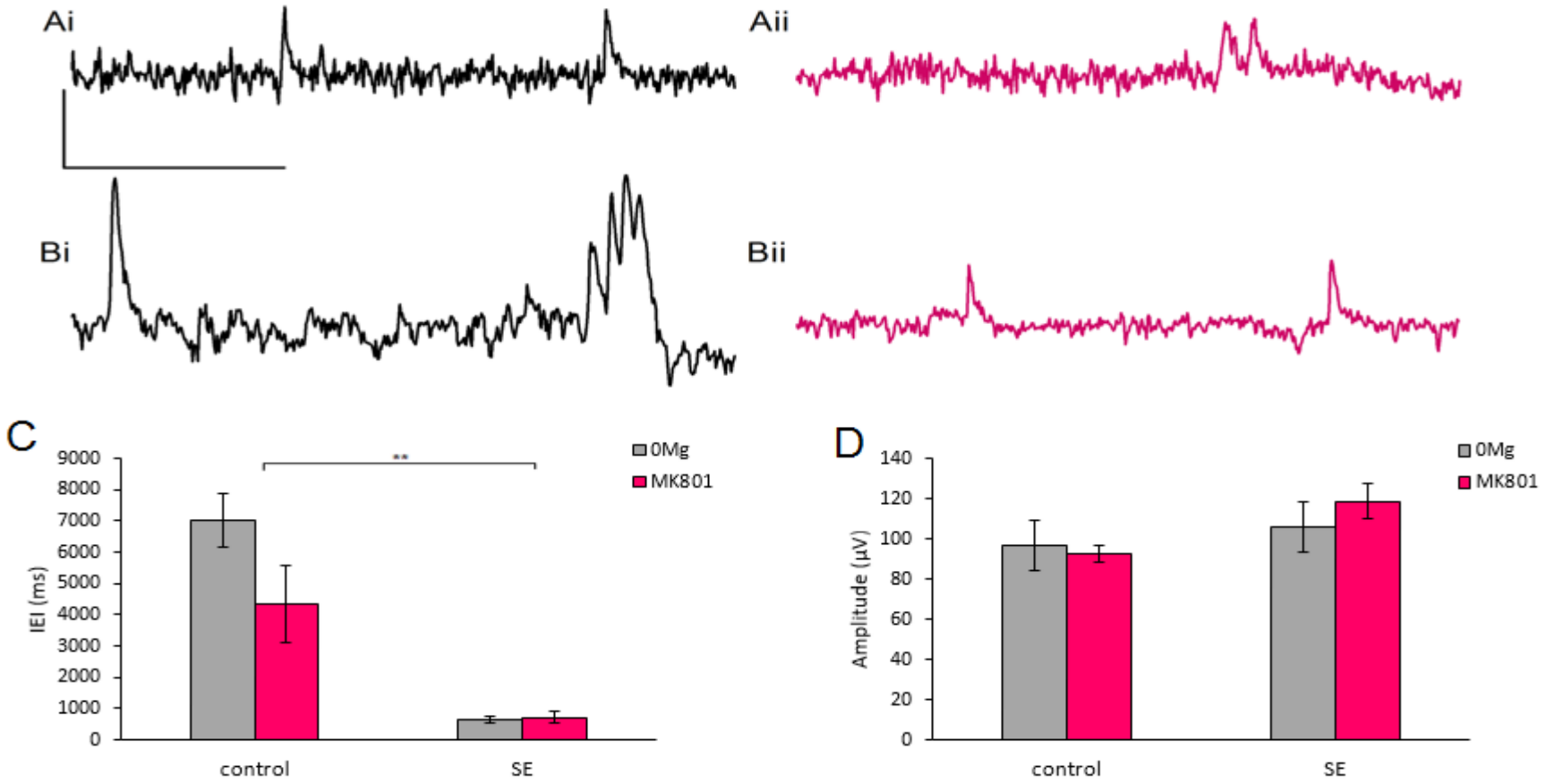

Figure 5-14. The effects of MK801 on field IPSPs in control and SE slices at 5 weeks post SE. Ai. Field IPSPs in a 300 g control slice in 0[Mg]$^{2+}$ aCSF. (Bandpass second order filter 1-300 Hz). Scale: 50 μV x 200 ms. Aii. The effect of MK801 (300 nM) on field IPSPs in 3000 g control slice. Bi. Field IPSPs in a 5 week SE slice in 0[Mg]$^{2+}$ aCSF. Bii. The effect of MK801 (300 nM) on field IPSPs in a 5 week SE slice. C. The effects of MK801 on the IEIs between field IPSPs in control (0[Mg]$^{2+}$ aCSF: 7032.68 ms ± 873.31, MK801: 4326.68 ms ± 1238.60) and 5 week SE slices (0[Mg]$^{2+}$ aCSF: 662.14 ms ± 118.33, MK801: 715.87 ms ± 185.23). D. The effects of MK801 on the amplitude of field IPSPs in control (0[Mg]$^{2+}$ aCSF: 96.42 μV ± 12.50, MK801: 92.58 μV ± 3.83) and 5 week SE slices (0[Mg]$^{2+}$ aCSF: 106.11 μV ± 12.47, MK801: 118.67 μV ± 8.67).

5.3 Discussion

The current study assessed how NMDA drive of inhibition changes during epileptogenesis in comparison to controls. Secondly the role of the NO pathway in mediating NMDA drive of inhibition during epileptogenesis was evaluated. Overall it was illustrated that IDs could be readily evoked by application of low concentrations of MK801 (0.1- 0.3 μM) in control and SE slices of all ages. There was a developmental trend for excitability in response to low concentrations of MK801 to decrease in controls and conversely excitability remained elevated in epileptic animals. Low dose MK801 ID inducing effects were not mediated by NO, as pre-treatment of slices with the NO synthase inhibitor, 7- nitroindazole, failed to exert any significant effects on IDs. Finally, MK801 did not exert any significant effects on field IPSPs, either. Overall, changes in NMDA receptor activity is evident during epileptogenesis but the mechanisms through which this alteration affects excitability remains to be elucidated as shall be discussed.

5.3.1 Developmental NMDA drive of inhibition in controls and epileptic slices

Numerous studies have demonstrated the ability of glutamate in modulating inhibition. Glutamate receptors have been well documented to be involved in plasticity associated with LTP and epilepsy, therefore understanding the mechanisms behind excitatory modulation of inhibition can also provide insights into how LTP and epilepsy can be manipulated (Belan & Kostyuk, 2002); Castillo et al., 2011; Moreau & Kullmann, 2013).

Initial investigations of this study demonstrated IDs could be readily evoked by application of low concentrations of MK801 (0.1- 0.3 μM) in control and SE slices of all ages. This supports many studies which have illustrated low dose MK801 administration increases locomotion (Deutsche et al., 1997; Wu et al., 2005; Wegnener et al., 2011) and seizure activity (Starr & Starr, 1993).

More interestingly, however, this study showed there was a developmental trend for excitability, in response to low concentrations of MK801, to decrease in controls and conversely remain elevated in epileptic animals. This supports studies which have demonstrated NMDA receptors are altered in epileptogenesis, either by changes in expression or by redistribution of receptors (Avanzini & Franceschetti, 2003; Dalby & Mody, 2001; Morimoto et al., 2004; Yang et al., 2006). These findings further support the idea that epileptogenesis recapitulates earlier development (e.g. excitatory actions of GABA), when susceptibility to epileptogenesis is heightened (Cohen et al., 2003; Cossart et al., 2005).

Moreover, Santucci and Raghavachari (2008) acknowledged during development NR2B receptors are displaced by NR2A receptors, which were suggested to be likely mediators of homeostatic stability of AMPA receptors. Additionally, the opening probabilities of NR2A and NR2B receptors were shown to be very different. Specifically, NR2B receptors opened for a

longer period of time but not as frequently in comparison to NR2A receptors. NR2B receptors are also sensitive to glutamate concentrations, reliably involved in LTP and are probably more extrasynaptically located following displacement by NR2A. As it has been suggested here and shown by others (Avanzini & Franceschetti, 2003; Dalby & Mody, 2001; Morimoto et al., 2004; Yang et al., 2006) the NR2B receptor does not undergo downregulation in epileptogenesis, therefore the enhanced excitability of networks and maintenance of seizures could be attributed to altered NR2B/NR2A ratio. Whether this alteration is a cause or effect of seizure activity remains to be established.

As demonstrated by Siesjo et al. (1985) the induction of seizures reduces extracellular pH. This acidification was suggested to occur as a result of diffusion of lactic acid and fast transcellular Na^+/H^+ exchange. Dravid et al. (2007) showed NR2 subunits are differentially affected by pH changes. Lowering pH results in greater potency of (-) MK801 and (+) MK801 at NNR2A receptors, thus more effectively reducing excitability. Changes in pH will affect the ionisation state of MK801 but protons can have a much larger effect as a multitude of amino acid residues, protein-associated lipids and carbohydrates can be ionised. It was suggested that this potency shift occurs as MK801 reacts to the ionisation state of the proton sensor. This was demonstrated through the increased potency of (-) MK801 and (+) MK801 at NR2A receptors in the presence of extracellular Zn^{2+} which increases proton affinity. This increased potency resembles activity at pH 6.9. On the other hand NR2B subunits are not associated with enhancements of MK801 potency, and the greater presence of these subunits receptors may enhance network excitability and LTP.

Based on these studies it would be beneficial for future studies to confirm alterations of NR2 subunits in this pilocarpine model of epilepsy. Others have suggested alterations in other NR2 subunits such as NR2A (Pratt et al., 1993) and NR2C (Bo et al., 2004) also occur as a result of epileptogenesis. It would be of further interest to explore the placement of NR1/NR2B receptors on different types of neurons (pyramidal cells, interneurons, astrocytes, etc). Do NR1/NR2B receptors remain as equally distributed over the synapse, as in the immature state or are the congregated extrasynaptically? Does conductivity of these receptors deviate from normal physiology in the epileptic state? These are important questions which can help develop a theory of how excitability and LTP are altered in epileptic pathology to promote recurring seizures.

To some extent some of these questions have begun to be explored. For example, Woodhall et al., (2001) demonstrated NR2B containing receptors are present presynaptically at excitatory synapses in layer V of the EC, but not at those in layer II of the EC. Additionally, Ca^{2+} entry was shown to be the mechanism underlying facilitation of glutamate release by presynaptic NMDA receptors. It would be of interest to conduct similar experiments in epileptic animals for comparison. Moreover, Frasca et al. (2011) investigated changes in

NR2B subunit phosphorylation, membrane localisation and cellular expression during epileptogenesis in the hippocampus. Expression of NR2B was activated in astrocytes and was increased at extrasynaptic and presynaptic neuronal compartments via redistribution. It was proposed the increased presence of NR2B containing receptors at extrasynaptic sites may modulate synaptic transmission by increasing synchronisation through slow inward currents and increased expression at presynaptic sites may lead to increased glutamate release from neurons and consequently contribute to hyper-excitation via over-activation of post synaptic glutamate receptors. Finally, blockade of NR2B with ifenprodil during epileptogenesis significantly reduced pyramidal cell loss in the hippocampus. Collectively these findings show NR2B changes during epileptogenesis contribute to enhanced excitotoxicity. Further supporting these findings, Muller et al. (2013) demonstrated chronic epilepsy leads to enhanced synaptic transmission and LTP, but unaltered LTD in the hippocampus through upregulation of NR2B but not NR2A. It was suggested that upregulation of NR2B may act as a compensatory mechanism that aims to reduce cognitive deterioration but actually leads to deleterious effects by yielding higher calcium influx. Interestingly in some cases NR2B up regulation has been associated with reduced hippocampal excitability (Gabriel et al., 2004; Kohling et al., 1995) thus further suggesting NR2B up regulation attempts to act as a form of seizure control.

5.3.2 NO and the effects of NMDA receptor activation

Having established NMDA activity is altered during epileptogenesis, it remained to be evaluated how subtle changes in NMDA activity can promote seizure activity. As suggested MK801 is a useful tool in this investigation given its variable activity at different NMDA subunit receptors and different concentrations. Whilst several studies have documented seizure inducing effects of MK801, the mechanisms through which this activity is initiated is largely unknown. However, suggestions as to the mechanistic action through which MK801 induces IDs includes: selective inhibition of interneurons (Stafstrom et al., 1997), differences in anatomical localisations of NMDA receptor subtypes (Tang et al., 2006), and through NO signalling pathways. Star and Starr (1993) showed NO synthase inhibitor, L-NAME, was proconvulsant. However, NO itself has been shown to have opposing effects depending on its concentration and place of action. For example, NO can act to enhance inhibition (Nugent et al., 2009; Yang & Cox, 2007) and suppress inhibition (Makara et al., 2007) (see Ferraro & Sardo, 2004; Banach et al 2011 for reviews). Given the prominent neuromodulator role of NO in physiological and pathological conditions, the role of the NO pathway in mediating NMDA drive of inhibition during epileptogenesis was investigated further.

Interestingly the current study showed that low concentration MK801 ID inducing effects were not mediated by NO, as pre-treatment of slices with the NO synthase inhibitor, 7-nitraindazole, failed to significantly affect IDs. The most common inhibitors of NOS include:

N-nitro-L-arginine methyl ester (L-NAME), 7-NI, NG –nitro-L-arginine (NNA) and 1-[2-(trifluoromethyl) phenyl] imidazole (TRIM). In support of the findings of this study, acute *in vivo* studies have shown 7-NI to be ineffective on altering seizure threshold in maximal electroshock (MES) models (25-50mg/kg) (Borowicz et al., 1997), amygdala kindled rats (100 mg/kg) (Borowicz et al., 2000a), PTZ models (30-50 mg/kg) (Borowicz et al., 2000b; Luszcki et al., 2007) and the KA model (50mg/kg) (Penix et al., 1994). However, when co-administered with certain AEDs, 7-NI did show anticonvulsant effects.

In contrast, other studies in the KA model have shown proconvulsant effects of 7-NI (20-60 mg/kg) (Kirkby et al., 1996). Furthermore, other studies have shown proconvulsive effects of 7-NI in the MES model (100-200 mg/kg) (88), PTZ model (50mg/kg) (Han et al., 2000), picrotoxin model (25-50 mg/kg) (Rajasekaran et al., 2003) and pilocarpine model (25-100mg/kg) (Van Leeuwen et al., 1995).

Moreover, in the pilocarpine model different NOS inhibitors have different effects. Starr and Starr (1993) showed L-NAME potentiated pilocarpine induced seizures, whereas Van Leeuwen et al. (1995) showed 7-NI exerted anticonvulsant effects. 7-NI has been identified as an inhibitor of nNOS (Babbedge et al., 1993; Moore et al., 1993), but could act as an inhibitor of constitutive NOS *in vitro* (Wolff & Gribin et al, 1994). L-NAME acts primarily on the eNOS isoform (Dwyer et al., 1991; Moncada et al., 1991) and is also an antagonist of muscarinic receptors (Buxton et al., 1993). It would be of interest to re-evaluate the role of the NO pathway in mediating NMDA drive of inhibition during epileptogenesis, with other NOS inhibitors, and even NO donors such as L-arginine as different results maybe yielded.

5.3.3 The effects of MK801 on GABAergic activity during the latent period

As this study showed, low dose MK801 ID inducing effects were not mediated by NO, the possibility that MK801 alters GABAergic activity was investigated. Bazelot et al. (2010) demonstrated interneurons, but not pyramidal cells, were capable of evoking field potentials at monosynaptic latencies. These inhibitory fields were shown to be GABAergic in nature and originated from postsynaptic sites as they were suppressed by low external Cl-. In a similar fashion, this study showed slow field events are inhibitory in nature. Field IPSPs are significantly reduced in SE slices during the early latent period in comparison to controls. These findings support numerous studies demonstrating attenuated inhibition in epileptogenesis (Gibbs et al., 1997; Kapur and Macdonald, 1997). MK801 did not exert any significant effects on field IPSPs in control or SE slices.

GABA receptors undergo cell internalisation and insertion on a continual bases. Internalisation has been shown to occur via clathrin-dependent endocytosis (Connolly et al., 1999; Kittler et al., 2000; Tehrani and Barnes, 1993, 1997), which can be initiated by PKC (Chapell et al., 1998) and BDNF (Cheng & Yeh, 2003; Jovanovic et al., 2004). Goodkin et al.

(2007) demonstrated $GABA_A$ receptor internalisation is accelerated by prolonged epileptiform activity resulting in reduced inhibition. Additionally, inhibition of neuronal activity reduced the rate of internalisation. Similar findings have been illustrated by Blair et al. (2004). Based on these results and other findings which have shown heightened activity of NMDA receptors during epileptogenesis, it was hypothesised that low concentrations of MK801 reduce inhibition, possibly through reduced internalisation, leading to increased interneuron inhibition, hence precipitating IDs. However, this study showed MK801 did not exert any differential effects on field IPSPs evoked by interneurons in controls and SE slices during the latent period, therefore this proposed MK801 ID inducing mechanisms is unlikely.

5.3.4 Conclusion

In conclusion, the current study demonstrated low concentrations of MK801 induced IDs in control and SE slices, emphasising previous demonstrations which show glutmatergic activation can have opposing effects depending on its concentration and place of action. Moreover, it was shown there was a developmental trend for excitability, in response to low concentrations of MK801, to decrease in controls and conversely remain elevated in epileptic animals. Again supporting previous evidence showing NMDA receptors are altered in epileptogenesis, either by changes in expression or by redistribution (Avanzini & Franceschetti, 2003; Dalby & Mody, 2001; Morimoto et al., 2004; Yang et al., 2006). Finally, this study investigated the NO and GABA mechanisms of MK801 ID inducing effects.

Future studies further investigating how NR2 subunits are altered in epileptogenesis in terms of localisation on different neurons and electrophysiology would greatly improve our understanding of pathological LTP in epileptogenesis. Moreover, despite results suggesting NO and GABA are not involved in IDs induced by low concentrations of MK801, variable effects of different NOS inhibitors in the literature suggest other NOS inhibitors and even NO donors should be investigated throughout epileptogenesis in order to draw firmer conclusions.

Moreover, whilst this study has focused on NMDA drive of inhibition, it has also been suggested that the interplay between different glutamate receptors (e.g. mGluRs, KARs and NMDA receptors), at different glutamate concentrations overall modulate inhibition (Belan & Kostyuk, 2002). The signalling mechanisms of different glutamate receptor subtypes may also interact. For example, it has been low concentrations of NO have been reported to activate BDNF (Xiong et al., 1999). NO also regulates neurotransmission of acetylcholine (Prast et al., 1998), noradrenaline, dopamine and serotonin (Feldman & Weidenfeld, 2004; Lorrain & Hull, 1993) in some brain areas. Evidently, there are a bewildering number of mechanistic possibilities with varying levels of complexity, which further need exploring in order to elucidate MK801 ID inducing effects in terms of excitatory regulation of inhibition during epileptogenesis.

Chapter 6 General discussion and future work

6.1 Introduction

A third of epilepsy patients are resistant to anti-epileptic drug (AED) treatment, leading to reduced quality of life, increased treatment costs and complexities surrounding polytherapy. It remains an important challenge of epilepsy research to understand the pathogenesis of TLE, in order to develop better treatments and alter the progression of the condition. The overall aim of this project was to explore dynamic network changes in the excitability and efficacy of AEDs in: acute models of epileptiform activity, chronic models of epileptogenesis and in resected human tissue, *in vitro*. Additionally, this project aimed to explore the underlying mechanisms of DRE.

Several limitations of epilepsy research have prevented the development of better treatments and understanding of underlying mechanisms. Firstly, many acute and chronic models of epilepsy are extremely damaging and induce gross neuronal loss (e.g. via chemical insult or damaging slice preparatory methods), therefore do not accurately imitate the human condition (Modebadze et al., 2016; Sloviter & Bumanglag, 2013). Secondly, numerous AED screening models often only assess the effects of one AED, when the definition of DRE explicitly states DRE is a failure to respond to two or more drugs (Berg et al., 2001; Cowan, 2002; Kwan & Brodie, 2006). Finally, studies aiming to explore the mechanisms behind epileptogenesis and DRE, have had a tendency to oversimplify complex pathology by proposing epilepsy is a mere imbalance between neuronal inhibition and excitation. This outlook has promoted the development of 'me too' drugs which do alter disease progression, which simply manage secondary effects by increasing inhibition or decreasing excitation. It is highly likely there are a variety of mechanisms underlying epileptogenesis and DRE, which are imperative to explore to improve prognosis for patients (Loscher & Schmidt, 2002; Margineanu & Klitgaard, 2009).

6.2 The effects of brain slice preparations on excitability and efficacy of AEDs

The initial investigation of this project exemplified the first of these limitations, by exploring the effects of two different brain slice preparations on the excitability and viability of layer II of the MEC. Standard prepared slices were much more excitable than sucrose prepared slices, and immunohistochemistry follow-up investigations suggested this enhanced excitability is due to a poorly preserved inhibitory network in standard slices in comparison to sucrose slices. Differences in oscillatory activity and intracellular properties have been previously demonstrated between standard and modified methods of brain slice preparations (Kuenzi et al., 2000; Modebadze, 2014; Prokic, 2012), but this is a novel demonstration of how brain slice preparation affects results when used in acute models of seizure-like events (SLEs). Additionally, this study showed there was little difference in response to combination AEDs in different brain slice preparations, but this could be due to increased latency to first seizure in sucrose prepared slices. LTP was suggested to play a role in the resistance to AEDs, as pre-

treatment with the protein synthesis blocker, cycloheximide significantly increased latency to first seizure, and induction of IDs were almost completely blocked with the PKC inhibitor, GF109203X. These results suggest sucrose prepared slices better preserve the neuronal network *in vitro*, and serve as a better acute model for assessing AEDs and mechanisms of resistance.

6.3 Excitability and efficacy of AEDs in the RISE model of epilepsy and in epileptic human tissue

The next investigation of this project explored changes in neuronal network excitability and efficacy of combination AEDs during epileptogenesis (24hrs and 1, 5 and 12 weeks post status) in the refined Li-Pilocarpine (RISE) model and in resected tissue from human epileptic patients. Ictal-like discharges (IDs) were seen in significantly greater numbers of slices from RISE animals (range 45.05- 53.57 %) compared to age- matched controls (range 18.75- 47.92 %). Additionally, RISE slices showed a consistently shorter latency to first seizure (LFS) across all time points (control LFS range 4801-8310s; RISE LFS range 2505-3290 seconds). These findings demonstrated immediate network changes, as following SE induction immediate network excitability can be seen SE slices in comparison to controls. Investigations exploring the efficacy of different AED combinations during epileptogenesis showed that the TGB+CBZ combination was most effective in reducing measures of ictal activity whilst the combination of LTG+GPT was least effective. The resistance toward different drug combinations was also variable depending on the stage of epileptogenesis. Specifically, resistance to combination AEDs was the lowest at 5 weeks post SE. Nevertheless, resistance at this stage remained elevated towards CBZ+ZNS and LTG+GPT. Resistance remained between 20-33 % of slices at all stages of epileptogenesis. These findings suggest that vulnerable networks show underlying hyperexcitability even at stages when chronic behavioural seizures are not yet developed, and that the RISE model may provide insights into the variable efficacy of AEDs.

It would be interest for future studies to further investigate antiepileptic potential of AEDs at different stages of epileptogenesis that do not work through classical mechanisms, such as LEV. On a related note, CBD has been shown to have antiepileptic potential, although the mechanism through which it has effect is debatable (Devinsky et al., 2014). Here, it was shown that although CBD is not the most effective AED it does become more efficient as epileptogenesis progresses. Developing a better understanding of CBD mechanisms, may provide better insights into underlying mechanisms of epilepsy. Furthermore, as evidenced in chapter 3 and by several others (Gabriel et al., 2004; Modebadze et al., 2016; Sloviter & Bumanglag, 2013), the level of damage imposed by models of epilepsy plays a vital consequential role in the outcomes and explanations of epilepsy research. Given the subtle network changes imposed by the RISE model in comparison it would be of interest to re-

examine earlier *in vivo* studies by Turski et al. (1987) which explored protective effects of AEDs.

Investigations of excitability in resected human tissue showed tissue from temporal lobe regions were more excitable in comparison to the motor cortex and frontal lobe regions. However, resected tissue required more excitation in order to induce IDs in comparison to epileptic rodent tissue. Preliminary investigations of AED efficacy illustrated all combination AEDs were effective in reducing IDs, with some resistance toward VPA and CBD. Discrepancies in excitability could be attributed to the likelihood that damage within human tissue is likely to be subtle, hence require more stimulation to induce ictal-like activity (Gabriel et al., 2004). One of the main criticisms of many kainate and pilocarpine models is that they are often severe and do not imitate human pathology, however the refined Li-pilocarpine model used within our laboratory has been demonstrated to show subtle alterations in network dynamics, without significant loss of neuronal network function and therefore provides adequate imitations of the human condition in comparison to harsher models (Modebadze et al., 2016). Additionally, discrepancies in the excitability of rodent and human epileptic tissue could also be attributed to differences in the architecture of rodent and human brains. As Buzsaki (2002) discussed, the amplitude of LFPs in the hippocampus decrease from rodent to cat and from cat to primate as a result of changes in cellular spatial alignments caused by differences in brain size and the presence of gyri. Such circumstances are difficult to avoid, as the opportunity to investigate human resected tissue remains scarce, rodent models provide a reliable alternative to gain insight in pathological mechanisms. On the other hand, methodological advancements are continually being made and others have begun attempting to maximise the utility of valuable human tissue through organotypic cultures (see Jones et al., 2016 for a review), which could be of particular interest in investigating epileptic changes.

6.4 The effects of the NO signalling pathway and GABA mediating latent period mechanistic alterations in epilepsy

Studies conducted here on the RISE model of epilepsy clearly showed benefit of AEDs affecting GABAergic transmission. Much of epilepsy research has focused on the imbalance between excitation and inhibition leading to the disruption of the brain's equilibrium and resulting in the epileptic seizures. This approach has only been partially fruitful, and this imbalance has been suggested to be a secondary effect of underlying pathological mechanisms (Loscher & Schmidt, 2002; Margineanu & Klitgaard, 2009). The final investigation of this project, explored how the NMDA drive of inhibition changes during epileptogenesis in comparison to controls. Secondly, the role of the NO pathway in mediating NMDA drive of inhibition during epileptogenesis was evaluated. Numerous studies have demonstrated glutamate-receptors modulate GABAergic inhibition through a variety of

mechanisms (Belan & Kostyuk, 2002). In particular, NMDA receptors have been extensively documented to be involved in seizure generation and LTP. Pre- and postsynaptic NMDA receptor activation of interneurons and pyramidal cells play an intricate role in regulating the excitation of neuronal networks. Many studies have also suggested NMDA receptors are altered significantly over the course of epileptogenesis. A prominent mechanism through which NMDA receptors exert control over excitability is via activation of NO signalling pathways which also has pro- and anticonvulsant action and has been proposed to play a fundamental role in the development of epileptogenesis.

Low concentrations of MK801 (100-300nM) induced IDs in control and SE slices, thus supporting previous demonstrations which show glutamatergic activation can have opposing effects depending on its concentration and place of action. Moreover, it was shown there was a developmental trend for excitability, in response to low concentrations of MK801, to decrease in controls and conversely remain elevated in epileptic animals.

During normal development NR2B receptors are displaced by NR2A receptors. These receptors have different characteristics as NR2B receptors remain open for longer but do not open as frequently as NR2A receptors. NR2B receptors are also sensitive to differences in glutamate concentrations and are more reliably involved in LTP (Santucci & Raghavachari, 2008). Changes in pH that often occur with seizures (Siesjo et al., 1985) also affect the potency of MK801 at NR2 subunits differently. The potency of (+) MK801 is enhanced at NR2A but not NR2B receptors, hence NR2B receptors promote excitability (Dravid et al., 2007). As it has been suggested in this study and shown by others (Avanzini & Franceschetti, 2003; Dalby & Mody, 2001; Morimoto et al., 2004; Yang et al., 2006) the NR2B receptor does not undergo downregulation in epileptogenesis, therefore the enhanced excitability of networks and maintenance of seizures could be attributed to altered NR2B/NR2A ratio.

Further investigation into altered placement of NR2B receptors in relation to NR2A on different cells (e.g. pyramidal cells, interneurons and astrocytes) as well synaptic and extrasynaptic locations and altered conductivity of receptors in the epileptogenesis could aid the development of a theory explaining how excitability and LTP are altered in epileptic pathology to promote recurring seizures. To some extent, some studies have begun to explore these possibilities (Frasca et al., 2011; Muller et al., 2008; Woodhall et al., 2005).

Finally, this study suggested NO and GABA are not involved in IDs induced by low concentrations of MK801. Pre-treatment of SE slices with the NOS inhibitor, 7-nitraindazole had no effect on IDs. Low concentrations of MK801 had no significant effects on the frequency and amplitude of field IPSPs in control and SE latent period slices. However, variable effects of different NOS inhibitors in the literature suggest other NOS inhibitors and even NO donors should be investigated throughout epileptogenesis in order to draw firmer

conclusions. Future investigations could also further explore how interactions between different glutamate receptors affect inhibition (Belan & Kostyuk, 2001). Moreover, the signalling mechanisms of different glutamate receptor subtypes may also interact. For example, it has been low concentrations of NO have been reported to activate BDNF (Contestabile & Ciana, 2004; Xiong et al., 1999). NO also regulates neurotransmission of acetylcholine (Prast et al 1998), noradrenaline, dopamine and serotonin (Feldman & Weidenfeld, 2004; Lorrain & Hull, 1993) in some brain areas. These interactions should be further considered in order to elucidate MK801 ID inducing effects.

6.5 Conclusion

Overall, this project has demonstrated variable levels of damage imposed by brain slice preparatory methods influence the excitability and response to AEDs in acute models of seizures. Specifically, standard prepared slices are less resistant to AEDs in comparison to sucrose prepared slices, due to loss of inhibitory interneurons. Sucrose slice resistance towards AEDs in the 0$[Mg]^{2+}$ aCSF model was suggested to occur as a result of LTP. Secondly, exploration of the excitability and efficacy of RISE model slices showed vulnerable networks show underlying hyperexcitability even at stages when chronic behavioural seizures are not yet developed, and that the RISE model may provide insights into the variable efficacy of AEDs. The RISE model was also an appropriate tool for comparative human tissue investigations. Finally, enhanced excitability with application of low concentration MK801 (100-300 nM) during the latent period, confirmed the importance of NMDA receptor changes during epileptogenesis. This was not mediated by the NO signalling pathway or changes in GABA. The future of epilepsy research would benefit in terms of the reliability of findings and explanations of epileptogenesis by using conservative acute and chronic models. Finally, further exploration of how pathological LTP effects inhibition could provide useful insights into the disease progression.

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Appendix

Appendix 1


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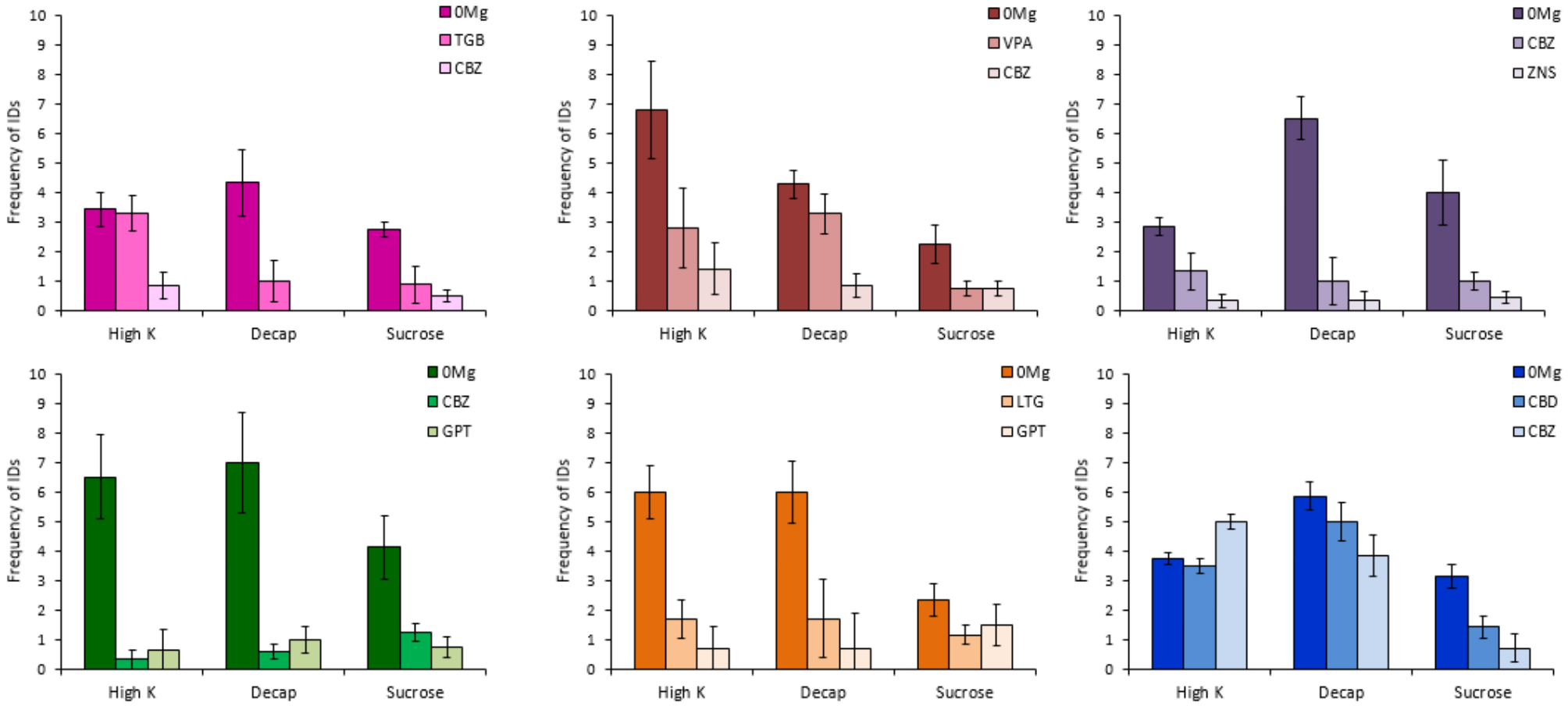
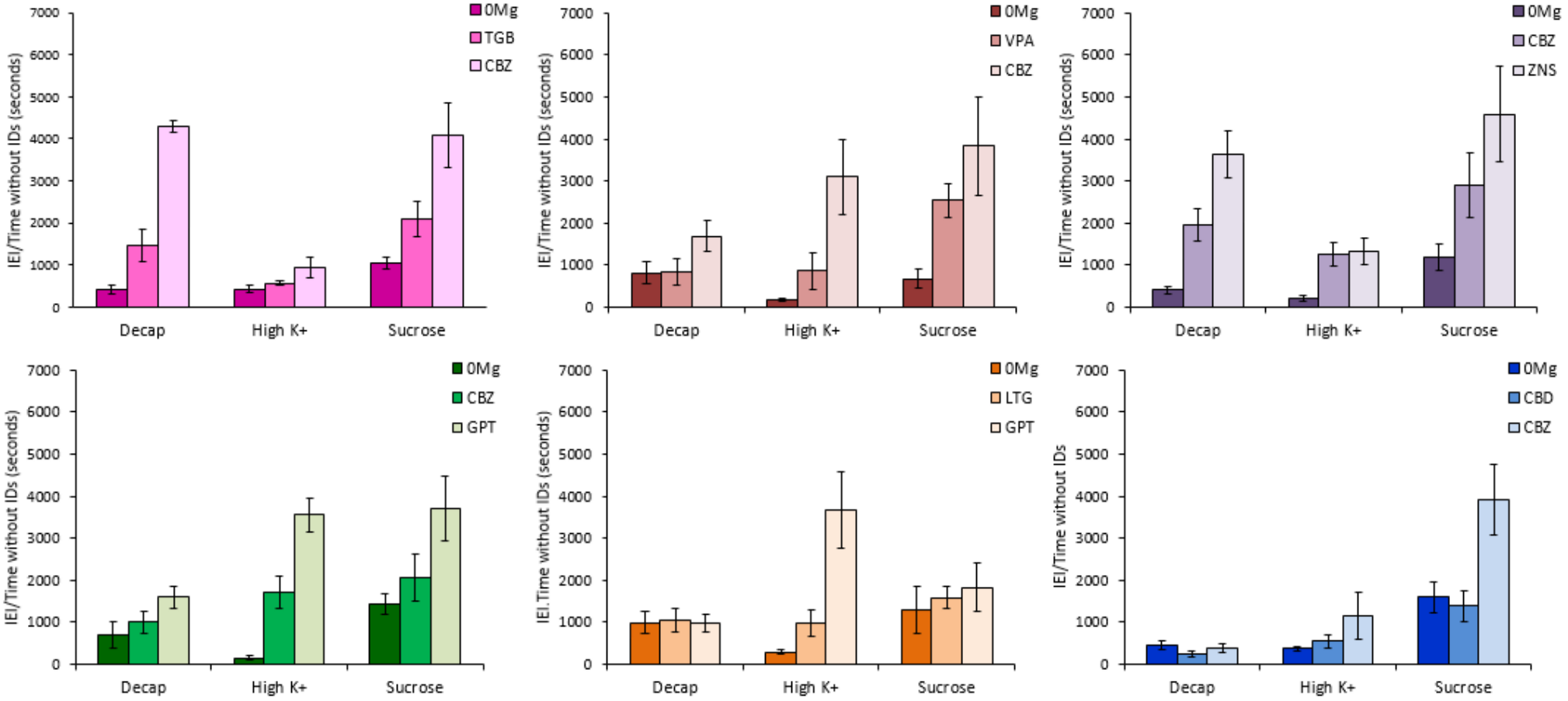
Appendix 3



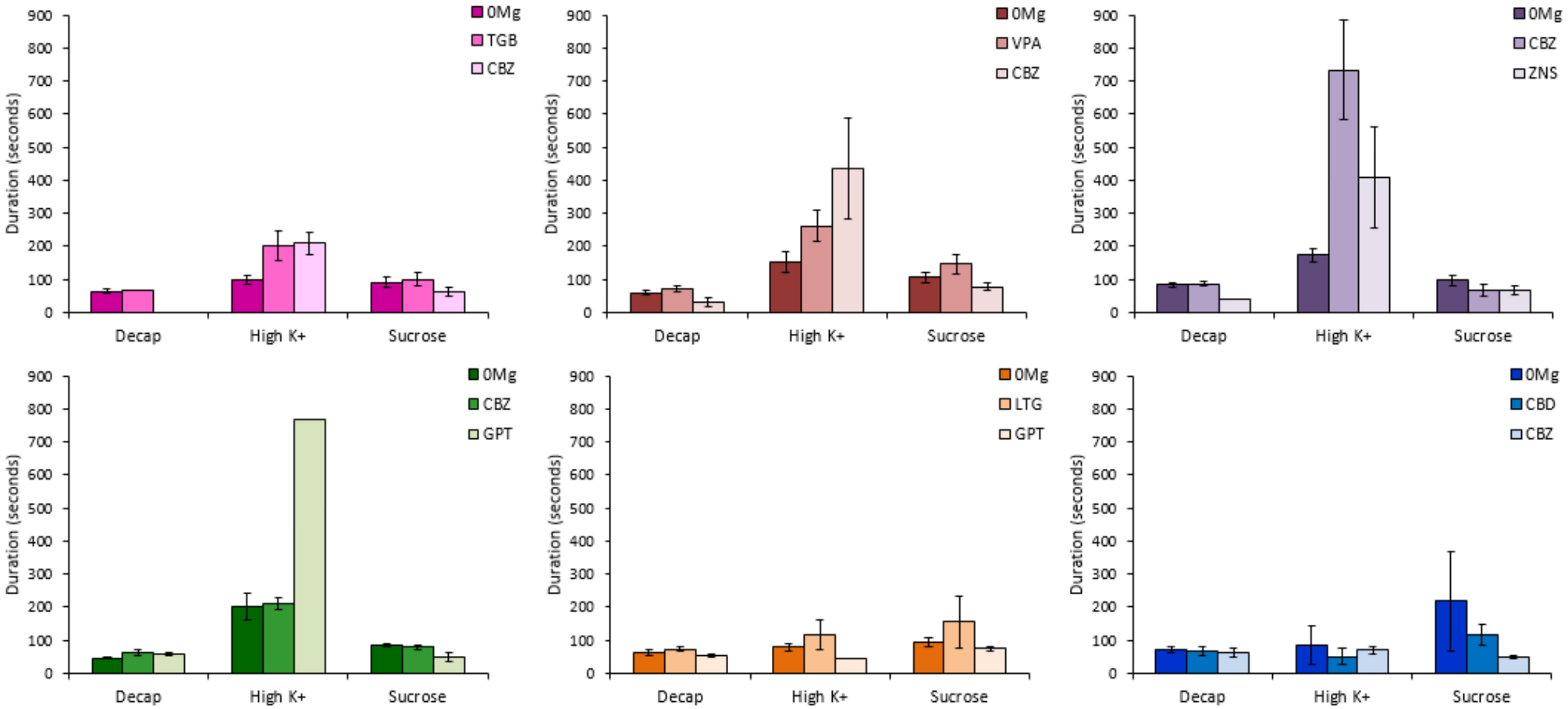
Raw data of the effects of AEDs on the frequency of IDs in 3 brain slice preparations presented in figure 3-8.

Appendix 4



Raw data of the effects of AEDs on the IEIs between IDs in 3 brain slice preparations presented in figure 3-9.

Appendix 5



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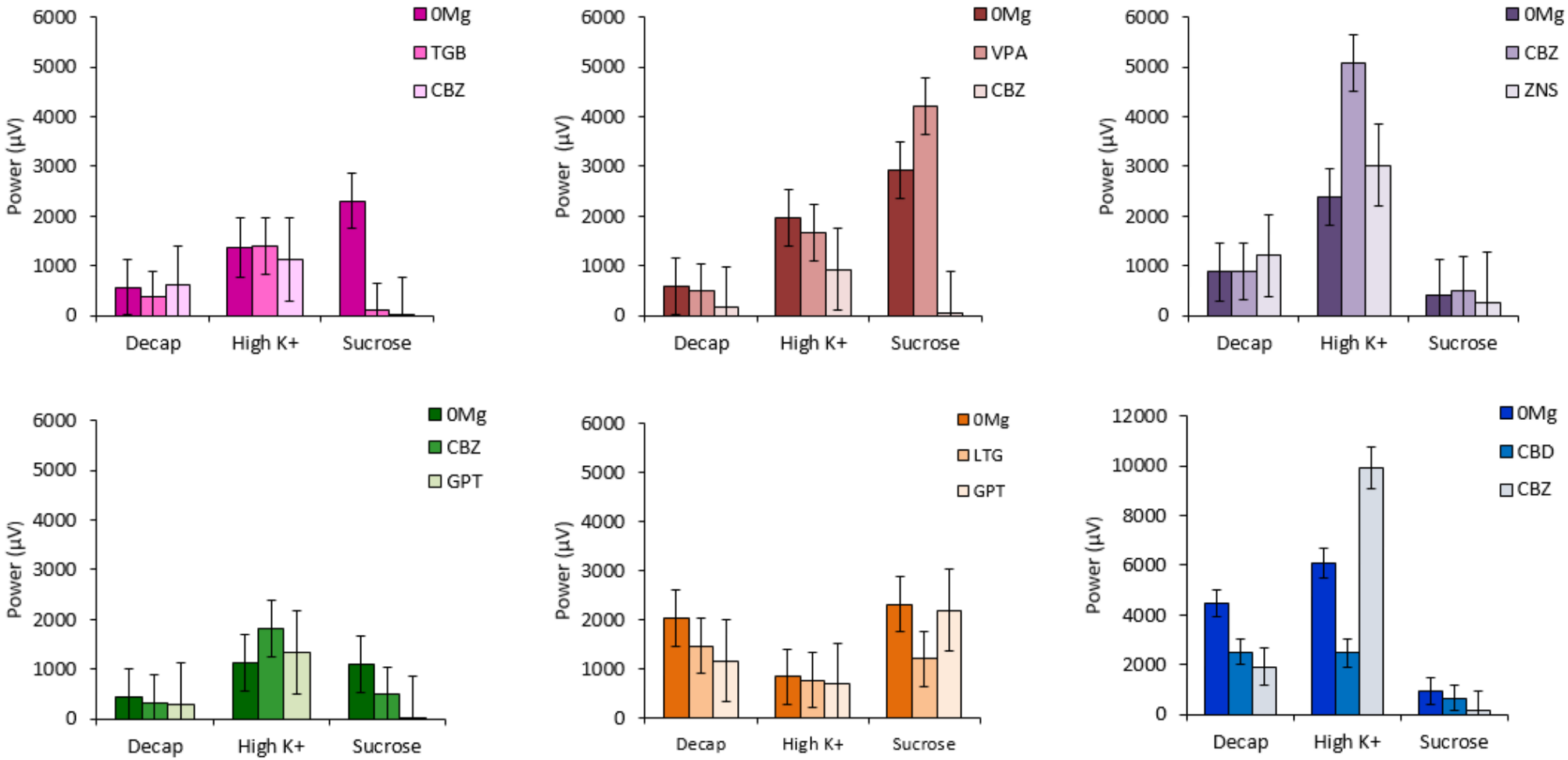
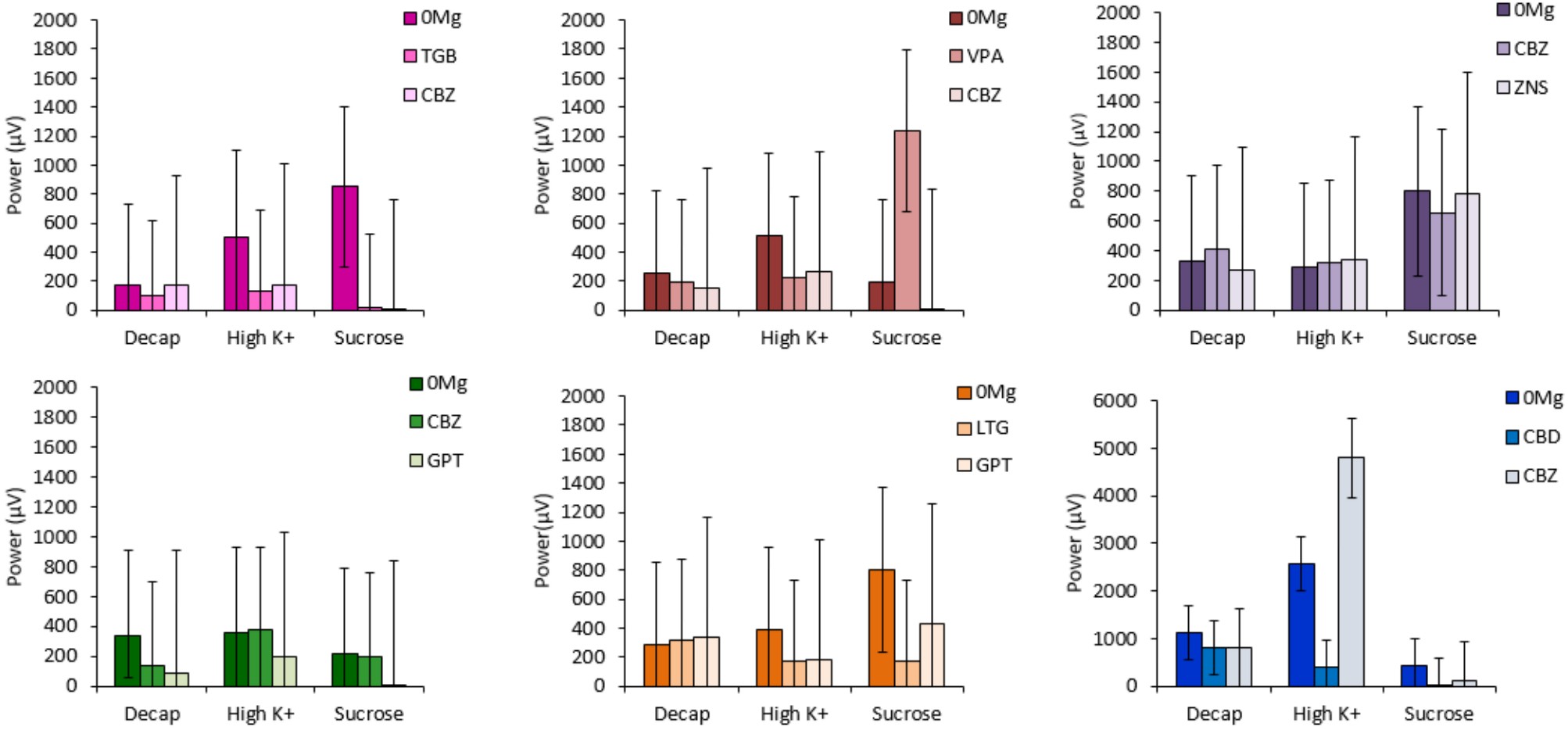
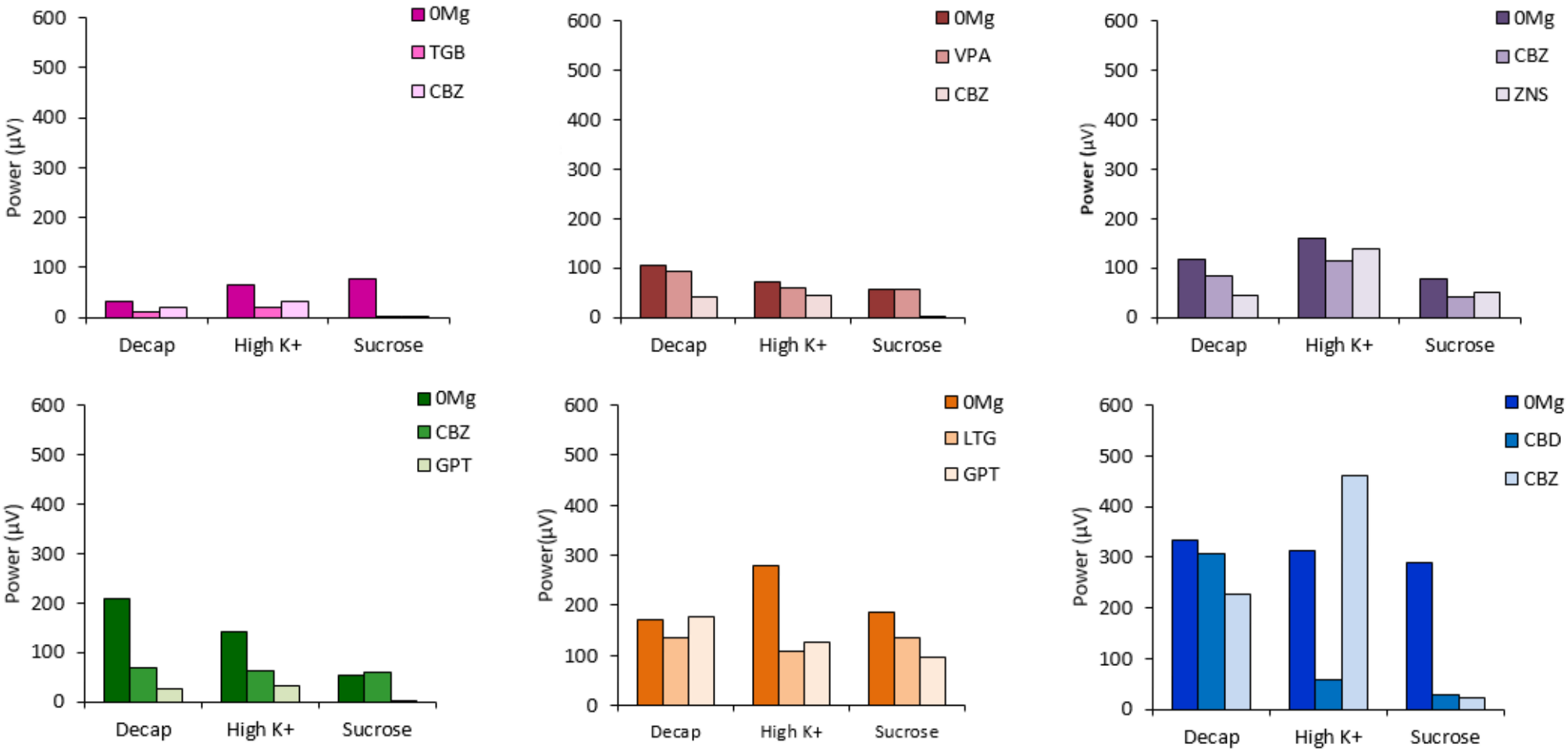
Appendix 6



Power of IDs at SW frequency



Power of IDs at delta frequency



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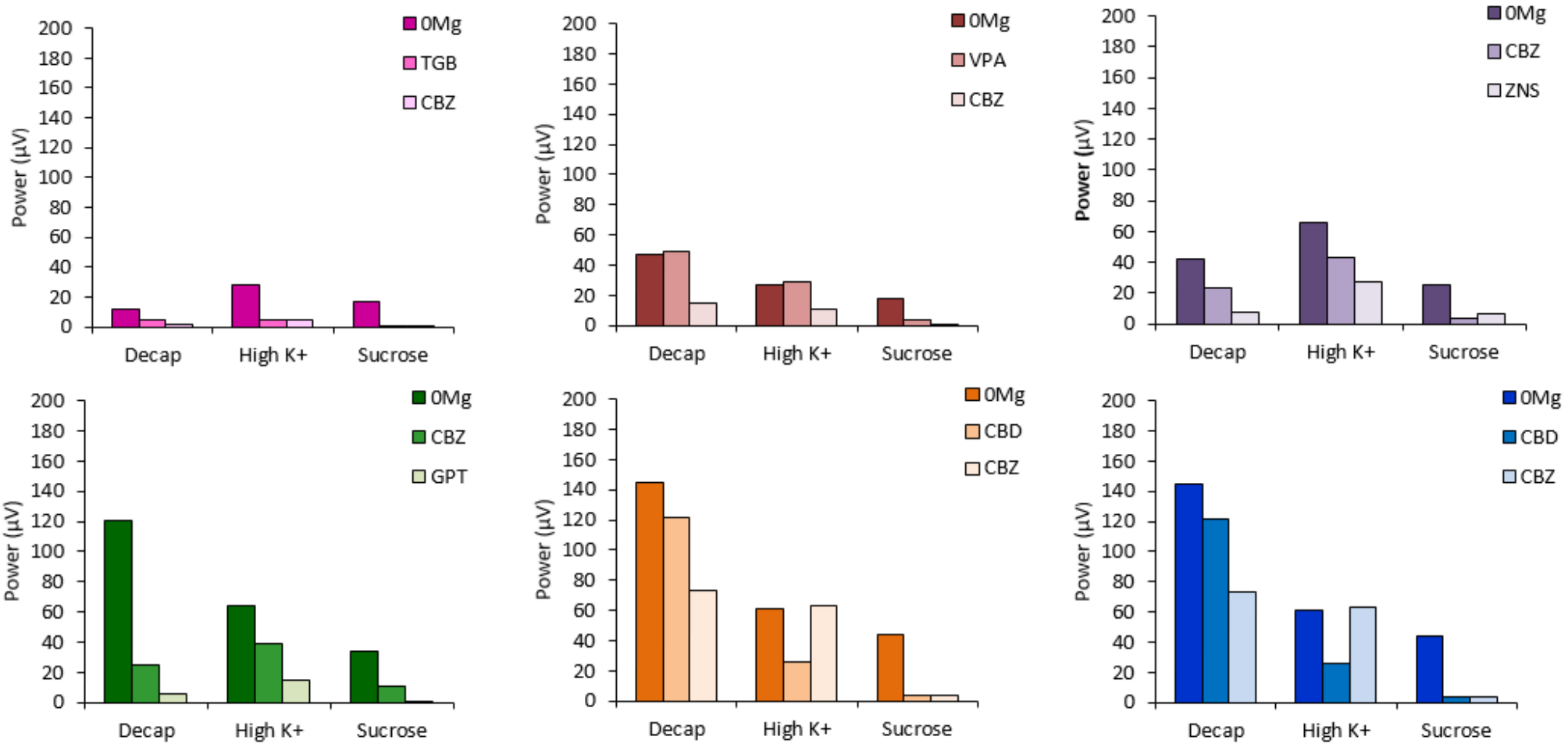
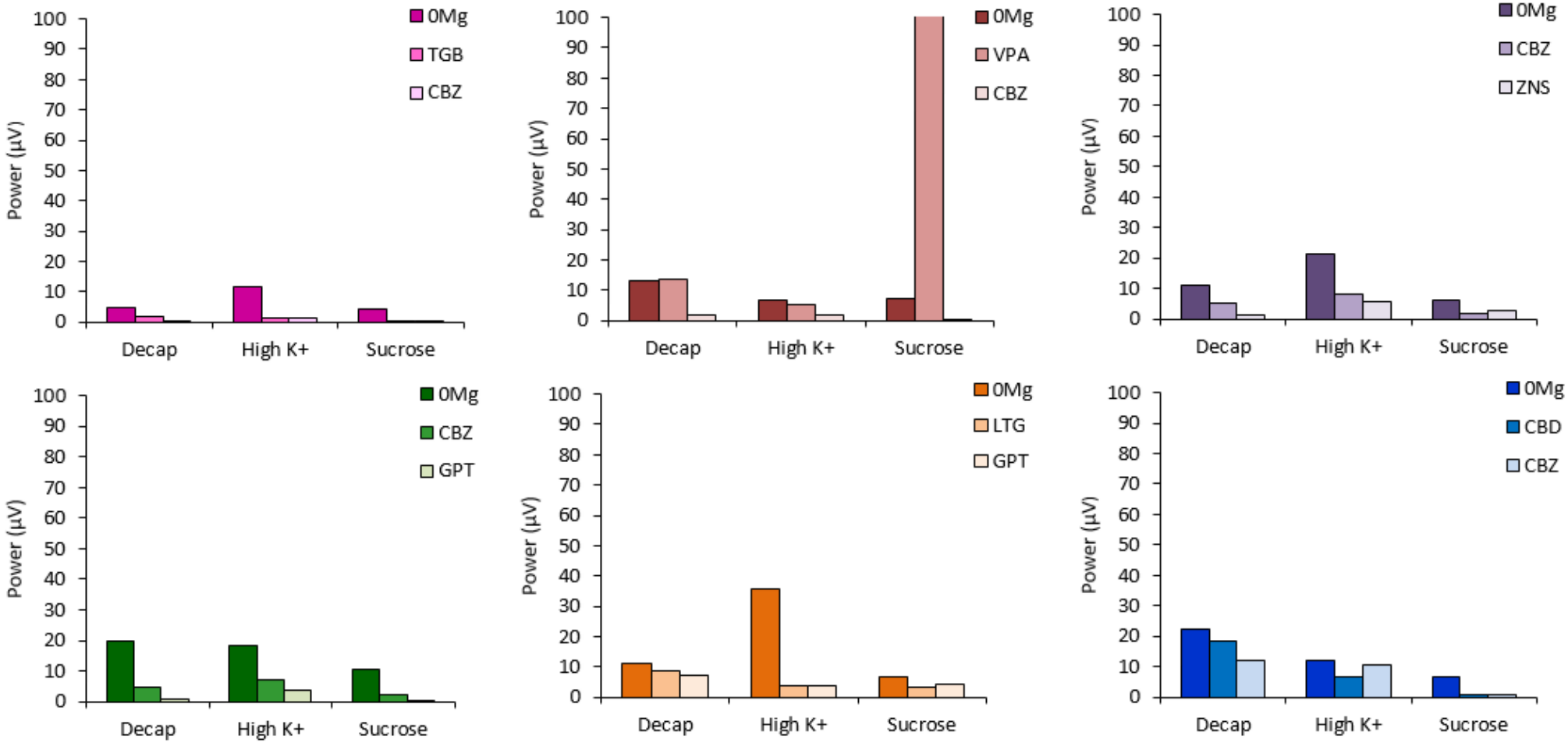
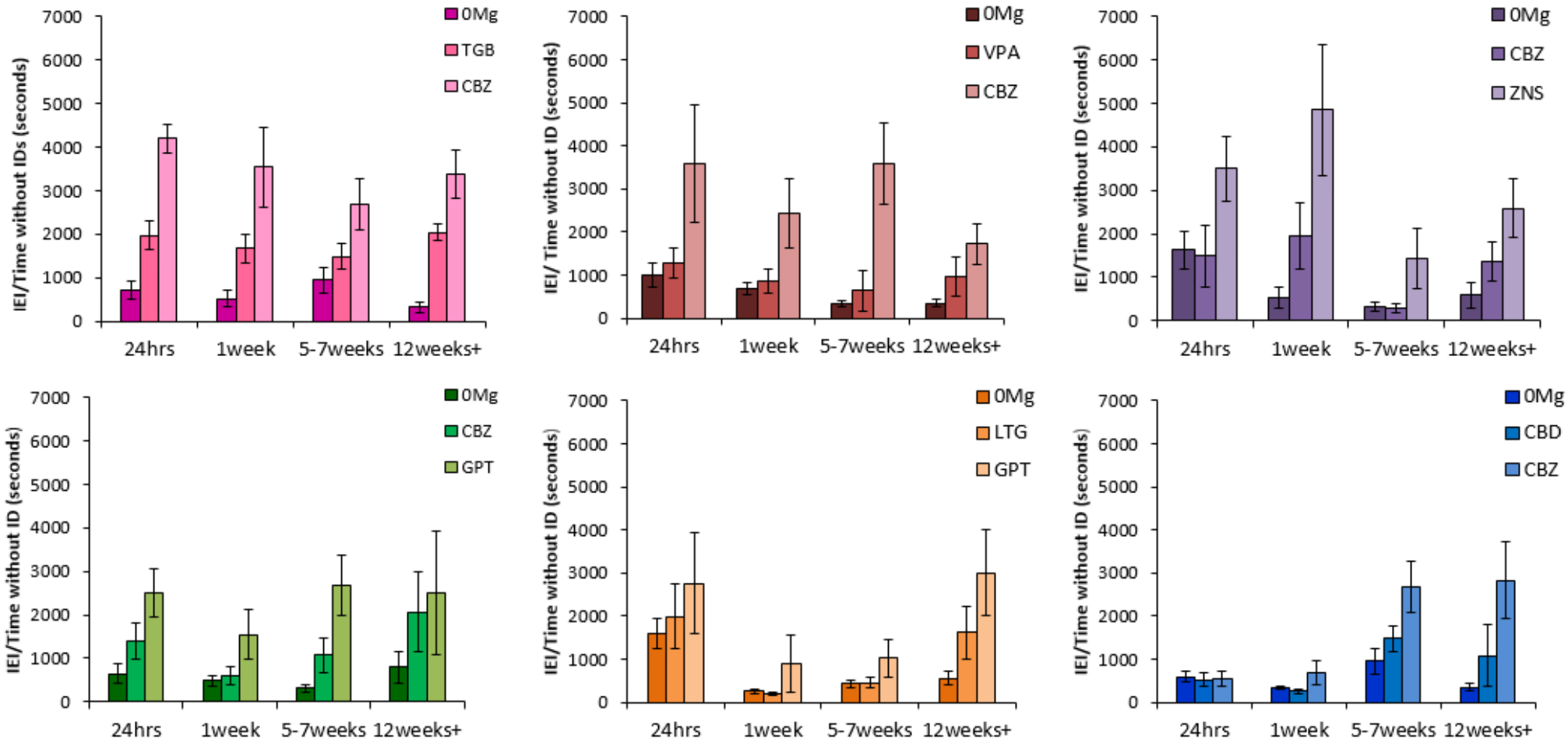


Power of IDs at beta frequency



Power of IDs at gamma frequency

Appendix 7



Raw data of the effects of AEDs on IEIs during epileptogenesis represented in figure 4-4.