Antidepressant pharmacotherapy in epilepsy:
The effects of chronic fluoxetine and citalopram treatments in a rat model of epileptogenesis

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Just keep swimming. Just keep swimming.

Just keep swimming, swimming, swimming.

Dory, Finding Nemo
ABSTRACT

Introduction

In patients with epilepsy there is a high incidence of comorbid psychiatric illnesses, especially mood and anxiety disorders, which have been associated with lower quality of life, impaired function and an elevated risk of suicide. In fact, the occurrence of these illnesses in patients with epilepsy has been reported to be a stronger predictor of quality of life than epilepsy variables such as illness duration or seizure frequency. In addition, there is evidence that the depressed state itself may predispose to seizures and epilepsy. For these reasons, effective management of depressive and anxiety symptoms and syndromes in epilepsy is essential. Selective serotonin reuptake inhibitors (SSRIs) are commonly used to treat depression in epilepsy, therefore it is important to consider their impact on epilepsy. To date, many studies have suggested that SSRI are safe for use in epilepsy, but the majority of these studies administered SSRIs only acutely or for short periods, and investigated effects only on acute seizure endpoints. There is no indication of the effect that chronic SSRI treatment may have on epileptogenesis and the associated neurobiological changes that continue after seizures emerge. This thesis aimed to investigate the effects of chronic SSRI treatment in a rat model of epileptogenesis, as well as investigating common neurobiological substrates of SSRI treatment and epileptogenesis that may also influence the disorder. It was hypothesised that chronic SSRI treatment would slow the rate of kindling epileptogenesis, as well as mitigate effects on common neurobiological substrates.

Methods

The amygdala kindling model was used to assess the effects of chronic SSRI treatment (with fluoxetine or citalopram) on epileptogenesis. 9-11 week old male Wistar rats were surgical implanted with a bipolar electrode into the left amygdala for electrical kindling and a subcutaneously implanted osmotic pump filled with fluoxetine (10mg/kg/day, n=19) or vehicle (50% DMSO, n=22) or citalopram (10mg/kg/day, n=26) or vehicle (50% DMSO, n=22), comprising two separate cohorts. All rats were given 30 stimulations and then kindling rate, seizure duration and seizure threshold before and after kindling were monitored. Effects on anxiety- and depressive-like behaviours were also investigated after kindling using two well-validated tests, the elevated plus maze and forced swim test respectively, as well as assessing the corticosterone response to stress and dentate gyrus neurogenesis.
Results

The key finding of this study was that rats chronically treated with SSRIs, either fluoxetine or citalopram, demonstrated accelerated rates of kindling epileptogenesis, showing a more rapid progression through the different stages of kindling compared to vehicle treated rats. The increase in seizure duration was also accelerated in the early stages of kindling in both cohorts of SSRI treated rats, however seizure threshold was not significantly different between vehicle and fluoxetine or vehicle and citalopram treated rats, either before or after kindling. This indicates that while epileptogenesis itself progressed at a faster rate during chronic SSRI treatment, accelerating the increase in seizure severity and duration, the local excitability and the threshold at which a seizure occurred was not affected by SSRI treatment. In order to investigate potential mechanisms underlying this, neurobiological alterations common to epileptogenesis and SSRI treatment were also investigated. Behavioural analyses found that both fluoxetine and citalopram treatments did not affect anxiety- or depressive-like behaviours, while kindling increased anxiety-like behaviour, but only in the fluoxetine treated cohort. Dentate gyrus neurogenesis was not significantly affected by kindling or drug treatment while stress-induced corticosterone levels were significantly reduced only by fluoxetine treatment. These investigations do not suggest that these alterations are associated with accelerating kindling rate during chronic SSRI treatment, however how these are affected during or immediately after kindling was not investigated.

Conclusions

Chronic treatment with fluoxetine and citalopram, at clinically relevant doses, accelerated kindling epileptogenesis in rodents. This highlights the need to investigate the effects of SSRI treatment on epileptogenesis over time in both animal models and people with epilepsy, rather than focusing solely on acute seizure time points. While the investigations in this study do not suggest that alterations in behaviour, neurogenesis or neuroendocrine responses are associated with accelerating kindling rate during chronic SSRI treatment, how these are affected during or immediately after kindling was not investigated. Therefore, future studies should further investigate the mechanisms underlying the effects of SSRIs on epileptogenesis at appropriate time points, such as during kindling epileptogenesis and also in complementary animal models of epilepsy, such as post-status epilepticus and post-traumatic models. It is essential to treat the depressive symptoms that manifest in people with epilepsy; however whether these medications affect the course of epilepsy and how they may do so should become priority areas for future research.
DECLARATION

This is to certify that:

i. the thesis comprises only my original work towards the Masters,

ii. due acknowledgement has been made in the text to all other material used,

iii. the thesis is no more than 50,000 words in length, exclusive of tables, maps, bibliographies and appendices.

Signature ______________________________ Date ________________
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# ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>5HT</td>
<td>Serotonin</td>
</tr>
<tr>
<td>8-OH-DPAT</td>
<td>8-hydroxy-2-(di-(n)-propylamino)tetralin</td>
</tr>
<tr>
<td>ACTH</td>
<td>Adrenocorticotropic hormone</td>
</tr>
<tr>
<td>ADT</td>
<td>Afterdischarge threshold</td>
</tr>
<tr>
<td>AED</td>
<td>Antiepileptic drug</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>BrdU</td>
<td>5-bromo-2'-deoxyuridine</td>
</tr>
<tr>
<td>BUP</td>
<td>Bupropion</td>
</tr>
<tr>
<td>CA</td>
<td>Cornu ammonis</td>
</tr>
<tr>
<td>CIT</td>
<td>Citalopram</td>
</tr>
<tr>
<td>CLV</td>
<td>Clovoxamine</td>
</tr>
<tr>
<td>CRH</td>
<td>Corticotrophin releasing hormone</td>
</tr>
<tr>
<td>DES</td>
<td>Desipramine</td>
</tr>
<tr>
<td>Dex</td>
<td>Dexamethsone</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DOI</td>
<td>2,5-dimethoxy-4-iodoamphetamine</td>
</tr>
<tr>
<td>DPX</td>
<td>Dibutylphthalate polystyrene xylene</td>
</tr>
<tr>
<td>ECT</td>
<td>Electroconvulsive therapy</td>
</tr>
<tr>
<td>EEG</td>
<td>Electroencephalogram</td>
</tr>
<tr>
<td>EPM</td>
<td>Elevated plus maze</td>
</tr>
<tr>
<td>FLV</td>
<td>Fluvoxamine</td>
</tr>
<tr>
<td>FLX</td>
<td>Fluoxetine</td>
</tr>
<tr>
<td>FST</td>
<td>Forced swim test</td>
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<tr>
<td>GABA</td>
<td>Gamma-aminobutyric acid</td>
</tr>
<tr>
<td>GEPR</td>
<td>Genetically Epilepsy Prone Rats</td>
</tr>
<tr>
<td>GR</td>
<td>Glucocorticoid receptor</td>
</tr>
<tr>
<td>HPA</td>
<td>Hypothalamo-pituitary adrenal</td>
</tr>
<tr>
<td>ILAE</td>
<td>International League Against Epilepsy</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>KA</td>
<td>Kainic acid</td>
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<tr>
<td>LH</td>
<td>Learned helplessness</td>
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<tr>
<td>MAOI</td>
<td>Monoamine oxidase inhibitor</td>
</tr>
<tr>
<td>MAP</td>
<td>Maprotiline</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>MDD</td>
<td>Major depressive disorder</td>
</tr>
<tr>
<td>MES</td>
<td>Maximal electroshock</td>
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<tr>
<td>MIR</td>
<td>Mirtazepine</td>
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<tr>
<td>MR</td>
<td>Mineralocorticoid</td>
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<tr>
<td>PAR</td>
<td>Paroxetine</td>
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<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PTZ</td>
<td>Pentylenetetrazol</td>
</tr>
<tr>
<td>SERT</td>
<td>Serotonin transporter</td>
</tr>
<tr>
<td>SRS</td>
<td>Spontaneous recurrent seizures</td>
</tr>
<tr>
<td>SRT</td>
<td>Sertraline</td>
</tr>
<tr>
<td>SNRI</td>
<td>Serotonin and noradrenaline reuptake inhibitor</td>
</tr>
<tr>
<td>SSRI</td>
<td>Selective serotonin reuptake inhibitor</td>
</tr>
<tr>
<td>SUDEP</td>
<td>Sudden unexpected death in epilepsy</td>
</tr>
<tr>
<td>REB</td>
<td>Reboxetine</td>
</tr>
<tr>
<td>TBI</td>
<td>Traumatic brain injury</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricyclic antidepressant</td>
</tr>
<tr>
<td>TLE</td>
<td>Temporal lobe epilepsy</td>
</tr>
<tr>
<td>VEN</td>
<td>Venlafaxine</td>
</tr>
<tr>
<td>VGAT</td>
<td>Vesicular GABA transporter</td>
</tr>
</tbody>
</table>
TABLE OF CONTENTS

Chapter 1: Literature Review

1.1. General introduction ............................................................................................................. 1

1.2. Epilepsy................................................................................................................................. 2
  1.2.1 Definitions and epidemiology ......................................................................................... 2
  1.2.2 Seizures .......................................................................................................................... 2
  1.2.3 Temporal lobe epilepsy .................................................................................................. 3
    1.2.3.1 Brief introduction to temporal lobe epilepsy ......................................................... 3
    1.2.3.2 Anatomical connectivity of the temporal lobe ...................................................... 4
    1.2.3.3 Aetiology of temporal lobe epilepsy ........................................................................ 6
    1.2.3.4 Pathology of temporal lobe epilepsy ....................................................................... 6
    1.2.3.5 Treatment of temporal lobe epilepsy ................................................................. 7
  1.2.4 Epileptogenesis ................................................................................................................ 7
  1.2.5 Animal models of temporal lobe epilepsy ..................................................................... 9
    1.2.5.1 The post-status epilepticus model of temporal lobe epilepsy ............................... 10
    1.2.5.2 The kindling model of temporal lobe epilepsy .................................................... 10

1.3. The comorbidities of epilepsy ......................................................................................... 12
  1.3.1 Historical and current perspectives of the comorbidities of epilepsy ......................... 12
  1.3.2 Prevalence and epidemiology of psychiatric disorders in epilepsy ............................ 14
  1.3.3 Depression and anxiety in epilepsy ............................................................................. 14

1.4. Antidepressant pharmacotherapy in epilepsy ................................................................. 16
  1.4.1 The discovery and use of first-generation antidepressants ......................................... 16
  1.4.2 The discovery and use of selective serotonin reuptake inhibitors ............................ 18
  1.4.3 Use of antidepressant pharmacotherapy in epilepsy .................................................. 20
    1.4.3.1 Human studies of SSRI pharmacotherapy in epilepsy ........................................ 21
    1.4.3.2 SSRI pharmacotherapy in animal models of epilepsy ....................................... 25

1.5. Antidepressants and epileptogenesis: shared neurobiological substrates .................... 30
Chapter 2: Methods

2.1. Overall study design ................................................................. 43
2.2. Experimental animals ....................................................................... 43
2.3. Live animal experimental procedures ............................................. 44
   2.3.1 Osmotic pump preparation .......................................................... 44
   2.3.2 Osmotic pump implantation ........................................................ 46
   2.3.3 Bipolar and recording electrode implantation ................................ 47
   2.3.4 Seizure threshold testing .............................................................. 48
   2.3.5 Amygdala kindling ..................................................................... 49
   2.3.6 Osmotic pump re-implantation .................................................. 50
   2.3.7 5-bromo-2-deoxyuridine injections ............................................ 50
   2.3.8 Elevated plus maze .................................................................... 50
   2.3.9 Forced swim test and corticosterone stress response ................ 51
   2.3.10 Corticosterone radioimmunoassay ............................................. 53
2.4. Post-mortem experimental procedures ....................................... 53
   2.4.1 Cardiac blood sampling, transcardial perfusion and tissue fixation 53
   2.4.2 Cryosectioning ........................................................................ 54
   2.4.3 Thionin staining and determination of electrode placement .......... 54
   2.4.4 BrdU immunohistochemistry ...................................................... 55
   2.4.5 BrdU cell number quantification ................................................ 56
Chapter 3: Pilot study - Tolerability of osmotic pumps and 
fluoxetine and the effects on behaviour, stress-induced 
corticosterone response and neurogenesis

3.1. Introduction

3.2. Materials and methods

3.2.1 Statistical analyses

3.3. Results

3.3.1 Fluoxetine administered by osmotic pumps does not affect weight gain

3.3.2 Rats treated with fluoxetine show serum levels within an appropriate therapeutic range

3.3.3 Fluoxetine-treated rats do not display an anxious phenotype in the elevated plus maze

3.3.4 Fluoxetine treated rats display a depressive-like phenotype

3.3.5 Fluoxetine treated rats show a trend towards a suppressed stress response

3.3.6 BrdU cell counts do not differ between fluoxetine and vehicle treatments

3.4. Discussion

Chapter 4: Chronic SSRI treatment and its effects on kindling 
epileptogenesis, behaviour, stress-induced corticosterone 
response and neurogenesis

4.1. Introduction

4.2. Materials and methods

4.2.1 Statistical analyses

4.3. Results

4.3.1 Rats treated with fluoxetine had serum levels within an appropriate therapeutic range

4.3.2 Seizure threshold is reduced by kindling, but is not affected by chronic SSRI treatment
4.3.3 Chronic fluoxetine and citalopram treatments accelerate kindling epileptogenesis. 77
4.3.4 Chronic SSRI treatments accelerate the progression of increase in seizure duration early in epileptogenesis .......................................................... 77
4.3.5 Chronic fluoxetine and citalopram treatments differentially affect anxiety-like behaviours ........................................................................................................... 81
4.3.6 Kindling, fluoxetine or citalopram treatments do not affect depressive-like behaviour ........................................................................................................... 81
4.3.7 Chronic fluoxetine, but not citalopram, treatment reduces the corticosterone response to swim stress ........................................................................................................... 84
4.3.8 Neither kindling nor chronic fluoxetine treatment affect dentate gyrus neurogenesis 85

4.4. Discussion .................................................................................................................. 86
4.4.1 Kindling epileptogenesis is accelerated by chronic antidepressant treatment .......... 86
4.4.2 Chronic fluoxetine and citalopram treatments increase seizure duration in the early stages of epileptogenesis ........................................................................................................... 89
4.4.3 Chronic fluoxetine and citalopram treatments do not affect seizure threshold .......... 90
4.4.4 Interim conclusion of kindling data ................................................................................. 91
4.4.5 Chronic fluoxetine and citalopram treatment and effects on anxiety- and depressive-like behaviours ........................................................................................................... 91
4.4.6 Chronic fluoxetine, but not citalopram, treatment reduces the corticosterone response to swim stress ........................................................................................................... 92
4.4.7 Neurogenesis is not affected by kindling or chronic fluoxetine treatment .............. 94
4.4.8 Final conclusions .............................................................................................................. 94

Chapter 5: General discussion

5.1. Key findings...................................................................................................................... 97
5.2. Potential mechanisms by which fluoxetine and citalopram accelerated kindling rate .. 99
5.2.1 Serotonin receptor alterations ...................................................................................... 100
5.2.2 Alterations in other aspects of neuroplasticity .............................................................. 101
5.2.3 Modulation of excitatory and inhibitory neurotransmission ......................................... 102
5.2.4 Overall conclusion of potential mechanisms .............................................................. 102
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.3.</td>
<td>The potential clinical implications of an accelerated rate of epileptogenesis</td>
<td>103</td>
</tr>
<tr>
<td>5.4.</td>
<td>Methodological considerations and limitations</td>
<td>106</td>
</tr>
<tr>
<td>5.4.1</td>
<td>Validity of the kindling model for comparison to epileptogenesis in humans</td>
<td>106</td>
</tr>
<tr>
<td>5.4.2</td>
<td>Normal rats versus the pathological state</td>
<td>108</td>
</tr>
<tr>
<td>5.4.3</td>
<td>Time point of analysis of common neurobiological substrates</td>
<td>108</td>
</tr>
<tr>
<td>5.4.4</td>
<td>Behavioural testing and comparison to human behaviours</td>
<td>109</td>
</tr>
<tr>
<td>5.5.</td>
<td>Future directions</td>
<td>110</td>
</tr>
<tr>
<td>5.5.1</td>
<td>Investigating the mechanisms involved in accelerated kindling epileptogenesis during chronic SSRI treatment</td>
<td>110</td>
</tr>
<tr>
<td>5.5.2</td>
<td>Investigating the effects of SSRIs in a chronic epilepsy model</td>
<td>111</td>
</tr>
<tr>
<td>5.5.3</td>
<td>Investigating the effects of SSRIs in a model of epilepsy and depression</td>
<td>111</td>
</tr>
<tr>
<td>5.5.4</td>
<td>Clinical directions</td>
<td>112</td>
</tr>
<tr>
<td>5.6.</td>
<td>Final conclusions</td>
<td>112</td>
</tr>
<tr>
<td></td>
<td>References</td>
<td>114</td>
</tr>
</tbody>
</table>
**LIST OF FIGURES AND TABLES**

Figure 1.1: Anatomy of the human temporal lobe...............................................................5
Figure 1.2: Location and structure of the rodent hippocampus..................................................5
Figure 1.3: Anatomical connectivity of the rodent hippocampus..................................................6
Figure 1.4: A schematic representation of epileptogenesis..........................................................9
Figure 1.5: Schematic representation of epileptogenesis in animal models of temporal lobe epilepsy.................................................................................................................................13
Figure 1.6: The time course of epileptogenesis and the typical stages at which antidepressants may be clinically introduced........................................................................................................17
Figure 1.7: The effects of SSRIs at the serotonergic synapse.........................................................19
Figure 1.8: The hypothalamo-pituitary-adrenal axis........................................................................32
Figure 1.9: The process of adult hippocampal neurogenesis in the dentate gyrus.........................34
Figure 2.1: Timeline of experimental procedures..........................................................................43
Figure 2.2: Internal structure of the osmotic pumps......................................................................45
Figure 2.3: The sequence of procedures for osmotic pump implantation........................................47
Figure 2.4: Location of recording and bipolar electrodes...............................................................48
Figure 2.5: An example of a seizure induced by electrical stimulation of the amygdala..................49
Figure 2.6: The elevated plus maze............................................................................................51
Figure 2.7: An example of the behaviours displayed in the forced swim test.................................52
Figure 2.8: Examples of sections cut on the cryostat.....................................................................54
Figure 2.9: An example of BrdU stained cells..............................................................................57
Figure 3.1: Timeline of experimental procedures..........................................................................61
Figure 3.2: Average weekly weight of fluoxetine- and vehicle-treated rats......................................62
Figure 3.3: Fluoxetine and norfluoxetine levels versus vehicle treatment as measured by mass spectroscopy........................................................................................................................................63
Figure 3.4: The effects of fluoxetine treatment on anxiety-like behaviours in the elevated plus maze........................................................................................................................................64
Figure 3.6: Change in corticosterone concentration from pre-stress levels....................................66
Figure 3.7: BrdU cell counts in the dentate gyrus and the hilus......................................................66
Figure 4.1: Timeline of experimental procedures..........................................................................75
Figure 4.2: Fluoxetine and norfluoxetine levels as measured by mass spectroscopy....................76
Figure 4.3: The effects of kindling and chronic fluoxetine and citalopram treatment on seizure threshold before and after kindling........................................................................................78
Figure 4.4: The effect of chronic fluoxetine and citalopram treatments on kindling epileptogenesis.........................................................................................................................................79
Figure 4.5: The effect of chronic fluoxetine and citalopram treatments on seizure duration...... 80
Figure 4.6: The effects of kindling and fluoxetine or citalopram treatment on anxiety-like behaviours................................................................. 82
Figure 4.7: The effects of kindling, fluoxetine and citalopram treatments on depressive-like behaviours............................................................................. 83
Figure 4.8: The effects of kindling and fluoxetine or citalopram treatments on corticosterone response following swim stress.................................................................................. 84
Figure 4.9: Total number of BrdU labelled cells in the subgranular zone of the dentate gyrus and hilus.............................................................................................................................. 85

Table 1.1: Studies of antidepressant use in patients with epilepsy................................................................................................................................. 24
Table 1.2: Studies of antidepressant administration in animal models of seizures and epilepsy................................................................................................................................. 27
Table 2.1: Total sample size included in each experiment................................................................................................................................................. 44
CHAPTER 1: LITERATURE REVIEW

1.1. General introduction

There is a high incidence of psychiatric comorbidity in patients with epilepsy, the most prevalent being depression (Gaitatzis et al., 2004, Kanner et al., 2012). Depression contributes to a large burden of the disability in patients suffering from epilepsy, more so than other factors such as illness duration or seizure frequency (Boylan et al., 2004, Gilliam et al., 2004, Kanner et al., 2010). Accordingly, considerable efforts have been made to improve the detection and diagnosis of depression in epilepsy, resulting in many patients being treated with antidepressant medication. Therefore, examining the effects of these medications in epilepsy is essential. Older generations of antidepressants, such as tricyclic antidepressants (TCAs), have been shown to induce seizures in some non-epileptic patients (Wroblewski et al., 1990, Preskorn and Fast, 1992) and increase seizure frequency in patients with epilepsy (Pisani et al., 1999). Therefore, although they are not contraindicated, TCAs need to be employed in patients with epilepsy with caution. Currently, there is growing evidence to suggest that the newer classes of antidepressants, in particular the selective serotonin reuptake inhibitors (SSRIs), have markedly less effects on seizure susceptibility, and may in fact lead to improvements in the severity of epilepsy itself. Studies in humans and in animal models of epilepsy suggest that SSRI treatment does not adversely affect seizures (Harmant et al., 1990, Wada et al., 1999, Hovorka et al., 2000, Kuhn et al., 2003, Borowicz et al., 2007), and in some cases may even improve seizure outcomes (Favale et al., 1995, Favale et al., 2003, Specchio et al., 2004), with only a few reports of proconvulsant effects (Gigli et al., 1994, Kanner et al., 2000, Thome-Souza et al., 2007). However, in all of these studies, the only investigations have been to examine the effects of SSRIs on short-term seizure outcomes. This includes assessing seizure threshold or seizure frequency over a short time period, mostly following short periods of treatment or following acute doses of SSRIs. To date, no studies have examined the effects of chronic SSRI exposure on epileptogenesis, the underlying and progressive neurobiological alterations that lead to the development of spontaneous seizures. This is essential to address, as SSRIs may be affecting the neurobiological processes leading to epilepsy, rather than just affecting the occurrence of seizures themselves.

This literature review first describes epilepsy and the pathological alterations occurring in epileptogenesis, with a description of animal models of epilepsy. The comorbidities of epilepsy are then discussed, examining their prevalence, epidemiology and theories of causation,
focusing on depressive disorders. Next, the use of antidepressant pharmacotherapy in epilepsy is reviewed, describing the different antidepressants available, focusing on SSRIs, and reviewing studies of SSRI use in patients and animal models of epilepsy. Finally, common neurobiological substrates between antidepressant treatment and epileptogenesis are described, to give further understanding of the effects antidepressants may have on epileptogenesis.

1.2. Epilepsy

1.2.1 Definitions and epidemiology

Epilepsy is a brain disorder that has been recognised from the early times of medical diagnosis. It is highly prevalent, affecting approximately 50 million people worldwide (World Health Organisation, 2006) with a bimodal distribution of seizure incidence, peaking in children and the elderly, although it can occur at any age (Hauser et al., 1991, 1993, Kotsopoulos et al., 2002). In fact, up to 10% of the population will experience a seizure during their lifetime, which can be described as a period of abnormal and synchronous excitation of a population of neuronal cells. Epilepsy itself is characterised by a history of a seizure that causes an enduring alteration in the brain, increasing the likelihood of developing further seizures, with associated neurobiological, cognitive and psychosocial conditions (Fisher et al., 2005).

The disorder poses a significant burden on quality of life, not only on the individual but also on their families and society at large. While epilepsy is a widely recognised condition in the present day, many issues still exist for patients with epilepsy. It can be a source of social discrimination and stigma, results in an increased mortality rate, economically contributes to 0.5% of the global burden of disease (Leonardi and Ustun, 2002), and importantly for this thesis, is associated with a higher incidence of cognitive, social and psychiatric comorbidities, which will be discussed in detail below.

1.2.2 Seizures

The definition of seizures has evolved throughout medical history with improvements in neuroimaging, genomic and molecular biology technologies. John Hughlings Jackson proposed one of the first definitions of a seizure in 1870 as "an occasional, an excessive and a disorderly discharge of nerve tissue" (Fisher et al., 2005, Scharfman and Pedley, 2006). Presently, the International League Against Epilepsy (ILAE) defines a seizure as "a transient occurrence of
signs and/or symptoms due to abnormal excessive or synchronous neuronal activity in the brain” (Fisher et al., 2005). While all seizures arise from an imbalance of excitation and inhibition in the brain, the initial causes vary, and therefore classification of seizures and epilepsy types has evolved. Currently, the ILAE classifies seizures as either focal or generalised: focal seizures originate in a specific region of the brain and are limited to a specific area and only one hemisphere, while generalised seizures do not have a localised onset and rapidly engage bilateral, and sometimes asymmetrically, cortical and subcortical networks (Berg and Scheffer, 2011).

The underlying causes of seizures can be divided into their origins: genetic, where the epilepsy is a direct result of a known or presumed genetic alteration from which seizures manifest; structural or metabolic, where there is a distinct condition or disease that is associated with the development of recurrent seizures, which may be acquired (e.g. stroke, brain injury) or genetic (e.g. channelopathies); or unknown, where the origin of seizures is due to an unrecognised genetic, structural or metabolic defect or a combination of these factors (Berg and Scheffer, 2011). Within these categories, many forms of epilepsies can be identified. The focus for this thesis is temporal lobe epilepsy (TLE), which is classified as focal epilepsy with structural/metabolic origins. It is one of the most common types of epilepsy in adults and is of particular interest due to the high comorbidity of psychiatric conditions, and as such, treatment of these conditions with pharmacotherapies.

1.2.3 Temporal lobe epilepsy

1.2.3.1 Brief introduction to temporal lobe epilepsy

Temporal lobe epilepsy, named so because seizures originate in temporal lobe structures (Van Roost et al., 1998), accounts for approximately 80% of focal epilepsies in adults (Hauser et al., 1991). It is frequently drug-resistant (Engel, 1996, 2001, Kwan et al., 2010) in that appropriate pharmacological interventions fail to provide adequate seizure control (Cascino, 1994). Patients typically experience focal seizures arising from the temporal lobe with or without impairment of consciousness, evolving to bilateral convulsive seizures (Engel, 1996, Sharma et al., 2007). Prior to discussing the pathophysiology of TLE, the normal structure and connectivity of the temporal lobe will be described, as it is relevant to the understanding of TLE, antidepressant action and their inter-relationship.
1.2.3.2 Anatomical connectivity of the temporal lobe

The temporal lobe is located bilaterally on the lateral sides of the brain, ventral to the Sylvian fissure and anterior to the parietal lobes (Figure 1.1). Its main structures, consisting of a majority of structures found in the limbic system, include the parahippocampal gyrus (including the parahippocampal, perirhinal and entorhinal cortices), the hippocampus and the amygdala. The latter two structures will be further discussed due to their involvement in emotional processing and in seizure development.

The hippocampus can be identified as a “C-shaped” structure in both the human and rodent brains (Figure 1.2), containing the hippocampus proper, consisting of Cornu Ammonis (CA) 1, 2 and 3 and the dentate gyrus. While the structures anatomical location is different in the human and rodent brains, the morphology and afferent and efferent connections are highly conserved. Figure 1.3 pictorially displays hippocampal connectivity. The granule cells of the dentate gyrus receive inputs from the entorhinal cortex via the perforant pathway. Mossy fibres, the axons of the granule cells, project from the dentate gyrus and synapse onto the pyramidal neurons of CA1, also synapsing onto CA3, from which the pyramidal neurons project via Schaeffer collaterals onto CA1 or directly out of CA3 to the fornix. Pyramidal cells of the CA1 region then project to the subiculum, which in turn projects out of the hippocampus to the hypothalamus via the fornix or to the entorhinal cortex, the latter of which contains reciprocal connections with the rest of the temporal lobe, serving as a gateway between the hippocampus and temporal lobe (Amaral et al., 1984, Witter, 1993). The amygdalae are located rostral to the hippocampi (Figure 1.1), comprising of multiple nuclei, including the basolateral, medial and central nuclei. The amygdala also has many reciprocal connections with the hippocampus (Amaral et al., 1987) and various cortical areas.
Figure 1.1: Anatomy of the human temporal lobe. Within the temporal lobe (red) reside the hippocampus (blue) and the amygdala (purple). From http://images.elephantjournal.com/wp-content/uploads/2011/08/amygdala-hippocampus.jpg

Figure 1.2: Location and structure of the rodent hippocampus. The rodent hippocampus is a C-shaped structure (red) located within the temporal lobe. O'Mara et al (2001).
1.2.3.3 Aetiology of temporal lobe epilepsy

The causes of TLE vary greatly, and in many cases are unknown. It may occur as a result of tumours, traumatic brain injuries or cortical and cerebrovascular malformations (Le Blanc and Rasmussen, 1974), amongst other causes, all of which can be exacerbated by external influences including stress (Haut et al., 2007, Koutsogiannopoulos et al., 2009), exercise (Dhanushkodi and Shetty, 2008), drugs of abuse (Gordon and Devinsky, 2001) or pharmacotherapies (Preskorn and Fast, 1992). However, the pathology of TLE remains largely similar regardless of the causation.

1.2.3.4 Pathology of temporal lobe epilepsy

The hippocampal formation remains the main area of investigation of TLE pathogenesis. This can be attributed to (1) electrographic recordings identifying the hippocampus as one of the main structures where seizures originate (Van Roost et al., 1998), (2) in patients with drug-resistant TLE, surgical removal of regions including the hippocampus ipsilateral to seizure onset reduces and often abolishes seizures (Zentner et al., 1995) (3) the pathological alterations in TLE patients largely involve the hippocampus. This includes hippocampal sclerosis, which is one of the most commonly described neuropathologies (Armstrong, 1993, de Lanerolle et al., 2003,
Sharma et al., 2007, Bae et al., 2010, Zheng et al., 2011), defined as a selective loss of neurons in the CA3 and CA1 regions, with relative sparing of CA2 and the granule cells of the dentate gyrus; mossy fibre sprouting, which involves the granule cell axons that usually synapse onto CA3, aberrantly projecting back onto granule cell apical dendrites and forming reciprocal excitatory projections which can contribute to seizure generation (Houser et al., 1990, Mathern et al., 1995, Buckmaster and Dudek, 1999, Gorter et al., 2001); gliosis, which is not entirely restricted to the hippocampus having also been demonstrated in the amygdala (Feindel et al., 1952, Feindel and Penfield, 1954, Pitkanen, 2002, Sharma et al., 2007, Shapiro et al., 2008). Whether these neuropathological alterations are a cause or a consequence of epilepsy remains unclear.

1.2.3.5 Treatment of temporal lobe epilepsy

In all epilepsies, treatment relies upon seizure suppression with pharmacotherapy. However, as mentioned above, up to 30% of patients become drug-resistant (Kwan and Brodie, 2000, Berg, 2008), while approximately 40% suffer significant drug-induced side effects (Kwan and Brodie, 2000). In drug-resistant patients, the only effective treatment option available to alleviate seizures is surgical removal of the epileptogenic region. However, in patients who are drug-responsive, treatment with antiepileptic drugs (AEDs) does not cure the condition. AEDs merely suppress seizures affording a better quality of life, with many patients continuing to take medication for the remainder of their lives. There is no alteration of the underlying pathology causing TLE despite much investigation into the processes leading to and continuing after seizures develop, that is, the process of epileptogenesis.

1.2.4 Epileptogenesis

Epileptogenesis is a dynamic process during which a variety of biochemical, anatomical and physiological changes occur in the brain that alter neuronal excitability and transform a normal brain into one that is capable of generating spontaneous seizures (Herman, 2002, Loscher, 2002, Pitkanen, 2002, Stables et al., 2002, Pitkanen et al., 2007, Engel and Pedley, 2008, Pitkanen, 2010). While epileptogenesis is most often associated with focal epilepsies resulting from structural lesions and focal seizures (Engel, 2001), it has also been suggested to occur in generalised epilepsies, perhaps through alterations in gene expression over time that increase seizure susceptibility (Zara and Bianchi, 2009). In the ‘three stage model’ of epileptogenesis, following an initial, precipitating insult to the brain, for example head trauma (Stage 1), a cascade of events are initiated that lead to altered excitability in the hippocampus and related structures. This is followed by a seizure-free latent period (Stage 2) which can last from months to years during which structural and biochemical changes occur, followed by the emergence of
spontaneous recurrent seizures (Stage 3) that can worsen epilepsy over time, further contributing to the neurobiological changes and psychiatric comorbidities (Figure 1.4, Scharfman, 2007). Additionally, the processes that underlie epileptogenesis do not cease once seizures commence; the neurobiological alterations continue and may even contribute to the progression of the disorder.

The progressive nature of epilepsy in humans is becoming more widely accepted, especially for TLE (O’Brien et al., 1999, Tasch et al., 1999, Bouilleret et al., 2000, Cole, 2000, Pitkanen and Sutula, 2002, Nearing et al., 2007, Cascino, 2009, Yang et al., 2010). This is due to evidence from humans and animal studies showing the progression of changes associated with epileptogenesis. As the epileptic disorder progresses, seizures may become more frequent and severe (Sillanpaa et al., 1998, Kwan and Sander, 2004, Shorvon and Luciano, 2007). In the more severe cases, as the disorder progresses, seizures may become resistant to conventional medications, and more invasive procedures, such as surgery to remove the epileptic focus, may need to be considered (Zentner et al., 1995). In association with this, psychiatric comorbidities may manifest, which can include anxiety and depression, as well as psychoses, memory impairments or cognitive decline (Kwan and Brodie, 2001, Motamedi and Meador, 2003, Andersson-Roswall et al., 2010). The association of some of these alterations and the implications of treating these comorbidities is a focus of this thesis, and will be discussed in more detail below (Section 1.3 onwards).

Although there has been much study on the processes occurring during and associated with epileptogenesis, it is still poorly understood, primarily due to the difficulties of studying epileptogenesis in humans; for example, in the majority of cases, human brain tissue from TLE patients is only available from those who are medically refractory, and at the most advanced stages of their illness and therefore the epileptogenic process. Consequently, animal models have provided a very valuable tool for gaining insights into the pathological changes occurring in epileptogenesis, as the changes occur over a period of months after the initial insult (Tanaka et al., 1992, Hellier et al., 1998, Kharatishvili et al., 2006) while in humans, similar processes can take years (French et al., 1993, Mathern et al., 1995).
**Figure 1.4: A schematic representation of epileptogenesis.** Following an initial precipitating event, a number of neurobiological changes occur contributing to increased seizure susceptibility and a reduced seizure threshold, leading to the emergence of spontaneous, recurrent seizures. The neurobiological alterations associated with epileptogenesis are indicated in the grey box, a period which includes the time after the precipitating event during the latent seizure-free period and the period after the emergence of spontaneous, recurrent seizures. BDNF – Brain-derived neurotrophic factor. Figure adapted from (Scharfman, 2007).

1.2.5 Animal models of temporal lobe epilepsy

The value in animal models of any disorder lies in their ability to inform on the human condition. This can be assessed according to the models' construct validity, in which the sequences of pathological and molecular events occurring are similar to the human condition; face validity, that the symptomatology is reasonably analogous to humans; and predictive validity, that the animal model is responsive to the same medications as the human condition. There is a wide range of animal models of epilepsy described in the literature, both genetic and acquired. Many of these models meet the above criteria, and are therefore valuable tools for assessment of the biological processes and modulators of epilepsy development and progression. Both genetic and acquired models of epilepsy can mimic the human condition: there is good construct validity, in that there are similar pathological and molecular alterations as well as the involvement of similar circuitries that generate seizures; face validity, as there is the development of spontaneous seizures and similar affective changes resembling the anxiety
and depressive comorbidities of epilepsy and predictive validity, with similar responses to pharmacological interventions. Genetic models of epilepsy will not be discussed here; instead the focus will remain on models of acquired epilepsy, in which epileptogenesis is initiated by an external brain insult. The two most commonly used models of acquired epilepsy in epilepsy research are the post-status epilepticus model and the kindling model. The development of epilepsy in these models is shown in Figure 1.5. Each model provides a good representation of the human condition, as well as being complementary, giving insight into different features of human TLE. However, each model has its own advantages and disadvantages, as will be discussed below.

1.2.5.1 The post-status epilepticus model of temporal lobe epilepsy

The post-status epilepticus model of TLE is a good model of the later stages of epilepsy, due to the development of spontaneous seizures. In this model, animals are typically systemically injected with a chemoconvulsant such as kainate or pilocarpine, which induce an acute period of recurrent seizures (status epilepticus), which are then terminated with an anticonvulsant. Following a latent period, seizures may spontaneously occur.

While the development of seizures in the post-status epilepticus model mimics the human condition, there are some limitations. The expression of spontaneous seizures is variable between different animals, a substantial portion of animals can die during status epilepticus and there is extensive cell loss associated with the initial seizures following the chemoconvulsant, as well as severe and widespread damage post-status epilepticus (Covolan and Mello, 2000, Peredery et al., 2000). Thus, the kindling model was used instead for the work described in this thesis, the advantages of which will be discussed below.

1.2.5.2 The kindling model of temporal lobe epilepsy

The kindling model has a number of advantages as a model of temporal lobe epileptogenesis disease progression. Kindling itself is defined as the “progressive changes that result from repeated electrical stimulation” (Goddard et al., 1969). Kindling was first demonstrated by Alonso-DeFlorida and Delgado (1958) and thoroughly tested by Goddard (Goddard, 1967, 1969), primarily in rats, but has also been shown in other species (mice, dogs, cats and several primate species) (Goddard et al., 1969, Racine, 1972b). Various limbic structures can be kindled to generate seizures, including the hippocampus and the amygdala (Delgado and Sevillano, 1961, Goddard et al., 1969, Racine, 1972b). The amygdala is the site most commonly kindled, as comparatively few electrical stimulations are required to induce a kindling effect (McNamara,
Additionally, studies by Adamec (1998) have shown that amygdala kindling is associated with behavioural alterations including increases in anxiety-like behaviour (Adamec, 1998, Adamec and Young, 2000, Adamec et al., 2004, Adamec et al., 2005) and cognitive deficits (Peele and Gilbert, 1992). On the other hand, depression, although common, has not been consistently shown to occur (Corcoran et al., 1992, Helfer et al., 1996, Adamec et al., 2004).

Amygdala kindling involves the repeated induction of focal seizures by an electrical current delivered via an implanted electrode to a specific amygdala nucleus. This produces a progressive increase in seizure susceptibility and the epileptic response, with a spread of seizure activity from focal to extrafocal regions and the appearance of generalised convulsive seizures, a condition that persists for months to years (Goddard et al., 1969, Racine, 1972b).

In conjunction with the increase in seizure susceptibility with subsequent stimulations, increases in the severity of behavioural seizures are also observed. The progression of the convulsive behaviours associated with amygdala kindling was extensively studied by Racine (1972b), who published a standard grading of behavioural seizures, from class I to V, during kindling. In the initial stages, when a focal seizure is elicited, an electrographic response occurs without any behavioural changes (class 0). With subsequent stimulations, behavioural responses begin to manifest which may include freezing and facial clonus (class I) followed by ipsilateral eye twitching and closure and head nodding (class II). As stimulations continue more distant structures from the initial seizure focus are recruited and forelimb clonus occurs (class III). Eventually, seizures generalise to bilateral cortical regions, the motor cortices are recruited and animals exhibit bilateral clonic seizures. This involves bilateral limb clonus, postural alterations such as rearing and twisting (class IV), and loss of balance (class V). Additionally, with subsequent electrical stimulations there is also a reduction in seizure threshold (Racine, 1972a, Tress and Herberg, 1972) and an increase in seizure duration, amplitude, spike frequency and morphology (Racine, 1972a,b, 1975).

The kindling model reliably produces seizures of increasing severity with repeated stimulations and is therefore an excellent model to assess the progression of epileptogenesis. Furthermore, anecdotal reports suggested that kindling might occur in humans. For example, occasionally electroconvulsive therapy with repeated stimulations unintentionally may result in spontaneous seizures, increased seizure durations and seizure-associated behavioural automatisms (Morrell, 1985, Sato et al., 1990, Coulter et al., 2002). However, a disadvantage of the kindling model is that seizures must be induced, as there is rarely development of spontaneous seizures (unless a large number of stimulations are given, termed “over kindling”, in which approximately half the animals will experience spontaneous seizures (Pinel and
The kindling model is also associated with only minor pathological changes such as mild cell loss and gliosis, dentate gyrus neurogenesis (Bengzon et al., 1997, Parent et al., 1998, Scott et al., 1998) and mossy fibre sprouting (Sutula, 1990, Ebert and Loscher, 1995). This damage is not as extensive as occurs following the post-status epilepticus models or in human TLE. However, this is beneficial, as it allows for the study of the functional changes in epileptogenesis independent of the confounding effects of cell loss and pathological alterations. For these reasons, the kindling model was chosen for this study. Furthermore, if the effects of antidepressants were to be seen with kindling in this study, this would justify further experiments employing the post-status epilepticus model, which are technically more difficult and require greater numbers of animals.

1.3. The comorbidities of epilepsy

1.3.1 Historical and current perspectives of the comorbidities of epilepsy

The co-occurrence of epilepsy and psychiatric disorders has been recognised since antiquity, as observed by Hippocrates, around 400 B.C., (Lewis, 1934) that “melancholics ordinarily become epileptics, and epileptics melancholics: what determines the preference is the direction the malady takes; if it bears upon the body, epilepsy, if upon the intelligence, melancholy.” Hippocrates observed that epilepsy was a result of natural and not sacred causes, eventually leading to medical investigations of the causes of epilepsy during the Renaissance, and the first understanding of seizure origins by John Hughlings Jackson in 1870 (Fisher et al., 2005, Scharfman and Pedley, 2006). From this, further investigations by Briquet in 1859 (Swinkels et al., 2005) and Morel in 1860 (Swinkels et al., 2005) recognised that psychological disturbances, behavioural and cognitive alterations occur between seizures as well as during the seizures themselves (Swinkels et al., 2005). The introduction of electroencephalography (EEG), the discovery of a temporal lobe origin of some seizures, and the association of the limbic structures within the temporal lobe with emotional processing further added support to ideas about how and why psychiatric disorders may be linked to epilepsy, especially epilepsy of temporal lobe origin. Currently, the understanding of the link between epilepsy and psychiatric disturbances recognises that this relationship is bidirectional, with several population-based studies finding that patients with epilepsy have a 5 to 20 fold higher risk of developing affective disorders (Tellez-Zenteno et al., 2007) and patients with a history of affective disorders have a 3 to 7 fold
higher risk of developing epilepsy (Forsgren and Nystrom, 1990, Hesdorffer et al., 2000, Hesdorffer et al., 2006, Morgan et al., 2012).

Figure 1.5: Schematic representation of epileptogenesis in animal models of temporal lobe epilepsy. In the post-status epilepticus model of TLE (A), administration of a chemoconvulsant initiates a period of status epilepticus, comprising of several hours of continuous seizures, which is terminated. Following a latent seizure-free period, which typically lasts for several weeks, spontaneous seizures commonly develop. In the kindling model of TLE (B), repeated stimulations administered at a set current result in seizures that increase in severity over time. When repeated stimulations are administered over several weeks or months (over kindling), spontaneous seizures may emerge. Figure taken from Morimoto et al. (2004).

While recurrent, unprovoked seizures are a hallmark of epilepsy and can be distressing, even fatal, the psychiatric comorbidities greatly contribute to the disability experienced by patients and their reduced quality of life. While a proportion of the comorbidity of epilepsy include physical injury (e.g. burns, fractures, falls), cognitive impairments, impaired fertility, social and behavioural changes and adverse effects to medications, the psychiatric disturbances constitute a large proportion of the burden of this comorbidity (Gaitatzis et al., 2004, Swinkels et al., 2005, Hesdorffer and Krishnamoorthy, 2011, Ottman et al., 2011). Furthermore, the importance of these problems was highlighted by the National Institute of Health Epilepsy Research
Benchmarks which nominated the comorbidities of epilepsy, including predominantly psychiatric comorbidities, as priority areas of research (Kelley et al., 2009).

1.3.2 Prevalence and epidemiology of psychiatric disorders in epilepsy

Patients with epilepsy have been reported to have higher rates of psychiatric comorbidities compared to the general population (Hesdorffer and Krishnamoorthy, 2011). These disorders are still highly prevalent in patients with epilepsy even when adjusting for socioeconomic disadvantage and other health issues (Rai et al., 2012). Psychiatric disorders in epilepsy can be classified according to their temporal relationship with seizures: peri-ictal – related to the seizure, or inter-ictal – between seizures and unrelated to the seizures themselves. Peri-ictal symptoms occurring in relation to the seizure will not be discussed; instead the focus will be upon the psychiatric alterations occurring independent of seizures themselves, during the inter-ictal period.

1.3.3 Depression and anxiety in epilepsy

In patients with TLE, both depression and anxiety are the most prevalent of the psychiatric disorders, with 17-44% of patients being diagnosed with depression and 7-35% of patients with anxiety (Ettinger et al., 2004, Mensah et al., 2007, Tellez-Zenteno et al., 2007, Kanner, 2011b). Community- and clinically-based studies have shown that the rates of occurrence of depressive (McLaughlin et al., 2008, Fuller-Thomson and Brennenstuhl, 2009) and anxiety (Gaitatzis et al., 2004, Kobau et al., 2006, Mensah et al., 2007, Kanner, 2011a) disorders in epilepsy are clearly elevated above the rates observed in the general population (Tellez-Zenteno et al., 2007). Other common disturbances, though less prevalent, include attention-deficit hyperactivity disorder and psychoses (Gaitatzis et al., 2004, Hesdorffer and Krishnamoorthy, 2011).

Depression in epilepsy can take several forms, including major depressive disorder, dysthymic disorder, adjustment disorder and the depressive phases of bipolar disorder. Studies have shown that depression is associated with impaired quality of life, greater cognitive complaints and deficits, and greater health care utilisation (Lacey et al., 2009), with some studies indicating that depression is a greater predictor of quality of life over other epilepsy-related variables, such as illness duration or seizure frequency (Boylan et al., 2004, Gilliam, 2005, Kanner, 2009, Kanner et al., 2010). Depression has also been shown to be a strong risk factor for suicide and a possible risk factor for Sudden Unexpected Death in Epilepsy (SUDEP) (Ridsdale et al., 2011).

Depression has been widely recognised as a common comorbidity of epilepsy while anxiety disorders, although also highly prevalent in people with epilepsy, have been somewhat
neglected. In fact, some studies suggest that anxiety disorders may even be more prevalent than depression and could be related to many of the same adverse consequences as depression (Li et al., 2002, Shimizu et al., 2002, Kanner, 2011a). Anxiety disorders in epilepsy can include generalised anxiety and panic disorders, which may also constitute risk factors for the development of depression in epilepsy, similar to the greater likelihood of developing depression in non-epileptic people who experience anxiety disorders (Kanner, 2011a).

Various epileptic factors have been examined with regard to the development of anxiety or depression. These include factors such as age of onset of epileptic disorder, seizure frequency or severity (Robertson et al., 1987), type of epilepsy syndrome and anatomical location of seizure focus, all of which have been examined with largely inconclusive results (Adams et al., 2008, Filho et al., 2008, Asmussen et al., 2009, Babu et al., 2009, Desai et al., 2010). In contrast to the neurological factors, psychosocial factors such as life stress, coping mechanisms, social support, perceived stigma and personality have shown to be more consistent predictors of the development of a comorbid psychiatric condition in epilepsy (Ormel et al., 1993, Hermann et al., 2000). Various studies suggest that TLE is more greatly associated with psychiatric disorders (Torta and Keller, 1999, Hermann et al., 2000). However this was due, in part, to a preponderance of studies involving TLE patients in tertiary epilepsy centres, which mostly include patients who are the most severely affected by seizures and other comorbidities. However, other studies suggest that rates of psychiatric comorbidities are similar across all epilepsies, as these studies have shown that psychiatric comorbidities also occur in generalised and extra-temporal epilepsies (Victoroff et al., 1994, Lambert and Robertson, 1999, Christensen et al., 2007, Adams et al., 2008, Hermann et al., 2008).

The link between epilepsy and psychiatric comorbidities has also been demonstrated in a wide variety of animal models of epilepsy. Both genetic and acquired models have been found to be associated with behavioural features relevant to the psychiatric co-morbidities that are commonly present in patients with epilepsy. This includes depressive- and anxiety-like behaviours observed in models of acquired epilepsy such as kindling (Adamec and Young, 2000, Kalyanchuk, 2000, Post, 2002, Mazarati et al., 2007), post-status epilepticus (Groticke et al., 2007, Koh et al., 2007, Groticke et al., 2008, Mazarati et al., 2008, Muller et al., 2009a, Muller et al., 2009b), posttraumatic epilepsy (Jones et al., 2008a) and febrile seizure (Mesquita et al., 2006) models, as well as in genetic models of epilepsy (Jobe and Browning, 2007, Jones et al., 2008b, Bouilleret et al., 2009, Sarkisova and van Luijteljaar, 2011). Furthermore, various early life psychosocial exposures have also been shown increase susceptibility to epilepsy development,
such as maternal separation (Salzberg et al., 2007, Jones et al., 2009, Kumar et al., 2011, Koe, 2012) and cross-fostering (Gilby, 2009).

As depressive and anxiety disorders are highly prevalent in epilepsy and may have adverse consequences, treatment and management of these symptoms in patients with epilepsy should be considered as an essential component of treatment (Barry et al., 2008).

1.4. **Antidepressant pharmacotherapy in epilepsy**

As highlighted above, the psychiatric comorbidities of epilepsy, particularly depression, constitutes a large proportion of the burden of comorbidity (Gaitatzis et al., 2004, Hesdorffer and Krishnamoorthy, 2011). As such, treatment of these depressive symptoms is essential. When these psychopathologies are recognised, a common approach is to treat with antidepressant pharmacotherapy. However, the way in which these drugs interact with the disease processes of epilepsy have not been thoroughly investigated, and may not only be treating the comorbid disorder, but may also be impacting on the epilepsy. Antidepressants may be introduced at various stages of epilepsy, shown by the dashed lines in Figure 1.6. Currently, selective serotonin reuptake inhibitors (SSRIs) or serotonin-noradrenaline reuptake inhibitors (SNRIs) are most commonly prescribed to patients with epilepsy. The following section will briefly discuss antidepressant medications, before discussing studies addressing the effect of SSRIs in epilepsy.

1.4.1 **The discovery and use of first-generation antidepressants**

Prior to the mid-20th century, pharmacological intervention to treat depression was not commonly practiced. Early studies suggested that monoaminergic deficits caused depressive symptoms and drugs that reversed this alleviated depressive symptoms. However, it was not until the opportune discovery in the 1950s of drugs that unintentionally treated depressive symptoms that there emerged the hypothesis of a monoaminergic deficit of depression. These two drugs included iproniazid, used to effectively treat tuberculosis, and imipramine, used to treat patients with schizophrenia. While these drugs effectively treated these disorders, they were also found to alleviate depressive symptoms in the patients taking these drugs (Smith, 1953, Crane, 1957, Loomer et al., 1957, Kuhn, 1958, Sandler, 1990, Lopez-Munoz et al., 2007), drugs later being discovered to act via monoaminergic mechanisms.
Iproniazid was the first drug in the class of antidepressants now known as monoamine oxidase inhibitors (MAOI). MAOIs act by inhibiting the monoamine oxidases to prevent the breakdown of monoamines, including serotonin and noradrenaline, increasing their availability. While MAOIs were widely used in the 1950s, they fell out of favour as first line therapies due to adverse side effects and interaction with dietary substrates (Lopez-Munoz et al., 2007, Lopez-Munoz and Alamo, 2009). However, MAOIs are still prescribed today, in cases of intolerance to first line treatments, in patients with refractory depression or when electroconvulsive therapy (ECT) is inadvisable or rejected as a treatment option (Ban, 2001).

Imipramine was the first drug in the class of tricyclic antidepressants (TCA) (Lopez-Munoz and Alamo, 2009). TCAs specifically inhibit the serotonin and noradrenaline reuptake membrane transporters, increasing the synaptic levels of these monoamines (Manji et al., 2001, Nestler et al., 2002, Morilak and Frazer, 2004, Gillespie and Nemeroff, 2005). Beginning with small studies in the late 1950s (Kuhn, 1958) and later clinical trials (Ball and Kiloh, 1959, Rees et al., 1961,
Klerman and Cole, 1965), their effectiveness in treating depressive symptoms was clearly evident. However, many undesirable side effects were also reported, including lethargy, weight gain and lethality in overdose (Ball and Kiloh, 1959, Rees et al., 1961, Klerman and Cole, 1965). TCAs are still broadly used today for the treatment of disorders other than depression such as obsessive-compulsive disorder and chronic pain.

The discovery of MAOIs and TCAs defined a turning point for psychopharmacological research into depression. The use of these drugs to treat depressive disorders initiated the idea that depression was caused by impairments and chemical alterations within the brain, challenging the long-held belief that patients with psychiatric disorders, especially depression, were "deranged individuals with moral defects, in need of moral therapy" (Lopez-Munoz and Alamo, 2009). This not only removed a considerable amount of burden from patients and their families, but also provided an understanding for the neurobiological basis of depressive disorders, as well as how antidepressant drugs worked.

1.4.2 The discovery and use of selective serotonin reuptake inhibitors

During this period in the 1950s and 1960s, as analytical techniques improved, it became understood that one mechanism by which antidepressants exerted their effects was by actions on the serotonergic system (Carlsson et al., 1968). For the first time, antidepressant drugs were being developed with a clear rationale and design to target specific deficits in depression. This paved the way for the development of drugs to specifically target the serotonin reuptake transporter to increase serotonin availability, avoiding other target receptors to reduce adverse side effects. This led to the discovery and introduction into popular use of the selective serotonin reuptake inhibitors (SSRIs), and also selective noradrenaline reuptake inhibitors and serotonin and noradrenergic reuptake inhibitors (SNRIs): drugs that specifically target the serotonergic and/or noradrenergic systems.

Through the development and testing of a variety of compounds, fluoxetine hydrochloride was found to potently inhibit serotonin reuptake 300 times more than noradrenaline (Richelson, 2001) and to less potently affect α1, histamine or muscarinic cholinergic receptors (Stahl, 1996). Reports of positive outcomes in clinical trials on depressive symptoms, fewer side effects and better safety in overdose led to approval from the United States Food and Drug Administration and the introduction of the first SSRI, fluoxetine hydrochloride (Prozac), into the market in 1987. With further research into SSRIs, a range of successors followed, including citalopram hydrobromide (Celexa) by Lundbeck in 1989. Citalopram is the most selective in its serotonin reuptake action in both in vitro and in vivo studies, showing 3500 times more
potency in inhibiting serotonin reuptake than it is with noradrenaline reuptake (Richelson, 2001). While many other selective serotoninergic and/or noradrenergic compounds have been discovered and are used today, for relevance to this thesis the subsequent discussion will focus upon fluoxetine and citalopram.

It is now widely established that one of the mechanisms of action of SSRIs, including both fluoxetine and citalopram, is inhibition of the serotonin transporter (SERT, Barker and Blakely, 1995, Stahl, 1996, Stahl, 1998) resulting in increasing extracellular levels of serotonin in the synaptic cleft of many brain regions (Fuller and Snoddy, 1990; Figure 1.7). With an increase in serotonin levels, the somatodendritic autoreceptors on the presynaptic neuron become desensitised. These autoreceptors normally act to inhibit further serotonin release but desensitisation contributes to increased release of serotonin into the synapse (Stahl, 1996). While both fluoxetine and citalopram are highly selective in their actions of increasing serotonin, fluoxetine has also shown to inhibit the P450 liver breakdown enzyme (CYP2D6) (Stahl, 1996), potentially resulting in adverse effects when combined with other medications as it may interfere with their metabolism. Fluoxetine also has a mean half-life of 1-3 days and its active metabolite norfluoxetine, a half-life of 7-15 days (Hyttel, 1994), which increases the likelihood of adverse interaction with other drugs administered at the same time.

![Figure 1.7: The effects of SSRIs at the serotonergic synapse.](http://www.bluelight.ru/vb/threads/532617-How-does-an-SSRI-work)
Although fluoxetine was widely used as an antidepressant in earlier days, and is still commonly used in the experimental literature, it is currently not regularly prescribed to patients with depression, due to the availability of more selective and safe SSRIs, such as citalopram. Citalopram is now one of the first line medications for clinical use in patients with depression, due to its high specificity and low side effect profile. It has low drug-drug interactions as it is less strongly protein bound, and its metabolites are not as biologically active as that of fluoxetine (the metabolites of citalopram are 4-11 times less potent than citalopram itself (Roseboom and Kalin, 2011). Furthermore, its 36 hour half-life results in rapid elimination prior to trying another drug and citalopram does not inhibit the P450 liver isozymes, like fluoxetine (Mula and Trimble, 2003, Roseboom and Kalin, 2011).

When SSRIs are administered, increases in serotonin occur very quickly, however, several weeks of treatment are required for clinical benefits to manifest in patients (Goodnick and Goldstein, 1998, Rosebook and Kalin, 2011), often resulting in poor compliance. In early studies, this delayed time of action was thought to be due to effects on receptor adaptations and to the delayed time of desensitisation of the somatodendritic and terminal serotonin receptors that act to regulate serotonin release (Lopez-Munoz et al., 2007). Studies now suggest that SSRIs have other targets of action in addition to reuptake inhibition, which may also explain the delayed time of therapeutic effect. There are a number of mechanisms by which the delayed time of response may occur, some of which will be discussed below in Section 1.5. Briefly, this may be due to the delayed time to affect regulation of intracellular signal transduction pathways (Nestler et al., 1989, Perez et al., 1991) and alterations in gene expression (Nibuya et al., 1996, Frechilla et al., 1998), as well as alterations in neurotrophic mechanisms, especially BDNF (Nibuya et al., 1995, Chen et al., 2001) and potentially through delayed effects on neuroplasticity (Nestler et al., 1989, Duman et al., 1997, Fabel et al., 2003).

### 1.4.3 Use of antidepressant pharmacotherapy in epilepsy

For some time, antidepressants have been known to influence seizure susceptibility. The first-generation antidepressants, the TCAs, were noted to have the side effect of triggering seizures in non-epileptic patients (Wroblewski et al., 1990, Edwards and Wheal, 1992, Preskorn and Fast, 1992) as well as aggravating pre-existing epilepsy (Pisani et al., 1999). Furthermore, experimental electrophysiological studies corroborated this ability of TCAs to aggravate seizures in vivo (Trimble, 1978, Koella et al., 1979, Ago et al., 2006, Ago et al., 2007) and in vitro (Luchins et al., 1984). As a consequence, antidepressant medications were more carefully prescribed to patients with epilepsy, with an increasing reluctance from physicians to treat the depressive symptoms in patients with epilepsy with antidepressants (Cotterman-Hart, 2010).
Consequently, many depressed patients with epilepsy remained, and in fact, still remain undertreated or completely untreated for their mood disorder. However, as the newer, second-generation antidepressants, the SSRIs came into popular use, being known for their reduced side effects and relative safety in overdose compared to TCAs, their use in epilepsy also increased and are considered a first-line medication for the treatment of depression in epilepsy.

One point to consider is also the concurrent use of AEDs with antidepressants and the interactions that may result. Patients with epilepsy are commonly treated with AEDs, and as such considering the effect of antidepressants on AEDs is also essential. Some AEDs have been shown to increase clearance of antidepressants (e.g., carbamazepine) while some antidepressants can inhibit clearance of AEDs through interaction with the CYP-450 hepatic enzyme system (Barry et al., 2008). Additionally, various AEDs are also used to treat psychiatric disorders such as mania and bipolar depression, and act as mood stabilisers in bipolar and schizoaffective disorders (Schmitz, 2011). However, AEDs have also shown to possibly be a cause of depressive symptoms in some patients (Mula and Monaco, 2009; Schmitz, 2011). Therefore considering the interaction of antidepressants and AEDs is essential given the common targets and indications of both drugs.

Various studies have attempted to address the issue of AED interaction with antidepressants and the effect this may have on seizures (Borowicz et al., 2006, Borowicz et al., 2007, Borowicz et al., 2011) while other studies have also investigated the way in which antidepressants themselves may affect seizures, in both patients and animal models. However, while various studies in humans and animals have investigated the effects of antidepressants on seizures, a common theme is the lack of investigation of the effects of antidepressants on epileptogenesis. Epileptogenesis is an important aspect to investigate, rather than just investigating the effects on the symptoms of the disorder, the seizures. This is because there is evidence to suggest that epilepsy is a progressive disorder that evolves over years in humans (O’Brien et al., 1999, Tasch et al., 1999, Bouilleret et al., 2000, Cole, 2000, Cascino, 2009, Yang et al., 2010, Ono and Galanopoulou, 2012) and while seizures may be controlled by AEDs, the underlying disorder itself may continue to progress.

1.4.3.1 Human studies of SSRI pharmacotherapy in epilepsy

Currently, ten studies have reported on the effect of SSRI or SNRI treatment in patients with epilepsy (Table 1.1). These studies involved both children and adult patients with either focal or generalised epilepsies either with (Harmant et al., 1990, Hovorka et al., 2000, Kanner et al., 2000, Kuhn et al., 2003, Specchio et al., 2004, Thome-Souza et al., 2007, Okazaki et al., 2011) or
without (Gigli et al., 1994, Favale et al., 1995, Favale et al., 2003) affective disorders. All patients in the study groups were receiving AED therapy concurrently with antidepressant medications. The antidepressants that were administered to the patients included various SSRIs and SNRIs as well as TCAs in one study (Okazaki et al., 2011). In all studies, the baseline seizure incidence (from 1-12 months prior to antidepressant administration) was obtained from medical records or seizure diaries, while during treatment seizure incidence was monitored at regular intervals for a time period of 1 to 15 months.

Of these ten studies, seven studies reported no change in seizure frequency (Harmant et al., 1990; Gigli et al., 1994; Hovorka et al., 2000; Kanner et al., 2000; Kuhn et al., 2003; Thome-Souza et al., 2007; Okazaki et al., 2011) and three studies reported an improvement in seizure frequency, with some patients reporting complete seizure freedom during antidepressant treatment (Favale et al., 1995; Favale et al., 2003; Specchio et al., 2004). While in some studies patients reported an increase in seizure frequency (Gigli et al., 1994; Kanner et al., 2000; Specchio et al., 2004, Thome-Souza 2007). However, these were rare occurrences and seizures were returned to pre-antidepressant treatment rates by increasing AED medications or removing the antidepressants.

Overall, these studies suggest that antidepressant medication administered to patients with epilepsy does not adversely affect seizure frequency, which in itself is an important outcome. Additionally, in the studies in which depressive symptoms were also monitored (Harmant et al., 1990, Hovorka et al., 2000, Kanner et al., 2000, Kuhn et al., 2003, Specchio et al., 2004, Thome-Souza et al., 2007, Okazaki et al., 2011), improvements were largely observed, which is important, given the impact of depression on quality of life (Boylan et al., 2004, Gilliam et al., 2004).

However, as with many clinical studies, various limitations exist when interpreting the results. Firstly, no studies were performed with a double blind, randomised-control design. Secondly, assessing the effects of the antidepressants on seizures may have been confounded by other factors such as medication compliance, stress, insomnia, brain damage or cognitive impairments. Thirdly, the magnitude of the effects may have been over- or underestimated due to fluctuations in seizure frequency, common throughout the course of the illness. Most studies were performed using small sample sizes resulting in low statistical power. Finally, in some studies, patients were chosen from highly selected population samples, such as patients from epilepsy clinics or patients being considered for epilepsy surgery.
Although there have been no placebo-controlled studies to assess the effect of antidepressants on epilepsy itself, one important and influential study assessed the seizure-inducing effects of antidepressant medications in non-epileptic patients (Alper et al., 2007). In this study, the effects of second generation antidepressants (mostly SSRIs) on seizures in over 75,000 patients from phase II and III randomly controlled clinical trials for treatment of depression were assessed. The results of this study suggested that in depressed (non-epileptic) placebo-treated controls, the rates of spontaneous seizures were considerably higher than known population rates and further suggested that this rate of spontaneous seizures was significantly reduced by antidepressant treatment. Although patients with epilepsy were excluded from analysis in this study, it does suggest an overall antiseizure effect for antidepressants when administered at therapeutic doses, which follows from the ten studies described above that investigated antidepressant medication and its effects on seizures in patients with epilepsy.

To date, there have been no clinical studies addressing the potential effects of antidepressant treatment on disease progression or disease-modification in epilepsy, as opposed to the anti-seizure effects. While it has been suggested in recent literature that such studies would be of value (Danzer, 2011, Igelstrom, 2012, Payandemehr et al., 2012, Vermoesen et al., 2012), the technical and logistic aspects make this a difficult undertaking. Such studies would require long-term treatment and follow up, large sample sizes, the need for appropriately matched placebo-treated controls, and the need to address the various manifestations of epilepsy progression that may be affected by antidepressant treatment. This includes not only seizure variables such as frequency, severity and duration but also other related factors such as type of AED administered and AED resistance, neuropsychiatric and neurocognitive deficits and associated structural brain changes. These factors are more readily addressed in animal models of epilepsy in which there is greater control of experimental settings, comparisons can be made before and after treatment, long-term follow up and assessment is more straightforward and achievable in a shorter time frame, and other aspects of the condition such as behavioural and neurochemical alterations can be more easily addressed.
<table>
<thead>
<tr>
<th>Study group</th>
<th>Antidepressants</th>
<th>Follow up</th>
<th>Seizure frequency in treatment group</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>9 PWE</td>
<td>FLX</td>
<td>3 months</td>
<td>4 Px increase in seizure frequency by &gt;50%; 5 Px not increased</td>
<td>Gigli et al., 1994</td>
</tr>
<tr>
<td>36 PWE and MDD</td>
<td>SRT, FLX</td>
<td>12 months</td>
<td>2 Px increase in seizure frequency; 34 Px not increased</td>
<td>Thome-Souza et al., 2007</td>
</tr>
<tr>
<td>100 PWE and depression or OCD</td>
<td>SRT</td>
<td>12 months</td>
<td>6 Px increase in seizure frequency*; 94 Px not increased (number of Px with reduced seizures not mentioned)</td>
<td>Kanner et al., 2000</td>
</tr>
<tr>
<td>39 PWE and depression</td>
<td>CIT</td>
<td>4 months</td>
<td>2 Px had 50% increase in seizures; 37 Px with 50% decrease in seizures</td>
<td>Specchio et al., 2004</td>
</tr>
<tr>
<td>11 PWE</td>
<td>CIT</td>
<td>8-10 months</td>
<td>All Px showed improvements: 64.1% mean reduction in seizure frequency</td>
<td>Favale et al., 2003</td>
</tr>
<tr>
<td>17 PWE</td>
<td>FLX</td>
<td>14 months</td>
<td>Seizures disappeared in 6 Px; in others, seizure frequency reduced by 30%</td>
<td>Favale et al., 1995</td>
</tr>
<tr>
<td>43 PWE and MDD</td>
<td>CIT</td>
<td>2 months</td>
<td>No change in seizure frequency</td>
<td>Hovorka et al., 2000</td>
</tr>
<tr>
<td>75 PWE and MDD</td>
<td>MIR, CIT, REB</td>
<td>7.5 months</td>
<td>No change in seizure frequency</td>
<td>Kuhn et al., 2003</td>
</tr>
<tr>
<td>28 PWE and depression</td>
<td>FLV</td>
<td>1-15 months</td>
<td>No change in seizure frequency</td>
<td>Harmant et al., 1990</td>
</tr>
<tr>
<td>121 PWE and affective or neurotic disorders</td>
<td>Various first and second gen. drugs</td>
<td>12 months</td>
<td>No change in seizure frequency compared to non-treated Px</td>
<td>Okazaki et al., 2011</td>
</tr>
</tbody>
</table>

Table 1.1: Studies of antidepressant use in patients with epilepsy. Patient dropouts are not included in sample sizes. Abbreviations: CIT – citalopram; FLV – fluvoxamine; FLX – fluoxetine; HAMD – Hamilton depression rating scale; MDD – major depressive disorder; MIR – mirtazapine; OCD – obsessive compulsive disorder; PWE – people with epilepsy; Px – patients; SRT – sertraline. *In 5/6 patients that had an increase in seizure frequency, there was only a probable cause of linking sertraline with an increase in seizure frequency.
1.4.3.2 SSRI pharmacotherapy in animal models of epilepsy

Various animal studies have investigated the effects of antidepressants in animal models of epilepsy. A recent review by Igelstrom (2012) which focused on determining the effects of antidepressants on animal models of seizures and epilepsy, found that antidepressants exerted an overall anticonvulsant effect. This follows the patient data in that antidepressants are not adversely affecting seizures. However, similarly to the human studies, most of the animal studies only address effects on short-term seizure outcomes (e.g., seizure threshold) and no longitudinal studies have been carried out.

The animal studies that have investigated the effects on SSRIs and SNRIs on seizures are summarised in Table 1.2. While human studies solely investigated seizure frequency, other measures were assessed in the animal models including number of spontaneous seizures, seizure frequency or duration, threshold to behavioural or electrical seizure, seizure severity, latency to first seizure and survival (see Table 1.2).

In summary, a majority of the studies found that SSRIs and SNRIs exert beneficial effects on seizures (anticonvulsant) or are without effect, indicated by reduced, or no change in, seizure frequency, duration, threshold, severity and increased latency to seizure between antidepressant-treated and vehicle-treated animals. However, a minority of studies did report a proconvulsant effect of antidepressants, indicated by reduced threshold to behavioural seizures, greater seizure severity, shorter latency and more frequent seizures. However this was a small number of studies (7 out of 38 studies), and in some cases, seizures were occurring at doses of antidepressants above the reported therapeutic range for animal models (Santos et al., 2002, Ahern et al., 2006, Payandemehr et al., 2012, Vermoesen et al., 2012). In fact, studies have reported a biphasic effect of serotonin (Clinckers et al., 2004a) and SSRI (Loscher, 2009, Payandemehr et al., 2012) concentrations on seizures, whereby low doses are anticonvulsant and higher doses are proconvulsant.

A strength of this collective data, suggesting that antidepressants have no adverse effect on seizure outcomes or may even be anticonvulsant, is that the evidence has been derived from a wide variety of different animal models including both chemoconvulsant- and electrically-induced seizure models as well as genetically predisposed models of epilepsy. However, there are some limitations to consider when interpreting this data. Firstly, in many of the models there were no behavioural abnormalities that were indicative of the comorbid psychiatric conditions that occur in patients with epilepsy. Secondly, many of the models employed were models of acute seizures, such as pentylenetetrazol or maximal electroshock, in which seizure
susceptibility can be tested in response to administration of antidepressants. However, the animals in these models are not epileptic and there would have been no associated pathological alterations, which are found in epileptic brains of humans and animals (described in section 1.2.3.4), and therefore the drugs may be having different effects. Thirdly, although the variety of animal models used is advantageous, it is also problematic to aggregate such data due to the wide variety of experimental designs used, particularly variations in the dose and timing of antidepressant administration relative to the seizure testing. Finally, in many of the studies, antidepressants were only administered as a single dose. This does not mimic the clinical situation, where several weeks of antidepressant treatment are required for clinically beneficial effects to manifest in patients and exposure may continue for months or years.

Absent from the current literature are any studies investigating the effects of chronic antidepressant treatment on epileptogenesis. Some studies have administered antidepressants for three weeks or greater (Dailey et al., 1992, Wada et al., 1995, Raju et al., 1999, Ferrero et al., 2005, Ahern et al., 2006). There have been three studies that investigated the effects of the chronic antidepressant treatment on long-term seizure outcomes (Hernandez et al., 2002, Mazarati et al., 2008, Vermoesen et al., 2012), all of which used a chemoconvulsant-induced model and investigated the occurrence of spontaneous seizures following SSRI treatment for 4-10 days. However, no studies have combined these measures to determine how chronic treatment with antidepressant drugs may impact epileptogenesis.
Antidepressants were found to be proconvulsant

<table>
<thead>
<tr>
<th>Model</th>
<th>Species</th>
<th>Drug</th>
<th>Dose (mg/kg)</th>
<th>Study protocol</th>
<th>Seizure outcome measures</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTZ</td>
<td>Mouse</td>
<td>CIT</td>
<td>25, 50</td>
<td>Injection 30min prior to sz test</td>
<td>Behavioural threshold</td>
<td>Payandemehr et al., 2012*</td>
</tr>
<tr>
<td>PTZ</td>
<td>Rat</td>
<td>FLX</td>
<td>10</td>
<td>Injection 1h prior to sz test</td>
<td>Severity</td>
<td>Zienowicz et al., 2005</td>
</tr>
<tr>
<td>PTZ</td>
<td>Rat</td>
<td>FLX</td>
<td>10</td>
<td>Sz tested after 21 days admin.</td>
<td>Behavioural threshold</td>
<td>Ferrero et al., 2005*</td>
</tr>
<tr>
<td>PTZ</td>
<td>Rat</td>
<td>VEN</td>
<td>75-100</td>
<td>Injection 30min prior to sz test</td>
<td>Severity and latency, frequency</td>
<td>Santos Jr et al., 2002</td>
</tr>
<tr>
<td>Lidocaine</td>
<td>Mouse</td>
<td>GBR 1293S, DES, MAP</td>
<td>5-20</td>
<td>Sz tested 2-5 after 5 days admin.</td>
<td>Behavioural threshold, latency</td>
<td>Arai et al., 2003*</td>
</tr>
<tr>
<td>Lidocaine</td>
<td>Mouse</td>
<td>CIT</td>
<td>10</td>
<td>Injection 30min prior to sz test</td>
<td>Frequency</td>
<td>Arai et al., 2003*</td>
</tr>
<tr>
<td>Pilocarpine</td>
<td>Mouse</td>
<td>CIT, REB, BUP</td>
<td>10, 30-300µM</td>
<td>Injection 1min prior to sz</td>
<td>Behavioural threshold</td>
<td>Vermoesen et al., 2011*</td>
</tr>
<tr>
<td>Flurothyl</td>
<td>Mouse</td>
<td>SRT, BUP</td>
<td>40,40</td>
<td>Injection 30min prior to sz test</td>
<td>Behavioural threshold, latency</td>
<td>Ugale et al., 2004</td>
</tr>
<tr>
<td>Flurothyl</td>
<td>Mouse</td>
<td>REB</td>
<td>20</td>
<td>Sz tested after 21 days admin.</td>
<td>Behavioural threshold, latency</td>
<td>Ahern et al., 2006*</td>
</tr>
</tbody>
</table>

Antidepressants were found to be anticonvulsant

<table>
<thead>
<tr>
<th>Model</th>
<th>Species</th>
<th>Drug</th>
<th>Dose (mg/kg)</th>
<th>Study protocol</th>
<th>Seizure outcome measures</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MES</td>
<td>Mouse</td>
<td>BUP</td>
<td>15-30</td>
<td>Injection 30min prior to sz</td>
<td>Behavioural threshold</td>
<td>Tutka et al., 2004*</td>
</tr>
<tr>
<td>PTZ</td>
<td>Zebrafish</td>
<td>CIT, REB, BUP</td>
<td>10-300µM</td>
<td>Injection 1min prior to sz</td>
<td>Behavioural threshold</td>
<td>Vermoesen et al., 2011*</td>
</tr>
<tr>
<td>PTZ</td>
<td>Mouse</td>
<td>REB, BUP</td>
<td>5-10, 10-40</td>
<td>Injection 30min prior to sz test</td>
<td>Behavioural threshold</td>
<td>Vermoesen et al., 2011*</td>
</tr>
<tr>
<td>PTZ</td>
<td>Mouse</td>
<td>FLX</td>
<td>20</td>
<td>Injection 60min prior to sz test</td>
<td>Survival</td>
<td>Kecskemeti et al., 2005*</td>
</tr>
<tr>
<td>PTZ</td>
<td>Mouse</td>
<td>FLX</td>
<td>1-20</td>
<td>Injection 30min prior to sz test</td>
<td>Behavioural threshold, latency</td>
<td>Ugale et al., 2004</td>
</tr>
<tr>
<td>PTZ</td>
<td>Mouse</td>
<td>CIT</td>
<td>0.5, 1</td>
<td>Injection 30min prior to sz test</td>
<td>Behavioural threshold</td>
<td>Payandemehr et al., 2012*</td>
</tr>
<tr>
<td>PTZ</td>
<td>Rat</td>
<td>VEN</td>
<td>25-50</td>
<td>Injection 30min prior to sz test</td>
<td>Severity and latency</td>
<td>Santos Jr et al., 2002</td>
</tr>
<tr>
<td>Pilocarpine</td>
<td>Zebrafish</td>
<td>CIT</td>
<td>10-300µM</td>
<td>Once epileptic, drug admin for 5 days, sz assessed for 72h</td>
<td>Behavioural threshold</td>
<td>Vermoesen et al., 2011*</td>
</tr>
<tr>
<td>Pilocarpine</td>
<td>Rat</td>
<td>FLX</td>
<td>20</td>
<td>Once epileptic, drug admin for 5 days, sz assessed for 72h</td>
<td>Severity</td>
<td>Hernandez et al., 2002</td>
</tr>
<tr>
<td>Pilocarpine</td>
<td>Rat</td>
<td>FLX</td>
<td>20</td>
<td>Once epileptic, drug admin for 10 days, sz assessed for 7 days</td>
<td>Behavioural threshold</td>
<td>Mazarati et al., 2008*</td>
</tr>
<tr>
<td>Flurothyl</td>
<td>Mouse</td>
<td>REB</td>
<td>20</td>
<td>Sz tested after 21 days admin.</td>
<td>Latency</td>
<td>Ahern et al., 2006*</td>
</tr>
<tr>
<td>Focal pilocarpine</td>
<td>Rat</td>
<td>CIT, GBR 12909</td>
<td>1µM, 1µM</td>
<td>Sz tested over 4h drug infusion</td>
<td>Behavioural threshold</td>
<td>Clinckers et al., 2004</td>
</tr>
<tr>
<td>Focal bicuculline</td>
<td>Rat</td>
<td>FLX</td>
<td>5-20</td>
<td>Injection 1h prior to sz test</td>
<td>Frequency, severity</td>
<td>Prendiville and Gale, 1993</td>
</tr>
<tr>
<td>Focal bicuculline</td>
<td>Rat</td>
<td>FLX</td>
<td>1.75-7nM</td>
<td>Injection 15min prior to sz test</td>
<td>Behavioural threshold</td>
<td>Pasini et al., 1992</td>
</tr>
<tr>
<td>Model</td>
<td>Species</td>
<td>Drug</td>
<td>Dose (mg/kg)</td>
<td>Study protocol</td>
<td>Seizure outcome measures</td>
<td>Reference</td>
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<td>---------------------------------------------</td>
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</tr>
<tr>
<td>PTZ, KA</td>
<td>Mouse</td>
<td>BUP</td>
<td>5-50</td>
<td>Injection 30min prior to sz test</td>
<td>Number of convulsions</td>
<td>Tutka et al., 2004*</td>
</tr>
<tr>
<td>PTZ, LH</td>
<td>Rat</td>
<td>FLX</td>
<td>10</td>
<td>Injection 1h prior to sz test</td>
<td>Behavioural threshold</td>
<td>Ferrero et al., 2005*</td>
</tr>
<tr>
<td>PTZ, pilocarpine</td>
<td>Mouse</td>
<td>CIT, REB, BUP</td>
<td>Various</td>
<td>Injection 30min prior to sz test</td>
<td>Behavioural threshold</td>
<td>Vermoesen et al., 2011*</td>
</tr>
<tr>
<td>PTZ</td>
<td>Mouse</td>
<td>FLX</td>
<td>20</td>
<td>Injection 60min prior to sz test</td>
<td>Latency</td>
<td>Kecskemeti et al., 2005*</td>
</tr>
</tbody>
</table>

Antidepressants resulted in no change:

- **Picotoxin Mouse FLX 20 Injection 65min prior to sz test Latency Pericic et al., 2005**
- **KA Mouse CIT 10 Sz tested after 7 days admin. Severity Jaako et al., 2011**
- **KA Rat CIT, REB 15, 20-30 Once epileptic, drug admin for 4 days and sz assessed Frequency, cumulative duration Vermoesen et al., 2012**
- **MES Mouse VEN 12.5, 25 Injection 30min prior to sz test Convulsive threshold Borowicz et al., 2011**
- **MES Mouse VEN 12.5, 25 Sz tested after 14 days admin. Convulsive threshold Borowicz et al., 2011**
- **MES Mouse FLX 10 Injection 60min prior to sz test Hind limb extension threshold Raju et al., 1999**
- **Ear shock Mouse FLX 15-25 Injection 30min prior to sz test Hind limb extension threshold Borowicz et al., 2006**
- **Hippocampal kindling Rat FLX 10nmol Injection 15min prior to sz test Electrical threshold Wada et al., 1993**
- **Hippocampal kindling Rat FLX 10 Sz tested after 21 days admin., 7 day wait, one injection Electrical threshold Wada et al., 1995**
- **Amygdala kindling Cat FLX 2-10 Injection 1-49h prior to sz test Electrical threshold Siegal and Murphy, 1979**
- **GEPR-3 and GEPR-9 Rat FLX 30 Injection 1-5h prior to sz test Severity Dailey et al., 1992**
- **GEPR-3 and GEPR-9 Rat FLX 7-20 Sz tested every 7 days over 28 day admin. Behavioural threshold Dailey et al., 1992**
- **GEPR-9 Rat FLX 15 Injection 1h prior to sz test Severity and latency, behavioural Yan et al., 1994**
- **El mice Mouse CIT 0.01, 0.02, 0.04% Sz tested after 14 days admin. Behavioural threshold Kabuto et al., 1994b**
- **El mice Mouse FLX 10 Sz tested after 3 or 7 days admin. Behavioural threshold Richman and Heinrichs, 2007**
<table>
<thead>
<tr>
<th>Drug</th>
<th>Species</th>
<th>Treatment</th>
<th>Dose</th>
<th>Time of Administration</th>
<th>Outcome</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTZ</td>
<td>Rat</td>
<td>FLX</td>
<td>2.5-20</td>
<td>Injection 30min prior to sz test</td>
<td>Latency and severity</td>
<td>Ceyhan et al., 2005</td>
</tr>
<tr>
<td>Lidocaine</td>
<td>Mouse</td>
<td>DES, MAP</td>
<td>20</td>
<td>Sz tested 5-9 after 5 days admin.</td>
<td>Frequency</td>
<td>Arai et al., 2003*</td>
</tr>
<tr>
<td>Lidocaine</td>
<td>Mouse</td>
<td>CIT</td>
<td>10-20</td>
<td>Sz tested after 5 days admin.</td>
<td>Frequency, threshold</td>
<td>Arai et al., 2003*</td>
</tr>
<tr>
<td>Pilocarpine</td>
<td>Zebrafish</td>
<td>REB, BUP</td>
<td>10-100</td>
<td>Injection 1min prior to sz test</td>
<td>Behavioural threshold</td>
<td>Vermoesen et al., 2011*</td>
</tr>
<tr>
<td>Pilocarpine</td>
<td>Rat</td>
<td>FLX</td>
<td>20</td>
<td>Once epileptic, drug admin for 10 days, sz assessed for 7 days</td>
<td>Seizure frequency</td>
<td>Mazarati et al., 2008*</td>
</tr>
<tr>
<td>Flurothyl</td>
<td>Mouse</td>
<td>VEN, REB</td>
<td>20-40,20</td>
<td>Injection 30min prior to sz test</td>
<td>Latency</td>
<td>Ahern et al., 2006*</td>
</tr>
<tr>
<td>Flurothyl</td>
<td>Mouse</td>
<td>VEN, BUP, SRT</td>
<td>20-40,40,40</td>
<td>Sz tested after 21 days admin.</td>
<td>Latency to first seizure</td>
<td>Ahern et al., 2006*</td>
</tr>
<tr>
<td>KA</td>
<td>Mouse</td>
<td>FLX</td>
<td>10-50</td>
<td>Injection 30min prior to sz test</td>
<td>Severity and duration</td>
<td>Jin et al., 2009</td>
</tr>
<tr>
<td>KA</td>
<td>Rat</td>
<td>CIT, REB</td>
<td>5-10,10</td>
<td>Once epileptic, drug admin for 4 days and sz assessed</td>
<td>Severity and duration</td>
<td>Vermoesen et al., 2012*</td>
</tr>
<tr>
<td>MES</td>
<td>Mouse</td>
<td>FLX</td>
<td>10</td>
<td>Sz tested after 21 days admin.</td>
<td>Hind limb extension threshold</td>
<td>Raju et al., 1999*</td>
</tr>
<tr>
<td>Ear shock</td>
<td>Mouse</td>
<td>FLX</td>
<td>10</td>
<td>Injection 30min prior to sz test</td>
<td>Hind limb extension threshold</td>
<td>Borowicz et al., 2006*</td>
</tr>
<tr>
<td>Ear shock</td>
<td>Mouse</td>
<td>FLX</td>
<td>7.5-20</td>
<td>Sz tested after 14 days admin.</td>
<td>Hind limb extension threshold</td>
<td>Borowicz et al., 2007</td>
</tr>
<tr>
<td>Hippocampal kindling</td>
<td>Rat</td>
<td>FLX</td>
<td>10</td>
<td>Injection 2h prior to sz test</td>
<td>Threshold and duration</td>
<td>Wada et al., 1999</td>
</tr>
<tr>
<td>Hippocampal kindling</td>
<td>Rat</td>
<td>FLX</td>
<td>1,10</td>
<td>Injection 1h prior to sz test</td>
<td>Electrical threshold</td>
<td>Wada et al., 1995*</td>
</tr>
<tr>
<td>EEG</td>
<td>Rat</td>
<td>FLV, CLV</td>
<td>10-30</td>
<td>Drugs infused and seizures assessed at 10min intervals</td>
<td>Epileptic activity on EEG</td>
<td>Krijzer et al., 1984</td>
</tr>
<tr>
<td>GEPR-3</td>
<td>Rat</td>
<td>FLX</td>
<td>3.5, 7.2, 14.1nmol</td>
<td>Injection 15min prior to sz test</td>
<td>Severity and latency</td>
<td>Statnick et al., 1996</td>
</tr>
<tr>
<td>GEPR-9</td>
<td>Rat</td>
<td>FLX</td>
<td>15</td>
<td>Injection 2h prior to sz test</td>
<td>Severity and latency</td>
<td>Browning et al., 1997</td>
</tr>
<tr>
<td>El mice</td>
<td>Mouse</td>
<td>CIT</td>
<td>80</td>
<td>Sz tested after 14 days admin.</td>
<td>Behavioural threshold</td>
<td>Kabuto et al., 1994a</td>
</tr>
</tbody>
</table>

Table 1.2: Studies of antidepressant administration in animal models of seizures and epilepsy.

Legend: *study is repeated within the table; #dietary supplement; ^no difference after 4 weeks. Abbreviations: ADT – afterdischarge threshold; BUP – bupropion; CIT – citalopram; CLV – clovoxamine; DES – desipramine; FLV – fluvoxamine; FLX – fluoxetine; GEPR – genetically epilepsy prone rats; h – hour; KA – kainic acid; LH – learned helplessness; MAP – maprotiline; MES – maximal electroshock; min – minute; PAR – paroxetine; PTZ – pentylenetetrazol; SRS – spontaneous recurrent seizures (as recorded on EEG); SRT – sertraline; sz – seizure; REB – reboxetine; VEN – venlafaxine.
1.5. Antidepressants and epileptogenesis: shared neurobiological substrates

Antidepressants affect a number of neurobiological processes in ways that could well affect epileptogenesis. This has only recently begun to be appreciated (Mazarati et al., 2010, Danzer, 2011, Jaako et al., 2011, Payandemehr et al., 2012) and as yet these processes have not been thoroughly investigated from this perspective. While determining how epileptogenesis may be affected by antidepressant exposure can be investigated to a limited extent by analysing the seizure outcomes that result from epileptogenesis, it is also essential to investigate the mechanisms of action common to the two disorders. As such, it is important to determine the common neurobiological mechanisms that are both affected by antidepressant treatment and implicated in epileptogenesis, as these factors may also be contributing to the effects of antidepressants in epilepsy. From the literature, it is clear that both epileptogenesis and antidepressant treatment share some common mechanisms of action, providing pathways by which antidepressants may potentially affect epileptogenesis. The most prominent of these are the effects on neuroplasticity and neurogenesis and alterations in the hypothalamo-pituitary-adrenal (HPA) axis, which are discussed below. There are also other factors and mechanisms common to the two, including monoamines, brain-derived neurotrophic factor and inflammatory processes, all of which will be briefly discussed but were not investigated in the experiments reported in this thesis.

1.5.1 Hypothalamo-pituitary-adrenal axis and glucocorticoids

Glucocorticoids, the end product of the hypothalamo-pituitary adrenal (HPA) axis, are steroid hormones that are released in a circadian and pulsatile rhythm, which become increasingly released in times of stress. Both circulating and stress-induced release of glucocorticoids, predominantly cortisol in humans and corticosterone in rodents, are homeostatically regulated by positive and negative feedback loops involving the hippocampus, amygdala, hypothalamus, frontal cortex and pituitary (Figure 1.8, Lupien et al., 2009). This occurs via activation of glucocorticoid receptors (GR) and mineralocorticoid receptors (MR) (Sapolsky et al., 1984, Jacobson and Sapolsky, 1991, Herman and Cullinan, 1997, Herman et al., 2003). The hippocampus and amygdala are also structures highly implicated in epileptogenesis, depression and antidepressant action. Therefore, considering the involvement of the HPA system in epilepsy and during antidepressant treatment may shed some light on possible mechanisms of their interaction.
Hyper-reactivity of the HPA axis has been reported to occur in greater than 50% of depressed patients (Holsboer, 2001, Pariante and Lightman, 2008), which is presumably due to dysfunctional negative feedback regulation of the HPA axis. This dysfunction has been shown to be alleviated by antidepressant treatment, particularly by SSRIs (Holsboer, 2001, Pariante, 2006, Himmerich et al., 2007). However, in a systematic review of studies investigating changes in cortisol levels in unipolar depression pre- and post-treatment, it was found that approximately 56% of patients had similar cortisol levels before and after treatment, regardless of depressive symptoms (McKay and Zakzanis, 2010). The ability of antidepressants to affect the HPA axis has also been investigated in animal studies, with a suppression of HPA axis function following treatment with fluoxetine or citalopram reported (Jensen et al., 1999, Jongsma et al., 2005, Mazarati et al., 2008). The mechanism by which antidepressants alleviate HPA dysfunction has been suggested to occur by increasing GR translocation in the hippocampus (Molteni et al., 2009) and regulation of CRH mRNA production in the paraventricular nucleus (Brady et al., 1991).

While hyper-reactivity of the HPA axis is a widely reported alteration in depressed patients (Pariante and Lightman, 2008), fewer, but increasing, reports suggest that HPA axis dysfunction also occurs in epilepsy. Various human and rodent studies have reported elevations in adrenocorticotrophic hormone (ACTH) and cortisol/corticosterone following seizures (Gallagher et al., 1984, Pritchard et al., 1985, Takeshita et al., 1986, Gallagher, 1987, Rao et al., 1989, Kumar et al., 2011). Humans with epilepsy, without comorbid depression or anxiety, were found to have a greater elevation in cortisol following the dexamethasone/corticotrophin-releasing hormone (Dex/CRH) test compared to controls, indicative of a hyper-reactive HPA axis (Zobel et al., 2004). In addition to this, animal studies suggest a cause/effect relationship between HPA axis dysfunction and epilepsy. For example, amygdala kindling epileptogenesis is accelerated with corticosterone supplementation (Karst et al., 1999, Taher et al., 2005, Kumar et al., 2007), and conversely, retarded kindling rates have been demonstrated in surgically adrenalectomised rats compared to sham-adrenalectomised rats (Cottrell et al., 1984, Weiss et al., 1993, Edwards et al., 1999). These findings are also consistent with a study using a rat model of epilepsy, in which HPA axis hyper-reactivity, assessed with the Dex/CRH test, was associated with the development of spontaneous seizures, and therefore epilepsy, which also correlated with the severity of depressive-like symptoms (Mazarati et al., 2009). Interestingly, in this study, HPA axis hyper-reactivity was also observed in rats that were not spontaneously seizing, indicating that the underlying epilepsy-associated pathological changes that contribute to the HPA axis changes may be unrelated to the seizures themselves.
Evidence suggesting a detrimental effect of glucocorticoids in epileptogenesis and the ability of antidepressants to influence HPA axis regulation indicates that the HPA system is a potential site of interaction where antidepressants may influence epileptogenesis and seizure susceptibility. While HPA axis dysfunction has not been shown to occur prior to epilepsy development in the absence of other causes (e.g., depression, other neuroendocrine disorders), it has been shown to be dysfunctional during the disorder. With animal studies suggesting that HPA hyper-reactivity may accelerate epileptogenesis (Edwards et al., 2002, Sadaghiani and Saboory, 2010, Ahmadzadeh et al., 2011), administration of antidepressants may in fact alleviate this and consequently slow the progression of the disorder. Various studies have investigated HPA axis alterations in epilepsy and following antidepressant treatment independently. However, to date, no studies have investigated how HPA axis function is affected in epilepsy during antidepressant treatment.

**Figure 1.8: The hypothalamo-pituitary-adrenal axis.** During times of stress, neurons in the paraventricular nucleus of the hypothalamus release corticotrophin-releasing hormone (CRH). This triggers the release of adrenocorticotrophic hormone (ACTH) from the anterior pituitary and subsequently activates the adrenal cortex to release glucocorticoids. In order to maintain homeostatic release of glucocorticoids, positive (amygdala) and negative (hippocampus) feedback loops regulate the release of glucocorticoids, through activation of the glucocorticoid receptors (GR) and mineralocorticoid receptors (MR). From Lupien et al. (2009).
1.5.2 Hippocampal neurogenesis

As discussed in Section 1.2.3.2, the hippocampus is an essential structure in TLE, being a common region of seizure initiation (Babb, 2001), as well as showing significant pathological alterations. This includes aberrant hippocampal plasticity, which is one of the key features of acquired epilepsy models and in human epilepsy, which may contribute to the generation of the hyperexcitable circuitry in the epileptic brain. The alteration of hippocampal neurogenesis may therefore potentially affect epilepsy development and progression.

In recent years, one of the most widely reported aspects of hippocampal plasticity has been hippocampal neurogenesis – the generation of newborn granule cells in the mammalian dentate gyrus, and their subsequent integration into the existing hippocampal circuitry (Altman and Das, 1965, Kaplan and Hinds, 1977, Cameron et al., 1993). Adult neurogenesis has been shown to occur in all mammalian species examined to date (humans, Eriksson et al., 1998; other species, Altman, 1962, Altman and Das, 1965, Gould et al., 1998, Kornack and Rakic, 1999) under normal conditions. Figure 1.9 shows the normal process of adult hippocampal neurogenesis that begins with neural progenitor cells giving rise to a pool of precursors. These precursor cells adopt a neuronal fate, and over several weeks undergo differentiation, maturation, migration and integration to become fully functional dentate granule cells (Eisch et al., 2008). Throughout this period, the newborn cells develop all the features of granule cells including the characteristic dendritic structure, axonal projections and afferent and efferent connections (Kaplan and Hinds, 1977, Markakis and Gage, 1999, Ambrogini et al., 2004, Overstreet et al., 2004, Laplagne et al., 2006, Ribak and Shapiro, 2007), becoming remarkably similar to cells produced earlier in development (Esposito et al., 2005, Zhao et al., 2006, Rahimi and Claiborne, 2007).
Figure 1.9: The process of adult hippocampal neurogenesis in the dentate gyrus. Progenitor cells (green) divide and multiply to give rise to a pool of precursor cells (blue) that adopt a neuronal fate (lavender) to become newborn neurons (purple). These immature neurons will develop over several weeks (pink) to eventually become fully integrated mature granule cells (orange). Figure adapted from Eisch et al (2008).

This process normally occurs in the brain continuously however insults to the brain may affect its progress. Studies have shown that seizures and epilepsy can be associated with both increases and decreases in neurogenesis. Acute seizures have been shown to stimulate hippocampal neurogenesis (Bengzon et al., 1997, Parent et al., 1997, Gray and Sundstrom, 1998, Parent et al., 1998), while chronic epilepsy in rodents resulted in a dramatic reduction in differentiation (Hattiangady et al., 2004, Hattiangady and Shetty, 2010). Integration of these cells into the hippocampal circuitry may also be affected by seizures. Following experimentally-induced status epilepticus, which over time often results in epilepsy, it has been found that newly generated neurons migrate ectopically into the dentate hilar region rather than into the normal hippocampal circuitry (Scharfman et al., 2000, McCloskey et al., 2006, Parent et al., 2006, Jessberger et al., 2007, Parent et al., 2007, Scharfman and Gray, 2007, Walter et al., 2007, Murphy et al., 2011). These cells have also shown to develop aberrant basal dendrites (Jessberger et al., 2007, Ribak and Shapiro, 2007, Shapiro et al., 2008) and contribute to mossy fibre sprouting (Parent et al., 1997, Danzer et al., 2008, Kron et al., 2010). This can create aberrant dendritic connectivity within the structure and potentially contribute to the generation of the epileptic circuitry (Parent et al., 1997, Scharfman et al., 2007, Kron et al.,
Several studies have also shown that blocking seizure-induced neurogenesis normalised the behavioural and electrophysiological effects of seizures (Jung et al., 2004, Jessberger et al., 2007, Raedt et al., 2007). However, other studies suggest that only a subset of newborn cells are affected (Jessberger et al., 2007, Walter et al., 2007) and that newly generated neurons may in fact protect the injured hippocampus from excessive excitability. These studies suggested that newborn cells receive less excitatory input than age-matched cells from control, non-epileptic animals (Jakubs et al., 2006, Murphy et al., 2011). Although it remains unclear, and controversial, how the neurogenic response to seizures may impact epileptogenesis, it is an important feature of epileptic pathology that is consistently observed and continues to be the focus of active research.

Adult neurogenesis is also sensitive to pharmacological interventions, as studies have shown that SSRIs, particularly fluoxetine but also citalopram and escitalopram, increase hippocampal neurogenesis in both humans (Boldrini et al., 2009) and animals (Malberg et al., 2000). In many studies neurogenesis was only observed after more than 14 days of SSRI treatment (Malberg et al., 2000, Kodama et al., 2005, Huang and Herbert, 2006, Marcussen et al., 2008), although a delayed time of action for SSRIs to increase neurogenesis is not observed in all studies (Himmerich et al., 2007, David et al., 2009, Hanson et al., 2011). In the study by Santarelli et al. (2003), it was found that neurogenesis was critical in mediating the positive behavioural response to antidepressants in animal models, however, this is still subject to debate (Miller et al., 2008, Zhao et al., 2008, Bessa et al., 2009). While neurogenesis may be a potential critical component required for the response to SSRIs, the mechanisms by which this occurs are not entirely clear. Studies have indicated that the enhancement of dentate gyrus neurogenesis following chronic antidepressant treatment may be mediated by increased serotonin and noradrenaline concentrations (Duman et al., 1997, Carlezon et al., 2005), an increased cAMP-CREB cascade (Nestler et al., 1989, Nibuya et al., 1996, Thome et al., 2000) or increased neurotrophic factors (Fabel et al., 2003, Khawaja et al., 2004).

There have been no studies specifically investigating the effect of long-term antidepressant treatment on neurogenesis in epilepsy. Nevertheless, a few studies have reported findings that may shed some light on this issue. For example, fluoxetine was able to reverse the maturation of a significant proportion of matured dentate granule cells into cells with immature neuronal properties (Kobayashi et al., 2010, Karpova et al., 2011). This may result in reduced synaptic connectivity with these immature cells and their subsequent projections into CA3, which may be relevant to the spread of seizure activity in the hippocampus. Other studies have investigated neuroplastic alterations following chemoconvulsant-induced status epilepticus during chronic...
treatment with citalopram. It was found that citalopram treatment reduced seizure-induced levels of reactive gliosis and aberrant cell proliferation (Jaako et al., 2009) as well as cell death and axonal sprouting (Jaako et al., 2011). This suggests that the pathological hippocampal circuit remodelling occurring in response to seizures can be alleviated by chronic SSRI treatment, which could potentially prevent the onset of epilepsy following an epileptic insult.

The importance of adult neurogenesis has also been debated. Although there is a high rate of neurogenesis occurring in the adult hippocampus, of approximately 9000 cells per day in a young adult rat, this is only 0.5% of the total mature granule cell population (Cameron and McKay, 2001). Therefore, the relative importance of these cells is questionable. However, these newborn cells possess greater levels of structural plasticity, especially in their immature stage (Hastings and Gould, 1999, Zhao et al., 2006, Toni et al., 2007), suggesting that they may be able to contribute to functionality more so than mature cells. As well as having greater plasticity, these cells also exhibit enhanced excitability and reduced threshold for long-term potentiation compared to mature granule cells (Snyder et al., 2001, Schmidt-Hieber et al., 2004, Saxe et al., 2006, Ge et al., 2007). Furthermore, with a greater level of structural plasticity comes the possibility of more robust changes in response to external influences, such as antidepressant treatment or brain insults such as epilepsy. However, no studies to date have addressed whether antidepressant treatment in epilepsy increases the production of normal or abnormal granule cells, and whether these alterations in neurogenesis contribute to epileptogenesis during antidepressant treatment. While increases in neurogenesis following antidepressant treatment appear to be beneficial, whether such increases in the epileptic brain are also beneficial is unknown. Addressing the alterations in neurogenesis in epilepsy and following antidepressant treatment may provide insight into how antidepressants affect the epileptic brain.

1.5.3 Other intersecting intermediaries

Alterations in the HPA axis and neuroplasticity, especially neurogenesis, are the focus in this thesis and are investigated as possible substrates by which antidepressants may affect epileptogenesis. However, there are other common substrates between antidepressant action and epileptogenesis that should also be highlighted such as alterations in monoamines, brain-derived neurotrophic factor (BDNF) and inflammation.
1.5.3.1 Monoamines

A broad literature describes monoamine abnormalities in both epilepsy and depression. With many of the newer-generation antidepressants targeting monoaminergic neurotransmission, this may be a possible candidate of interaction between epilepsy and antidepressant treatment. Studies have shown that serotonergic and noradrenergic alterations contribute to epileptogenesis. Wada et al. (1997) investigated kindling epileptogenesis following administration of serotonin receptor agonists and found that a 5HT$_{1A}$ receptor agonist, 8-hydroxy-2-(di-n-propylamino)tetrinal (8-OH-DPAT) inhibited kindling rate while a 5HT$_2$ receptor agonist, 2,5-dimethoxy-4-iodoamphetamine (DOI), facilitated kindling. The effect of DOI was blocked by the addition of a 5HT$_2$ receptor antagonist, ketanserin. This suggests a pivotal role of serotonergic neurotransmission in epileptogenesis. The importance of serotonin in epilepsy has also been highlighted in the Genetically Epilepsy Prone Rats (GEPR), a model of generalised seizures, in which widespread reductions in serotonin concentrations and uptake, as well as alterations in serotonergic receptor distributions have been reported (Statnick et al., 1996a). Other studies have also highlighted the involvement of serotonergic neurotransmission in modulating seizure susceptibility (Kilian and Frey, 1973, Buterbaugh, 1978, Hiramatsu et al., 1987, Dailey et al., 1992, Przegalinski et al., 1994, Gerber et al., 1998, Filakovszky et al., 2001) while other studies have investigated the effects of fluoxetine on the serotonergic system and its relationship to seizures. In GEPRs fluoxetine reduced the severity of sound-induced convulsions, the time course of which was paralleled by a fluoxetine-induced rise in serotonin levels (Dailey et al., 1992). Additionally, in the pilocarpine model of status epilepticus, chronic fluoxetine restored epilepsy-induced serotonergic deficits (Mazarati et al., 2008).

Noradrenergic alterations have also been shown to contribute to seizure susceptibility and epileptogenesis. In animals, lesioning noradrenergic inputs from the locus coeruleus accelerated amygdala kindling, implying an antiepileptogenic role for noradrenaline. Further, the GEPR-9 rats display seizure susceptibility concurrent with noradrenergic deficits (Jobe et al., 1995, Statnick et al., 1996b) while reversing these neurotransmitter deficits reduces the seizure susceptibility (Yan et al., 1993). Together, these studies suggest a role of monoamines in mediating the effects of antidepressants on epileptogenesis.

1.5.3.2 Brain-derived neurotrophic factor

BDNF is another factor that has been shown to be involved in epilepsy and in the actions of antidepressants. Studies have shown that BDNF is elevated in hippocampal tissue obtained from patients with epilepsy (Mathern et al., 1997, Takahashi et al., 1999, Murray et al., 2000), and its
synthesis is increased in response to seizures (Ernfors et al., 1991, Rudge et al., 1998). However, both proepileptogenic (Croll et al., 1999, Asztely et al., 2000, Zhu and Roper, 2001, Scharfman et al., 2002, Koyama et al., 2004) and antiepileptogenic effects (Larmet et al., 1995, Osehobo et al., 1999, Reibel et al., 2000a, Reibel et al., 2000b) of BDNF have been reported. As BDNF is a potentially an essential component to epileptogenesis, alterations in this factor may affect epilepsy.

SSRIs have also been shown to affect BDNF, with studies showing that BDNF and its receptor TrkB are required for the action of antidepressants (D'Sa and Duman, 2002, Nestler et al., 2002, Popoli et al., 2002). Clinical studies report increases in serum BDNF following antidepressant treatment in depressed patients, correlating with improvements in mood (Karege et al., 2002, Aydemir et al., 2005, Gervasoni et al., 2005, Gonul et al., 2005, Aydemir et al., 2006). Animal studies have also found increases in BDNF mRNA (Nibuya et al., 1995, Russo-Neustadt et al., 2000, Dias et al., 2003) and protein (Chen et al., 2001, Altar et al., 2003, Xu et al., 2003), as well as TrkB expression (Nibuya et al., 1995) and activation (Saarelainen et al., 2003) in the hippocampus and prefrontal cortex following antidepressant treatment, although other studies have failed to demonstrate such elevations (Miro et al., 2002, Coppell et al., 2003, Altieri et al., 2004). While there is a clear overlap of BDNF alterations following antidepressant treatment and in epileptogenesis, no studies to date have investigated the effects of antidepressants on BDNF during epileptogenesis.

1.5.3.3 Inflammation

Inflammation is another process implicated in epilepsy (Vezzani et al., 2012), depression (Miller et al., 2009) and in antidepressant action (Janssen et al., 2010). In epilepsy, increases in inflammatory mediators have been identified in resected brain tissue of TLE patients (Crespel et al., 2002, Zattoni et al., 2011, Hirvonen et al., 2012), and in experimental seizure models (Minami et al., 1991, Vezzani et al., 2000, Turrin and Rivest, 2004, Gorter et al., 2006, Aronica et al., 2007). In depression, increases in inflammatory mediators (Maes et al., 1995, Lanquillon et al., 2000, Mikova et al., 2001, Owen et al., 2001, Tiemeier et al., 2003, Alesci et al., 2005, Brietzke et al., 2009) have also been shown to be alleviated by antidepressant treatment (Kenis and Maes, 2002, Sutcuigil et al., 2007), although other studies have failed to replicate this (Hannestad et al., 2011). Furthermore, pro-inflammatory effects of antidepressants have also been demonstrated (Piletz et al., 2009, Fornaro et al., 2011). As such, antidepressants may play an anti-inflammatory role in epilepsy, but whether this is the case and if it is beneficial or detrimental has not been thoroughly investigated.
Two studies have investigated the interaction of fluoxetine with IL-1β in the pilocarpine-induced post-status epilepticus model. In these studies, rats rendered epileptic displayed depressive-like behaviours, dysregulation of the HPA axis and abnormal serotonin signalling. It was found that intrahippocampal infusion of an IL-1 receptor antagonist (IL-1ra) ameliorated depressive-like behaviours, with no effect on spontaneous seizures (Mazarati et al., 2010). In a follow up study, combined treatment of fluoxetine and IL-1ra improved the depressive-like behaviours and abnormalities in serotonin signalling, but only partially alleviated HPA axis hyper-reactivity while fluoxetine treatment in isolation had no effect (Pineda et al., 2012). These findings indicate that co-treatment of antidepressants with anti-inflammatory agents could reverse some of the pathological processes associated with epilepsy development.
1.6. Study rationale

As psychiatric disorders contribute to a major burden of disease in epilepsy, management of these disorders is of great importance. As such, many patients with epilepsy may be prescribed antidepressants at various stages of their disorder to treat comorbid psychiatric issues. This is an important area to investigate, as treatment with antidepressants may impact the progression of the epileptic disorder. To date, much of the literature has focused on the effects of antidepressants on short-term seizure endpoints, with most studies only investigating seizures (the occurrence, frequency or threshold to a seizure) rather than epilepsy (alterations in the pathological and biochemical changes that lead to seizures and the occurrence of spontaneous seizures). When taken together, these studies largely suggest that antidepressants are beneficial in epilepsy and are therefore considered safe for use in patients with epilepsy. However, many studies only administer antidepressants acutely or for short periods of time, which does not follow the clinical situation in which antidepressants are administered for several weeks to ensure a beneficial therapeutic effect. As such, there is no indication of the effect that chronic antidepressant treatment has on epileptogenesis and the associated neurobiological changes that continue even after seizures emerge. Investigation of long-term endpoints is needed to determine the long-term effects of antidepressant treatment in epilepsy. This includes investigation of seizure frequency over time and changes in the severity of the disorder during antidepressant treatment, as well as exploring the effects on common substrates. As highlighted, undertaking such studies in patient populations is technically and logistically difficult and time consuming. Investigating this in animal models provides a much more straightforward and achievable approach. If animal studies were to show effects of antidepressants on epileptogenesis, this would then be a strong argument for conducting human studies to further investigate this phenomenon.
1.7. **Hypotheses and aims**

The main research questions of this thesis are:

1. Does chronic treatment with the SSRIs fluoxetine and citalopram have effects on amygdala kindling epileptogenesis?

2. Does chronic treatment with fluoxetine and citalopram influence the severity of anxiety- and depressive-like behaviours, suppress the corticosterone response to stress and reduce aberrant neurogenesis associated with the kindling model of epileptogenesis?

*Study 1 – Pilot study: tolerability of osmotic pumps and fluoxetine and the effects on behaviour, stress-induced corticosterone and neurogenesis (Chapter 3).*

Prior to investigating the effects on epileptogenesis, the chosen method of drug delivery – SSRIs delivered by osmotic pumps – was piloted. Additionally, the behavioural, neuroendocrine and neuroplastic effects of fluoxetine were investigated.

**Hypotheses:**

1. Fluoxetine delivered by osmotic pumps will be an effective method of drug delivery to rodents.

2. Chronic treatment with fluoxetine will affect behaviour, suppress the corticosterone response to stress and increase hippocampal neurogenesis.

**Aims:**

1. To validate the use of osmotic pumps as an effective method to chronic deliver fluoxetine treatment in rats.

2. To investigate the changes in depressive- and anxiety-like behaviours, stress-induced corticosterone levels and hippocampal neurogenesis during treatment with fluoxetine.
Study 2 – The effects of chronic fluoxetine and citalopram treatment on kindling epileptogenesis and related behavioural and physiological alterations (Chapter 4).

Hypotheses:

1. Compared to vehicle-treated rats, fluoxetine- and citalopram-treated rats would have a slower rate of kindling epileptogenesis.

2. Kindling would increase anxiety and depressive-like behaviours and this would be alleviated with fluoxetine and citalopram treatments.

3. Kindling would increase the corticosterone response to stress, and this would be alleviated with fluoxetine and citalopram treatments.

Aims:

1. To investigate the effects of fluoxetine or citalopram on epileptogenesis using the amygdala kindling model.

2. To investigate the kindling-induced changes in depressive- and anxiety-like behaviours, stress-induced corticosterone levels and hippocampal neurogenesis and how these are affected by fluoxetine and citalopram treatment.
CHAPTER 2: METHODS

2.1. Overall study design

This study was designed to evaluate effects of chronic antidepressant treatment on the progression of epileptogenesis. A general timeline of experimental procedures is shown in Figure 2.1. To ensure continuous delivery of drugs throughout the experiment, all rats were implanted with osmotic pumps (each of four week delivery duration) at the beginning of the experiment (week 0) and at week 4. A pilot study (Chapter 3) was designed to validate the osmotic pumps as a method of drug delivery and to determine the tolerability of the osmotic pumps for the experimental time period. Therefore, this cohort of rats went through identical experimental procedures as set out in the timeline (Figure 2.1) excluding electrode implantations, seizure threshold testing and kindling stimulations. Chapter 4 describes the kindling studies that followed the timeline in Figure 2.1, with rats being treated with either fluoxetine or citalopram.

Figure 2.1: Timeline of experimental procedures

2.2. Experimental animals

9-11 week old male Wistar rats were used (n=10 for pilot study, n=63 for fluoxetine kindling study, n=69 for citalopram kindling study). Rats were bred and housed in the Department of
Medicine Biological Research Facility and maintained on a 12 hour light/dark cycle (lights on at 6am) at 19-24°C with ad libitum access to food and water.

Table 2.1: Total sample size included in each experiment. Reasons for exclusion: incorrect electrode placement, non-detectable levels of drug in plasma, culled due to loss of electrode cap, culled due to infection from osmotic pump, died during/post electrode surgery, kindling procedure incomplete (did not receive thirty stimulations).

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2.3. Live animal experimental procedures

All procedures were performed according to the guidelines set by the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (2004) and approved by the University of Melbourne Animal Ethics Committee (ethics number: 0911543).

2.3.1 Osmotic pump preparation

Osmotic pumps allow for the continuous systemic infusion of solutions into unrestrained rats. The internal structure of a pump is displayed in Figure 2.2. To prepare the pumps, the drug is injected into the impermeable reservoir. Upon implantation, water from the surrounding environment permeates the semi-permeable membrane and expands the osmotic layer (which contains a high osmolality solution, causing water to flux in). This displaces the solution from the impermeable reservoir at a predetermined rate via the flow moderator, which controls the release of the agent over the required time period (Theeuwes, 1975). This process begins
approximately 6 hours after implantation and continues uninterruptedly until removal. The drug is then taken up systemically, according to the site of implantation.

![Figure 2.2: Internal structure of the osmotic pumps.](http://www.alzet.com/products/ALZET_Pumps/howdoesitwork.html)

The pumps used in this study lasted between 32 and 35 days depending on the lot number (2ML4, Alzet Osmotic Minipump, Alza, USA). Weight gain over the experimental period was taken into account when calculating drug concentrations. The concentration of fluoxetine hydrochloride (Aurobindo Pharma, India) or citalopram hydrobromide (Biotrend Chemicals, Switzerland) to fill the pump was based on the predicted weight of the rat four weeks from date of implantation to ensure the target dose of 10mg/kg was delivered by the end of the experimental period. The average weight gain was predicted from a large inventory of data on weight gain of rats over time from several different experiments within the laboratory. The dose of 10mg/kg was chosen for both drugs on the basis of previous studies that have administered fluoxetine and citalopram to rats using pumps with therapeutic plasma levels being detected in rats that had received this dose (Czachura and Rasmussen, 2000, Castro et al., 2003, Hasegawa et al., 2005, Hesketh et al., 2005, Hesketh et al., 2008, Riad et al., 2008).

To prepare the solutions of fluoxetine and citalopram, the appropriate concentration was dissolved in a sterile 50% dimethyl sulfoxide (DMSO) and distilled water vehicle. In order to ensure pumps were correctly filled, the pump with its flow moderator was weighed, the solution injected into the pump under sterile conditions (performed in a laminar flow hood), and the filled pump was weighed again. If the filled weight was >90% of the mean fill volume (as specified from manufacturer's instructions) the pump was considered filled and ready for implantation.
2.3.2 Osmotic pump implantation

Pumps were prepared prior to commencement of surgical procedures. All rats were implanted with osmotic pumps with the appropriate drug or vehicle. Rats were anaesthetised by inhalation of isofluorane (5% induction, 1.5-2.5% maintenance, in 1:1 medical air:oxygen). Heart rate and blood oxygenation were monitored with a pulse oximeter throughout surgery. Once eye blink and footpad reflexes were absent, the site of incision was shaved and swabbed with a topical antiseptic (10% w/v povidone-iodine, Betadine) from the nape of the neck to 1cm below the shoulder blades along the midline and 2cm lateral to the spine on either side (Figure 2.3A).

The sequence of images in Figure 2.3 shows where the pump was implanted. A 2cm incision was made at the shoulder blades through the skin, avoiding the underlying muscle and connective tissue. Using haemostats, an area of subcutaneous tissue, 1cm lateral and parallel to the spine was made above the left flank, 6-7cm long for the pump to fit lengthways. This is an area slightly larger than the pump to allow the pump to move freely and not be of discomfort to the rat. The pump was then inserted (Figure 2.3B), with the flow moderator end pointed caudally, until it was at least 1cm away from the incision site (Figure 2.3C). This ensures the pump does not adhere to tissue under the incision site, break the skin and become exposed. The incision was closed with buried sutures using absorbable suture (Biovek 4/0, Dynek, Australia).

Rats in the kindling studies of Chapter 4 then underwent electrode implantation (Section 2.3.3). For rats in the pilot study (Chapter 3), the incision area was swabbed with topical antiseptic and the rat injected with 1ml saline (0.9%, subcutaneously) and an analgesic, 4mg/kg carprofen (intraperitoneally, Rimady1, Pfizer, Australia). Rats were recovered on a heat mat and weight was monitored for 5 days or until pre-surgery weight was obtained. At the end of the experimental period, pumps were removed and the remaining volume aspirated to ensure that over 85% of the solution had been released as per manufacturer’s instructions.

Rats were also monitored regularly (at least once per week) for any signs of inflammation or infection around the pump. A common post-surgery occurrence was seromas, an accumulation of clear, sterile fluid around area of tissue trauma. This was observed in 15/73 rats, but drained away naturally over time. Two rats were culled due to signs of infection, as the seroma did not reduce. Other issues that arose were scratching around the site of incision. This was due to irritation caused by the sutures and was treated with a fusidic acid/betamethasone gel (5mg/1mg, Fuciderm, Lyppard, Australia). These mild reactions to the pumps did not have an effect on the well-being of the rats, and were therefore not considered as a confounder to the results.
Figure 2.3: The sequence of procedures for osmotic pump implantation. (A) A 2cm incision is made and underlying tissue dissected from surrounding tissue. (B) The pump is initially inserted diagonally then parallel to the spine (C) to ensure it is comfortable for the rat. (D) At 4 weeks, the pump is removed and replaced with another on the opposite side, filled with the same solution as the first pump (E).

2.3.3 bipolar and recording electrode implantation

Bipolar and recording electrode implantations were only performed in kindling cohorts (Chapter 4). Following osmotic pump implantation, while still under anaesthesia, the head was shaved and cleaned with topical antiseptic and the rat placed into a stereotactic frame (David Kopf Instruments, USA). A single midline incision was made along the skin, from between the eyes to between the ears to reveal the surface of the skull, with bleeding stopped using diluted hydrogen peroxide. Using an engraving drill (300 series variable rotary tool, Dremel, Australia) with a 0.9cm drill bit attached (Australian Jewellers Supplies, Australia), five holes were made through the skull and just above the dura – three for extradural electroencephalogram (EEG) recordings and two for anchoring side screws (1.4x3mm, Mr Specs, Australia) to hold the head cap in place (Figure 2.4A). Extradural electrodes were custom-made by soldering a ‘male’ pin to a screw.

Once the anchoring screws and extradural electrodes were attached, a hole was drilled for stereotactic insertion of a bipolar stimulating electrode (MS303/1, Plastics One, USA) targeting the lateral amygdaloid nuclei (Figure 2.4B). Co-ordinates were 3mm caudal and 5mm lateral from bregma, and 6.5mm ventral from the dura (Paxinos and Watson, 1998). The lateral position was adjusted to 5.25mm for the citalopram cohort after evaluation of electrode placement in the fluoxetine cohort. This assembly was held in place by dental acrylic (Vertex Dental, The Netherlands) then the skin sutured (Dysilk 3/0, Dynek, Australia) around the headpiece and cleaned with topical antiseptic. Rats were then injected with 1ml saline (0.9%,
subcutaneously) and an analgesic, 4mg/kg carprofen (intraperitoneally). Rats were recovered on a heat mat and weight was monitored for 5 days or until pre-surgery weight was obtained. Rats were allowed at least one week of recovery before the next procedure commenced.

Figure 2.4: Location of recording and bipolar electrodes. (A) Diagram of a rat skull showing approximate placement of the recording electrodes (blue), anchoring screws (green) and bipolar electrode (orange). (B) An image of a coronal section of the rat brain showing the bipolar electrode (orange) and the target region for the electrode within the lateral amygdaloid nuclei (purple). Images adapted from Paxinos and Watson, 1998.

2.3.4 Seizure threshold testing

Prior to commencement of kindling procedures, another laboratory member blinded the treatment condition of all rats. Following this, the seizure threshold for all rats was determined, defined as the minimal current required to elicit an electrographic response of at least 6s. To do this, the bipolar electrode was attached to a stimulus generator and isolator (Accupulser Pulse Generator/Stimulator, A310, World Precision Instruments, USA). Additionally, recording cables were attached to each of the extradural electrodes, which were connected to an amplifier and computer running LabChart software (ADInstruments, USA). Then, starting at 20μA, seizure threshold was determined by applying trains of electrical current (60Hz, 1s duration, 1ms biphasic square wave pulse) via the bipolar electrode to the amygdala. This was increased by 20μA, 60 seconds apart, until an electrographic seizure lasting at least 6s was observed on the EEG (Figure 2.5). When seizure thresholds were successfully determined, rats were divided into either a kindling or sham-kindling group. In the event of no detectable electrographic response up to 400μA, the same procedure was repeated 24 hours later. However, if no electrographic
response was detected at this point, the electrode was assumed to be incorrectly placed within the amygdala and the rat assigned to the sham-kindling group. Seizure threshold was tested again post-kindling, 3-5 days prior to being culled to determine the effect of the kindling on seizure threshold.

![Figure 2.5: An example of a seizure induced by electrical stimulation of the amygdala.](image)

Seizures occurred immediately after the stimulation and the current eliciting this response was defined as the seizure threshold.

### 2.3.5 Amygdala kindling

Kindling procedures commenced one day after seizure threshold determination. All rats (both kindling and sham-kindling) were individually removed from their home cage, placed in another box and the stimulator cable attached to the bipolar electrode. Rats assigned to the sham-kindling group were allowed to freely move around the cage with the cable attached for approximately 5min while those in the kindled group were given an electrical stimulation at their seizure threshold. All other stimulation parameters were identical to that used to determine seizure threshold. All kindled rats received a total of 30 stimulations, with two stimulations delivered daily (5 days/week), at least 4h apart, as previous studies have shown that there is a period of post-seizure inhibition of amygdala kindled seizures, lasting up to 90min in which seizures cannot be elicited unless a higher current is used (Mucha et al, 1977). For each stimulation, an electrographic response of at least 6s was required. If an electrographic response was not elicited, the stimulation was not counted as one of the 30 stimulations, and an extra stimulation was given at the end until all rats received 30 stimulations with an electrographic response of at least 6s.
During kindling, behavioural seizures were graded by direct observation according to the Racine (1972) scale: Class I – facial clonus, class II – head nodding, class III – unilateral forelimb clonus, class IV – bilateral forelimb clonus and/or rearing, class V – rearing and falling. All seizures were video recorded for blind observation by a second reviewer if required. Total seizure duration was also recorded, defined as the period of the seizure from the end of the stimulation to the end of the total electrographic response. If the seizure exceeded 300s, a total seizure time of 300s was recorded.

2.3.6 Osmotic pump re-implantation

After the 29th stimulation, the osmotic pump was removed and replaced with another pump containing the same solution (Figure 2.3D and E), to ensure continuous delivery for the remainder of the experimental period. The reimplantation was performed after the 29th stimulation to allow the rats to recover for 2 days, and then receive the 30th stimulation followed immediately by BrdU injections (fluoxetine cohort only, Section 2.3.7). This was to ensure post-surgical recovery did not interfere with BrdU uptake. Although citalopram-treated rats did not receive BrdU injections, the same procedure for pump reimplantation was followed. Briefly, under anaesthesia, the first pump was removed via an incision made at the caudal end overlying the pump and closed with buried sutures. Then another incision was made 5mm below the incision for the first pump, and the second pump was implanted over the right flank following the same procedures as outlined in Section 2.3.2.

2.3.7 5-bromo-2-deoxyuridine injections

5-bromo-2-deoxyuridine (BrdU) is a chemical used to label dividing cells. It allows for post mortem quantification of newborn cells that can be detected immunohistochemically (Section 2.4.4). The BrdU solution was prepared by dissolving in 0.9% saline at a concentration of 10mg/ml. This solution was covered with aluminium foil to avoid exposure to light, stored at 4°C and checked daily to ensure the solution had not precipitated. Beginning 5min after the 30th stimulation, all rats received an injection of BrdU (100mg/kg, intraperitoneally) for 7 days, 22-26h apart.

2.3.8 Elevated plus maze

The elevated plus maze was used to assess anxiety-like behaviour, taking advantage of two opposing instincts in the rat: the fear of open spaces versus the desire to explore a new area. The ability of this test to assess anxiety-like behaviours is based on studies by Handley and
Mithani (1984) that showed that anxiogenic agents decreased open arm entries while anxiolytic agents increase entries. The maze consists of 1m x 1m plus-shaped platforms elevated 60cm off the floor with two opposite arms being enclosed and the other two arms open (Figure 2.7A). On the day of the test (performed between 10am and 12pm), the rat was brought into the testing room and allowed to habituate for 30min. Lighting in the room was adjusted to 90-100 lux in the open arms and centre area. Each rat was then placed individually into the centre of the maze and allowed to explore the arena for 5min, with its movement tracked from above (Figure 2.7B) by Ethovision tracking software (Noldus, The Netherlands). This software allows the objective assessment of the number of entries into and time spent in the open arms. An avoidance of the open arms is considered to be indicative of a heightened anxiety state (Rodgers and Dalvi, 1997).

Figure 2.6: The elevated plus maze. (A) Photograph of the elevated plus maze. (B) An image taken from Ethovision during an elevated plus maze acquisition period, with the red lines indicating the rat’s movement throughout the maze. Image A taken from http://www.mpipsyk.mp.de/en/institute/services/emolab/index.html.

2.3.9 Forced swim test and corticosterone stress response

The forced swim test (FST) was used to assess depressive-like behaviour. The test consists of a clear Perspex cylinder, diameter 30cm and height 40cm, filled with 25°C water to a height of 30cm. The rat was brought into the testing room and allowed to habituate for 30min (test performed between 10am and 12pm). The rat was then placed in the cylinder for 5min, during which its movements were video-recorded from a horizontal angle for off-line analysis of behaviours, examples of which are schematically shown in Figure 2.8. The time spent displaying each of the behaviours was summed for the trial: (i) immobility (the primary outcome) -
defined as passive floating with only slight movements to keep its head above water with a lack of movement of at least 3 paws, (ii) climbing – defined as vigorous active movements with all four paws while parallel to the wall and its head and shoulders above the water, (iii) swimming – all remaining time was considered swimming, with the rat making more vigorous movements than necessary to keep its head above water and usually in a horizontal position and (iv) first time immobile. At the end of the trial, rats were removed from the cylinder and towel-dried. Behaviours that persisted for 2s or greater were counted, and each trial was blindly assessed three times and the times averaged for each rat.

**Figure 2.7: An example of the behaviours displayed in the forced swim test.** Adapted from Cryan, 2005

According to established protocols (Porsolt et al., 1977), the FST is used to assess the efficacy of antidepressants. In the traditional protocol, the test is conducted over two days, where on day 1 the rat is allowed to swim for 15min, a habituation trial, followed 24h later by administration of the antidepressant and a 5min swim trial. It is during the 5min trial where the behaviours and therefore efficacy of the antidepressant are assessed – the time immobile, indicative of depressive-like behaviour, is compared between antidepressant and vehicle treatments. However, as this two-day protocol is moderately stressful to rats, affecting outcomes of this study such as neurogenesis, a one day, 5min trial was used for this study instead.

The corticosterone response to swim stress was measured by taking advantage of the mild stress response induced by swimming in an inescapable tank for 5min (Duncan et al., 1998). Prior to being placed into the FST, the rat was placed into a restraint tube with its tail protruding at one end. A small incision was made at the end of the tail and a baseline blood sample was collected (approximately 0.05ml into a heparinised syringe). This procedure took between 30s and 1min. At the end of the 5min swimming trial, the rat was removed from the FST, towel-dried for 1min and placed back into the restraint tube to take a “post-stress” sample.
within 2 min of completing the FST. All blood samples were transferred from the syringe into individual 3 ml Eppendorf tubes and stored on ice. Samples were then centrifuged at 4°C for 10 min at 10,000 rpm, after which plasma was collected, placed into a clean Eppendorf tube and stored at -20°C for later analysis.

2.3.10 Corticosterone radioimmunoassay

Plasma samples obtained from the stress test were analysed for levels of corticosterone, both at baseline and post-stress. This was performed with a rat specific corticosterone double antibody ¹²⁵I radioimmunoassay kit (MP Biomedicals, Australia). Plasma samples and a set of samples provided by the manufacturer to create a standard curve were prepared to manufacturer's instructions. Once complete, corticosterone levels were quantified using a Packard Cobra II Auto-Gamma counter (Perkin Elmer, USA). Counts per minute were averaged for duplicate samples, and using the values from the standard curve, corticosterone levels were calculated.

2.4. Post-mortem experimental procedures

2.4.1 Cardiac blood sampling, transcardial perfusion and tissue fixation

Prior to being culled, rats were given a 2000μA, 1 s continuous electrical stimulation via the bipolar electrode to induce the release of ferrous ions at the electrode tip. This allowed for determination of electrode placement, as the ferrous ions will interact with the cyanide in the fixative solution, resulting in a mark of ferrocyanide in the exact location of the electrode tip. Immediately after, rats were given a lethal injection of phenobarbital (Lethabarb, 1 ml/kg intraperitoneally) and once eye-blink and footpad reflexes were absent, the sternum and rib cage were cut open to expose the heart. For the determination of plasma fluoxetine and citalopram levels, a 3-5 ml blood sample was taken via the left ventricle into a heparinised syringe (stored on ice, centrifuged at 4°C for 10 min at 10,000 rpm, plasma collected and stored at -20°C for later analysis (Section 2.4.7). Immediately following this, rats were transcardially perfused using a blunt 21-gauge needle placed into the aorta via the left ventricle using a peristaltic pump (Cole Parmer Instruments, USA). This began with 200 ml 0.1 M phosphate-buffered saline (37°C, pH 7.4) then 450 ml 4% paraformaldehyde (PFA, dissolved in 0.1 M PBS, 4°C, pH 7.4) containing 0.05% each of potassium ferrocyanide and potassium ferricyanide. Brains were removed and post-fixed in 4% PFA overnight followed by 30% sucrose for 48h
(both at 4°C). Brains were wrapped in aluminium foil and frozen over dry ice for 10min and stored at -80°C until cryosectioning.

2.4.2 Cryosectioning

Using a cryostat, 20μm thick coronal sections were cut. Sections of the dorsal and ventral hippocampus were collected, beginning at bregma -1.60mm to -6.72mm (Paxinos and Watson, 1998). In this hippocampal region, every alternating section was collected on Superfrost Plus slides (Menzel-Glaser, Germany) culminating in 10 series, with 4 slides per series and 3 sections per slide. In this arrangement, each slide held every 20th section. For electrode placement and immunohistochemistry, the same series from each rat was allocated to each staining procedure.

Due to the high rate of neurogenesis in the subventricular zone (Zheng et al., 2004), sections from this region (between bregma +1.20mm and -0.40mm) were collected for positive controls for BrdU immunohistochemistry. All slides were air-dried and stored at -80°C.

![Figure 2.8: Examples of sections cut on the cryostat.](image)

(A) Rat brain atlas representation of the first hippocampal section collected and (B) the last hippocampal section collected. From Paxinos and Watson (1998).

2.4.3 Thionin staining and determination of electrode placement

In order to determine electrode placement, one series from each brain was stained for thionin. Thionin staining allowed for visualisation of the ferrocyanide marking at the site of the bipolar electrode tip. By staining the surrounding structures, this made them identifiable under the microscope so that the exact location of the electrode could be determined.
Slides were removed from the -80°C freezer and allowed to thaw for 45min or until all condensation had evaporated. Sections were then post-fixed in 10% potassium-buffered formalin (10min), then taken through the following solutions: distilled water (3x5min), 0.03% thionin working solution (5min), distilled water (2 dips in 3 changes of water), 96% ethanol (6 dips), 100% ethanol (2-4min, depending in darkness of stain), histolene (2x5min). Slides were then coverslipped with DPX (dibutylphthalate polystyrene xylene, BDH Chemicals, United Kingdom) and dried in a fumehood. Once dried, excess DPX was scraped off and slides cleaned with ethanol.

Images were taken of one slide per rat, in which the blue dot at the electrode tip or electrode track was visible. From these images, electrode placement was confirmed by comparing the image of the section to the rat atlas (Paxinos and Watson, 1998), using landmarks such as the 3rd ventricle, piriform cortex, rhinal fissure and shape of the section to mark the electrode’s placement on the appropriate section. With treatment blinded, two observers decided on inclusion according to set criteria (a standardised outline of the amygdala from sections in the rat atlas in which the marking should be located). An example of the target region is shown in Figure 2.4B. Incorrect electrode placement resulted in exclusion of that rat from all kindling, behavioural, corticosterone and histological analyses.

2.4.4 BrdU immunohistochemistry

Sections were stained to detect any newborn cells that were labelled with BrdU (i.e., cells born after kindling). For this protocol, one series was used for BrdU staining. In addition, a negative control slide (3 sections, omission of primary antibody) and positive control (one slide, three sections from the SVZ) were included in each batch.

All washes were performed in 0.1M Tris-buffered saline (TBS, pH 7.4, 4x5min each). Slides were removed from the -80°C freezer and thawed for 45min. Once thawed, slides were placed in 10% phosphate-buffered formalin (3min), washed and incubated in a 1% hydrogen peroxide/50% methanol solution to remove endogenous peroxidases (30min). Slides were washed again and incubated in 2M hydrochloric acid (37°C, 30min). This was performed to denature the DNA and expose the sites for recognition by the primary antibody (Wojtowicz, 2006). Following neutralisation in 0.1M sodium tetraborate (pH 8.5) and washing, slides were incubated for 2h in 10% normal goat serum to block non-specific binding sites of the secondary antibody. As previous studies in the laboratory have shown, BrdU staining produces a lot of non-specific background staining, making it difficult to distinguish BrdU-labelled cells. Therefore, an additional blocking step was included using CAS-block (10min, Invitrogen, Australia), a universal blocking agent for non-specific background staining. Slides were then incubated in the
primary antibody overnight (at least 16h, 4°C). This consisted of 2% NGS with 0.3% Triton X-100 containing the monoclonal rat anti-BrdU antibody (Accurate, USA) at a 1:400 dilution.

The next day, slides were washed to remove unbound primary antibody and incubated in secondary biotinylated antibody for 90min (rabbit anti-rat, 1:500, Vector Laboratories, USA). Following another wash, sections were incubated in avidin-biotin peroxidase for 90min (Vectastain ABC kit, Vector Laboratories, USA). After washing, staining was visualised by developing the peroxidase with a diaminobenzidine (DAB) substrate, activated with 3μl hydrogen peroxide. Each section was covered with DAB solution until a colorimetric reaction occurred, after which DAB was washed off with TBS into bleach (to deactivate DAB solution). Finally, sections were washed, counterstained in thionin (20-30s), dehydrated in ethanol (70%, 96% and 100%), cleared in histolene and coverslipped.

2.4.5 BrdU cell number quantification

Using the Cavalieri principles to estimate cell number, as described in Miki et al (2005), the number of BrdU-positive cells were quantified in the left and right granule cell layer and subgranular zone (defined as being within two cell bodies of the granule cell layer) of the dentate gyrus and the hilus (ectopic cells). Inclusion criteria for positive cells were those with diffuse, dark brown staining throughout the nucleus or punctuate staining (multiple distinct spots of staining within the nucleus) and a rounded shape (Figure 2.10). Elongated, diffusely dark brown stained endothelial-like cells, and small (<5μm diameter) BrdU-positive nuclei, indicative of glial cells were not counted.

Per brain, every twentieth section throughout the left and right hippocampus, were counted on an Olympus BX51 (Olympus, USA) microscope at x20 magnification, using StereoInvestigator software (MBF Bioscience, USA). In both the validation study (Chapter 3) and fluoxetine and kindling epileptogenesis study (Chapter 4), no significant difference in the total number of cells between the left and right dentate gyrus or hilus were found, therefore cell counts were combined from both sides to give a total dentate gyrus cell count and hilar cell count.
Figure 2.9: An example of BrdU stained cells. (A) The regions quantified for BrdU-positive cells, dentate gyrus outlined in green and the hilus located within (the end of the region marked by the dashed lines) and (B) examples of BrdU-positive cells (red circles).

2.4.7 Plasma sample analysis for fluoxetine levels

Plasma samples obtained at the end of the experiment were sent to Sydney Southwest Pathology where levels of fluoxetine and its metabolite, norfluoxetine, were measured using gas chromatography-mass spectroscopy with selected ion monitoring. The results of the analysis were provided as levels of fluoxetine and norfluoxetine in each rat in ng/ml. Those with plasma levels below the detectable limit were excluded from all analyses. For citalopram-treated rats, plasma citalopram levels were not analysed. This was due to two reasons: (i) there was no company within Australia that was able to perform this procedure for rodents and (ii) as only 3/63 rats in the fluoxetine-treated group had non-detectable plasma levels, the pumps were considered to be an effective method to deliver the drugs, and it was reasonable to assume that citalopram was also effectively delivered by the pumps to the large majority of rats.
CHAPTER 3: PILOT STUDY

Tolerability of osmotic pumps and fluoxetine and the effects on behaviour, stress-induced corticosterone response and neurogenesis

3.1. Introduction

There have been a number of studies that have investigated the effects of SSRIs in animal models of epilepsy (section 1.4.3.2); however in most studies SSRIs were only acutely administered. This is a limitation in terms of clinical relevance, as patients are administered SSRIs chronically rather than as a single dose, and several weeks of SSRI administration are required before therapeutic benefits manifest (Goodnick and Goldstein, 1998, Rosebook and Kalin, 2011). Throughout this period of delay, several biochemical and physiological changes occur that contribute to the observed therapeutic effects of SSRIs. This includes receptor adaptations such as desensitisation of the somatodendritic and terminal serotonin receptors that normally act to regulate serotonin release (Stahl, 1998), which results in an increase in serotonin (Fuller and Snoddy, 1990). While such effects of SSRIs occur soon after administration, other downstream alterations have been shown to occur that may also explain the delayed time for manifestation of therapeutic effects. This includes effects on intracellular signalling pathways (Nestler et al., 1989, Perez et al., 1991, Tardito et al., 2006), gene expression (Nibuya et al., 1996, Frechilla et al., 1998, Tardito et al., 2006), neurotrophic mechanisms (Nibuya et al., 1995, Chen et al., 2001) and potentially effects on neuroplasticity (Nestler et al., 1989, Duman et al., 1997, Fabel et al., 2003). In acute dosing paradigms, such effects would not occur rendering these results less clinically relevant when investigating the effects of SSRIs on epilepsy. Therefore, the effects of chronic SSRI treatment in animal models of epilepsy should be investigated however there have been few studies that have done so.

In this thesis, the next chapter (Chapter 4) describes a study in which chronic SSRI treatment was used in an animal model of epilepsy. However prior to this, the chosen method of chronic drug delivery, by osmotic pumps, was piloted and is the focus of this chapter (Chapter 3). Osmotic pumps allow for the continuous systemic infusion of solutions into unrestrained rats and are advantageous for two main reasons:

1. The pumps are implanted at one time point, after which the drug is infused continuously, eliminating the need for repeated daily injection and stressful handling procedures. This is important for this study, as factors that are affected by stress such as
kindling epileptogenesis, behavioural outcomes, corticosterone levels and neurogenesis are outcome measures.

2. Daily injections result in fluctuating drug plasma concentrations (Cremers et al., 2000). Infusion with osmotic pumps maintains the drug at constant therapeutic levels. While bolus injection does mimic human administration (i.e., one pill per day), the faster drug metabolism (Martignoni et al., 2006) and shorter half-life of drugs (Lemberger, 1972, Caccia et al., 1990) in rats compared to humans produces fluctuating levels rather than the chronically high doses seen in patients.

There are also some disadvantages to using osmotic pumps. However the negative attributes can be minimised:

- Plasma levels of the drug can only be assessed at the end and not throughout the experimental period unless a cannula is permanently implanted which would allow for regular blood sampling to monitor drug levels. However, it is sufficient to inspect the pump at the end of the experimental period to ensure it has emptied and therefore the solution has been absorbed systemically, and cardiac blood taken at euthanasia is a practical method for assessing the blood concentrations of the drug achieved.
- Drug concentration needs to be calculated at implantation. Although it is possible to take into account the rat’s weight to work out drug concentration (refer section 2.3.1), this is only an average predicted weight over the chronic treatment period. Therefore, individual rats may receive slightly higher or lower doses depending upon individual weight gain during the study.

The SSRI chosen for this pilot study was fluoxetine. This drug has been widely used for research in animal models of depression, and many of the studies investigating the effects of antidepressants in animal models of seizures have also used fluoxetine (Dailey et al., 1992, Prendiville and Gale, 1993, Raju et al., 1999, Ugale et al., 2004, Ferrero et al., 2005, Zienowicz et al., 2005, Mazarati et al., 2008).

In addition to assessing the tolerability of the osmotic pumps for the experimental time period, behavioural and physiological markers of the effects of fluoxetine were also assessed. As outlined in Chapter 1, both SSRI treatment and epilepsy have similar neurobiological targets and alterations (section 1.5). Chronic fluoxetine treatment has been shown to have effects on behaviour (Santarelli et al., 2003, Cryan et al., 2005, Mazarati et al., 2008), HPA axis function (Mazarati et al., 2009) and dentate gyrus neurogenesis (Malberg et al., 2000) all of which have also shown to be altered during epileptogenesis (Parent et al., 1997, Zobel et al., 2004, Jones et
al., 2008a). Therefore, this study was designed to assess the tolerability of osmotic pumps for the experimental period and to assess the effects of chronic fluoxetine treatment on affective behaviours, the corticosterone response to stress and dentate gyrus neurogenesis.

It was hypothesised that fluoxetine delivered by osmotic pumps will be an effective method of drug delivery to rodents and that chronic fluoxetine treatment will result in reduced anxiety- and depressive-like behaviours, suppressed corticosterone levels following stress and increased dentate gyrus neurogenesis.

### 3.2. Materials and methods

The number of animals used, details of surgical procedures, behavioural testing, blood sampling and post-mortem procedures are described in detail in chapter 2, sections 2.2 to 2.4. Briefly, 9-11 week old male rats (n=5 per group) were implanted with osmotic pumps filled with 10mg/kg/day fluoxetine or vehicle (50% DMSO in distilled water). Following 3 weeks of drug infusion all rats were given seven daily BrdU injections. Four weeks later, anxiety- and depressive-like behaviours were assessed, as well as the corticosterone response to swim stress. One day later, rats were culled, a cardiac blood sample taken for analysis of plasma fluoxetine and norfluoxetine (its major active metabolite) levels, and the brain extracted for post-mortem quantification of hippocampal neurogenesis (Figure 3.1).

![Timeline of experimental procedures](https://via.placeholder.com/150)

**Figure 3.1: Timeline of experimental procedures.** w – week.
3.2.1 Statistical analyses

Weight changes were analysed with a one-way repeated measures ANOVA (independent variables, IV: drug; dependent variable, DV: weight change over time). BrdU cell counts, corticosterone levels post-stress, elevated plus maze and forced swim test data were analysed with two-tailed t-tests. All data are presented as mean±SEM unless otherwise stated.

3.3. Results

3.3.1 Fluoxetine administered by osmotic pumps does not affect weight gain

Rats were weighed at least once per week. Figure 3.2 shows that all rats gained weight across the experimental time period. While there was no significant difference between fluoxetine- and vehicle-treated rats in weight gain over time ($F_{(1,10)}=0.59$, $p=0.46$), fluoxetine-treated rats showed a small, but non-significant ($p>0.05$) weight loss at four weeks.

![Figure 3.2: Average weekly weight of fluoxetine- and vehicle-treated rats.](image)
3.3.2 Rats treated with fluoxetine show serum levels within an appropriate therapeutic range

The dose received was calculated based on the weight of the rats at the end of the drug delivery period. The fluoxetine-treated rats received an average dose from the pumps at the end of the experimental period of 10.12±0.31mg/kg/day, approximating the target dose of 10mg/kg/day.

After rats were culled, a cardiac blood sample was taken to determine plasma levels of fluoxetine and norfluoxetine, its major active metabolite (analysis performed by Sydney Southwest Pathology), with the results shown in Figure 3.3. Plasma fluoxetine (0.60±0.47 μmol/L) and norfluoxetine (1.27±0.44 μmol/L) levels were at the bottom end of the therapeutic range reported in patients receiving 20-80mg/day fluoxetine (fluoxetine: 0.32±2.26μmol/L, Koran et al, 1996), and within the therapeutic range reported in a rat study in which the same dose of fluoxetine was administered (fluoxetine: 0.38±0.13μmol/L; norfluoxetine: 1.62±0.44μmol/L) (Czachura and Rasmussen, 2000). There were no detectable levels of fluoxetine or norfluoxetine in vehicle-treated rats.

![Figure 3.3: Fluoxetine (A) and norfluoxetine (B) levels versus vehicle treatment as measured by mass spectroscopy. Individual concentrations for each rat are represented in addition to the mean (line).](image)

3.3.3 Fluoxetine-treated rats do not display an anxious phenotype in the elevated plus maze

There was no significant difference between fluoxetine- and vehicle-treated rats in the time spent in (p=0.09) or number of entries into the open arms (p=0.59) or distance travelled (p=0.42), as shown in Figure 3.4.
Figure 3.4: The effects of fluoxetine treatment on anxiety-like behaviours in the elevated plus maze. There was no significant difference (p>0.05) between vehicle- and fluoxetine-treated rats in (A) percentage of open arm entries, (B) percentage of time spent in the open arms and (C) distance travelled.
3.3.4 Fluoxetine treated rats display a depressive-like phenotype

Fluoxetine-treated rats display a greater amount of behavioural despair, indicative of a depressive-like phenotype, compared to vehicle-treated rats in the forced swim test (FST), as indicated by significantly less climbing (p=0.04), more immobility (p=0.02) and shorter latency to the first immobile activity (p=0.03) (Figure 3.5).

![Graph showing effects of fluoxetine on depressive-like behaviours in the forced swim test.](image)

**Figure 3.5: The effects of fluoxetine on depressive-like behaviours in the forced swim test.** Fluoxetine-treated rats spent less time climbing and more time immobile, and displayed the first immobile activity earlier (*p<0.05) compared to vehicle-treated rats.

3.3.5 Fluoxetine treated rats show a trend towards a suppressed stress response

Tail vein blood samples were taken before and after five minutes of swim stress and corticosterone levels were assessed. As there was no significant effect of fluoxetine treatment in corticosterone levels before swim stress (p=0.41), data were analysed as the percentage change in corticosterone from pre-stress to post-stress levels (Figure 3.6). The results show a non-significant trend towards a lower corticosterone response in fluoxetine-treated rats (p=0.22).
**Figure 3.6: Change in corticosterone concentration from pre-stress levels.** The graph shows the effects of swim stress on corticosterone levels in vehicle- and fluoxetine-treated rats. There was no significant difference between fluoxetine- and vehicle-treated rats in corticosterone levels post-stress (p=0.22). Data are presented as a percentage change from prestress levels.

3.3.6 BrdU cell counts do not differ between fluoxetine and vehicle treatments

Figure 6 shows that there is no significant difference between fluoxetine- and vehicle-treated rats in the number of BrdU-positive cells in the dentate gyrus (p=0.63) or the hilus (p=0.11).

**Figure 3.7: BrdU cell counts in the dentate gyrus and the hilus.** There was no effect of fluoxetine treatment on the number of newborn cells (BrdU-positive) in the dentate gyrus or hilus (p>0.05) compared to vehicle treatment.
3.4. Discussion

This chapter reports on the validation pilot study for the use of osmotic pumps as a method to chronically deliver fluoxetine to rats. It was found that the osmotic pumps were well-tolerated for the experimental time period and that this method of delivery was able to achieve therapeutically relevant plasma concentrations. While fluoxetine treatment resulted in behavioural alterations contrary to the hypothesis, the effects of fluoxetine on the neuroendocrine system and on neurogenesis, albeit not statistically significant, trended to be consistent with the prior hypotheses, with the inability to demonstrate a significant effect potentially due to small sample sizes.

There was a wide range of plasma levels of fluoxetine found in this study, however these fell within the therapeutic range reported in a previous study in which the same dose of fluoxetine was administered, and therapeutically relevant plasma and cerebrospinal fluid fluoxetine and norfluoxetine concentrations were detected (Czachura and Rasmussen, 2000) (fluoxetine: 0.38±0.13µmol/L compared to 0.60±0.47µmol/L in this thesis; norfluoxetine: 1.62±0.44µmol/L compared to 1.27±0.44 µmol/L in this thesis). The variability range may be due to the small sample size used, variability in individual drug clearance or variability in the assay detection. However, there was no correlation found between plasma concentrations and any outcome measure including behaviour, corticosterone levels following stress or number of newborn cells (data not shown). This is also reported to be the case in humans, where one study has shown clinical response to be independent of plasma antidepressant concentrations (Kelly et al., 1989). Additionally, plasma fluoxetine concentrations were not related to health of the rats (assessed by weight gain over time).

Assessment of anxiety- and depressive-like behaviours both showed unexpected outcomes. It was shown that treatment with fluoxetine, while not significant, had a trend to increase anxiety and also resulted in significantly more time spent immobile during the FST, indicative of behavioural despair and a depressed-like state in fluoxetine-treated rats. While these results may be due to the small sample size used in this study, other factors may have had an effect. One point to consider is that normal rats were used in this study, rather than those already exhibiting a depressed-like phenotype. Previous studies suggest that fluoxetine does not reduce anxiety in non-anxious rats (Durand et al., 1999, File et al., 1999, Griebel et al., 1999, Silva and Brandao, 2000) while other studies suggest that chronic fluoxetine treatment can increase anxiety-like behaviour in mice (Oh et al., 2009, Kobayashi et al., 2011) and rats (Silva et al., 1999) in the elevated plus maze. In terms of depression, studies have also shown that fluoxetine does not act as a mood elevator or reduce depressive-like behaviours in otherwise normal rats,
as was the case in this study, or healthy patient controls (Gelfin et al., 1998, Geyer and Markou, 2002). Behavioural testing of subtle alterations in mood should be assessed by a battery of tests, and not just one test for each endophenotype as was performed in this study (i.e., EPM only for anxiety, FST only for depression). As behavioural changes were not the main outcome of this study, only one test was used to assess each of the behaviours. As these are subtle alterations, future studies should use a broader array of behavioural tests that will give a better indicator of the effects of fluoxetine in this study.

Measurements of changes in corticosterone levels following a stressor are another way to assess the biological effects of fluoxetine. In this study, it was found that following a brief stressor, fluoxetine-treated rats showed a trend for suppressed levels of corticosterone compared to vehicle-treated rats. This is consistent with data from previous studies in animals (Pariante, 2006) and humans (Linkowski et al., 1987, Souetre et al., 1989, Steiger et al., 1989) showing that fluoxetine normalises the HPA dysfunction together with recovery of depressive symptoms. However, there are several reasons that may explain why there was no significant difference in the studies reported in this thesis, such as the small sample size, the use of non-depressed rats, which do not exhibit a hyper-reactive HPA axis response, and the mild intensity of the stressor (swim stress). While swim stress is generally considered to be sufficiently stressful, studies have previously found that corticosterone levels post-stress are increased to 300 to 400ng/ml (Duncan et al., 1998, Bouilleret et al., 2011) while the highest level of corticosterone observed in this study was 288.58ng/ml (mean±SEM: 132.29±29.43ng/ml). While this may be due to the protocol itself, in that swim stress was not sufficiently stressful, it may also be due to the time point of analysis post-stress. Although previous studies have shown that peak corticosterone concentrations are found 30 minutes after stress (Joels et al., 2007, de Kloet et al., 2008), other studies contradict this, finding no change (Duncan et al., 1998, Mathews et al., 2008, Norcross et al., 2008) or increases (Gozen et al., 2007, Droste et al., 2008, Steiner et al., 2008) in corticosterone levels after swim stress during chronic fluoxetine treatment.

BrdU cell counts were also assessed post mortem to provide an indicator of the effects of fluoxetine on hippocampal neurogenesis. The results suggest that there was a large variability in the number of BrdU-positive cells, although there does appear to be a trend for fluoxetine-treated rats to have more BrdU-positive cells in the hilus. High cell counts in the hilus indicate that cells have ectopically migrated from the dentate gyrus. The reasons for this trend occurring in this study are not clear, as in other studies cells migrate ectopically only following a brain insult, for example following status epilepticus (Scharfman and Pedley, 2006, Jessberger et al., 2007, Walter et al., 2007, Kron et al., 2010) or stroke (Niv et al., 2012). However this was not the case here. Ultimately, the results suggest that there was no significant effect of fluoxetine on
dentate gyrus neurogenesis. While the small sample size may explain why there was no difference, methodological limitations should also be considered such as inadequate detection of BrdU-positive cells with the immunohistochemical methods used or inadequate uptake of BrdU from the intraperitoneal injection. Overall, conclusions regarding the effects of fluoxetine on newborn granule cell number cannot be drawn from this study.

Taken together, the results of this study show that osmotic pumps are a reliable method of drug delivery and that rats are able to tolerate the pumps for the experimental time period and receive the targeted dose of fluoxetine. Furthermore, although not significantly different, it is likely fluoxetine is having a biological effect in the rats, as it was shown to have effects on depressive-like behaviours and trends for effects on anxiety-like behaviours and suppression of the corticosterone response to stress.

Following the validation of osmotic pumps as a reliable method of fluoxetine delivery and indications of biological and behavioural effects, the next investigation will focus on the effects of fluoxetine, and a more clinically relevant SSRI, citalopram, on epileptogenesis.
CHAPTER 4

Chronic SSRI treatment and its effects on kindling epileptogenesis, behaviour, stress-induced corticosterone response and neurogenesis

4.1. Introduction

The high incidence of comorbid psychiatric illnesses, especially mood and anxiety disorders, in patients with epilepsy (Gaitatzis et al., 2004) can have a significant impact on quality of life and cognitive functioning as well as increasing the likelihood of suicide (Hesdorffer et al., 2006) and possibly Sudden Unexpected Death in Epilepsy (SUDEP) (Ridsdale et al., 2011). In fact, the manifestation of these illnesses in patients with epilepsy has been reported to be a greater predictor of quality of life compared to illness duration or seizure frequency (Boylan et al., 2004, Gilliam et al., 2004, Kanner et al., 2010). Therefore, management of these depressive and anxiety symptoms and syndromes is an essential part of comprehensive treatment of people with epilepsy.

Presently, the use of selective serotonin reuptake inhibitors (SSRIs) in patients with epilepsy is considered to be acceptably safe (Jobe and Browning, 2005, Bagdy et al., 2007, Kanner, 2009, Kondziella and Asztely, 2009), however antidepressants have not always been considered safe for use in epilepsy. This is mainly due to reports of seizures as a side effect of many ‘first generation’ antidepressants, particularly tricyclic antidepressants (TCAs) (Preskorn and Fast, 1992, Rosenstein et al., 1993, Salzberg and Vajda, 2001). The seizure aggravating effects of TCAs have been demonstrated in both in vitro (Luchins and Ananth, 1976) and in vivo (Trimble, 1978) experimental models. Furthermore, TCAs were recognised to have the capacity to induce seizures in non-epileptic patients (Wroblewski et al., 1990, Preskorn and Fast, 1992) and to aggravate seizures in pre-existing epilepsy (Pisani et al., 1999). Consequently, clinicians became hesitant to prescribe antidepressants to patients with epilepsy (Cottermann-Hart, 2010). Currently, there is still uncertainty amongst physicians regarding the use of antidepressant medications in epilepsy and, as such, many depressed patients with epilepsy remain either undertreated or completely untreated for their mood disorders.

However, there is a growing body of evidence to suggest that the newer, or ‘second generation’, antidepressants have less effects on brain excitability, and may in fact reduce it. The newer antidepressants, notably the SSRIs and serotonin and noradrenergic reuptake inhibitors
(SNRIs), have been shown to have a better safety profile in terms of seizure occurrence during treatment in patients with epilepsy. There have been many studies that have investigated the effect of SSRIs on seizures in both people with epilepsy and in animal models (reviewed in Cardamone et al., 2012). Studies conducted in patients with epilepsy have found that chronic treatment with SSRIs or SNRIs did not affect seizure frequency and, in some cases, even reduced seizure frequency (no effect: Harmant et al., 1990, Gigli et al., 1994, Hovorka et al., 2000, Kanner et al., 2000, Kuhn et al., 2003, Thome-Souza et al., 2007, Okazaki et al., 2011; reduction: Favale et al., 1995, Favale et al., 2003, Specchio et al., 2004). Similar patterns are found in animal models of epilepsy. Many of these studies report an anticonvulsant effect (Dailey et al., 1992, Kabuto et al., 1994a, Wada et al., 1995, Hernandez et al., 2002, Pericic et al., 2005, Ahern et al., 2006, Richman and Heinrichs, 2007, Mazarati et al., 2008, Borowicz et al., 2011, Jaako et al., 2011, Vermoesen et al., 2012) or no effect (Raju et al., 1999, Ahern et al., 2006, Borowicz et al., 2007, Mazarati et al., 2008, Choi et al., 2010, Vermoesen et al., 2012) of chronic treatment with SSRIs or SNRIs. While reports of a proconvulsant effect of SSRIs in patients with epilepsy are few, and occur primarily in those who have taken overdoses (Judge and Rentmeester, 2011), a proconvulsant effect has been observed in some animal studies after chronic administration of SSRIs or SNRIs at therapeutic doses (Arai et al., 2003, Ferrero et al., 2005, Ahern et al., 2006). However, in all of these studies, seizures were only assessed at one time point after SSRI/SNRI administration, rather than investigating the effects on longer-term epileptic outcomes such as changes in seizure frequency over time.

In both human and animal studies, much of the focus has been upon short-term outcomes, such as changes in seizure frequency and threshold in response to short-term, or even single-dose, antidepressant treatment. Together, the data from both human and animal studies largely suggest that SSRIs do not adversely affect seizure frequency and threshold, and as they alleviate depressive symptoms in patients with epilepsy, are considered safe to use in this context. However, absent from the literature are studies investigating the effects of chronic antidepressant treatment on epileptogenesis, the underlying neurobiological changes that are associated with the development of epilepsy that also continue even after seizures emerge.

In the human studies, the long-term effects on epilepsy beyond the period of assessment (1 to 15 months) have not been examined. Furthermore, only three animal studies have investigated the effects of chronic SSRI treatment on long-term seizure outcomes using post-status epilepticus models of epilepsy. Using the post-pilocarpine status epilepticus model, in which rats develop spontaneous recurrent seizures therefore allowing for long-term monitoring of seizure outcomes, Hernandez et al. (2002) found that five days of fluoxetine treatment inhibited
spontaneous recurrent seizures, while in the same model Mazarati et al. (2008) found no differences in spontaneous recurrent seizures following ten days of fluoxetine treatment. In the post-kainic acid induced status epilepticus model, Vermoesen et al. (2012) investigated the effects of four days of citalopram treatment on seizure frequency, severity and duration. In this study, it was found that when administered at therapeutically relevant doses, citalopram reduced seizure frequency and cumulative seizure duration, without affecting seizure severity. While these three studies provide some insight on the effects of chronic SSRI treatment on seizures, epileptogenesis was not investigated as antidepressants were administered after the emergence of spontaneous seizures. In addition, the periods of treatment used in these studies, between four and ten days, are relatively short and cannot be considered as chronic treatment. As such, investigation of the effects of chronic SSRI administration on epileptogenesis is needed. Long-term studies investigating changes in seizure frequency over time and severity of the disorder during antidepressant treatment would aid in understanding the impact of antidepressant treatment during epilepsy.

Investigating this critical issue in patient populations would be technically and logistically difficult and require very long-term studies. In contrast, using animal models to examine the effects of chronic SSRI treatment on epileptogenesis provides a much more practical approach. Investigating epileptogenesis in animals can be accomplished with the amygdala kindling model of epileptogenesis. In this model, a bipolar electrode is implanted into the amygdala through which an electrical current is delivered twice daily to induce focal seizures (Goddard, 1967, Goddard et al., 1969, Racine, 1978). This produces a progressive increase in seizure susceptibility and the epileptic response, with a spread of seizure activity from focal to extrafocal regions and the appearance of generalised convulsive seizures (Goddard et al., 1969, Racine, 1972b). During this kindling period, various aspects of seizures and epileptogenesis can be monitored, including seizure threshold before and after kindling, seizure duration with subsequent stimulations, and importantly for this study, the progression of seizure severity with repeated electrical stimulations. This will allow for close monitoring of the progression of epileptogenesis, and other kindling parameters, during chronic SSRI treatment.

The SSRIs used in the experiments discussed in this chapter were fluoxetine and citalopram, administered by osmotic pumps. The use of fluoxetine to affect behaviour and investigate its effects on acute seizure outcomes has been well-established in various animal models, allowing for relevant comparison of this study with the literature. Citalopram was chosen for this study as it is one of the first-line medications currently used in clinical practice for the treatment of depression in epilepsy, due to its low side effect profile and low levels of interaction with AEDs,
and has also been previously used in the experimental literature. As such, citalopram is considered safe for use in epilepsy with studies showing that it does not adversely influence seizure frequency (Hovorka et al., 2000, Favale et al., 2003, Specchio et al., 2004). The study presented in this chapter investigated the effects of chronic antidepressant treatment on epileptogenesis. Changes in behaviour, hypothalamo-pituitary-adrenal axis function and neurogenesis were also investigated, as these factors have been shown to be involved in epileptogenesis and are also affected by SSRI treatment.

It was hypothesised that:

1. Compared to vehicle-treated rats, fluoxetine- and citalopram-treated rats would have a slower rate of kindling epileptogenesis.

2. Kindling would increase anxiety- and depressive-like behaviours and this would be mitigated with fluoxetine and citalopram treatments.

3. Kindling would increase the corticosterone response to stress, and this would be mitigated with fluoxetine and citalopram treatments.

4.2. Materials and methods

The number of animals used, details of electrode implantation, surgical, kindling, behavioural testing, blood sampling and post-mortem procedures are described in detail in Chapter 2, Sections 2.2 to 2.4. Throughout this chapter, results are presented separately for the fluoxetine-treated cohort and their vehicle-treated counterparts, and for the citalopram-treated cohort and their vehicle-treated counterparts. As such, two separate vehicle-treated cohorts (treated with the same vehicle) were used for comparison with the respective SSRI treatment.

In this study, seizure threshold was assessed prior to and at the end of the kindling procedures: prior to kindling to determine the current at which to stimulate during kindling, as well as to determine any effects of SSRI treatment on seizure threshold at this time; at the end of kindling to assess the effects of kindling and as well as chronic SSRI treatment on seizure threshold. Kindling procedures commenced one day after seizure threshold determination during which all rats assigned to the kindling group received 30 stimulations, twice daily (5 days/week). Following kindling, rats in the fluoxetine cohort received seven daily BrdU injections. Three
weeks later, anxiety- and depressive-like behaviours were assessed in all cohorts, as well as the corticosterone response to swim stress. One day later, rats were culled, a cardiac blood sample was taken for analysis of levels of plasma fluoxetine and norfluoxetine (its major active metabolite; not performed for citalopram cohort), and the brain extracted for determination of electrode placement (both cohorts) or post-mortem quantification of hippocampal neurogenesis (fluoxetine cohort only) (Figure 4.1).

4.2.1 Statistical analyses

Seizure threshold and kindling rate were each analysed with a one-way repeated measures ANOVA (IV: drug; DV: threshold current, number of stimulations to reach seizure class). Individual data points for seizure duration were analysed with a Student’s t-test (seizure duration at each stimulation). Data for the elevated plus maze, forced swim test, corticosterone concentrations and BrdU cell counts were each analysed with a two-way repeated measures ANOVA (IV: drug, kindling; DV: time spent in open arms/number entries to open arms/distance travelled, time spent climbing/immobile/time first immobile, plasma corticosterone concentrations and cell counts respectively). All data is presented as mean±SEM unless otherwise stated.

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![Timeline of experimental procedures](image)

*Figure 4.1: Timeline of experimental procedures.* w – week.
4.3. Results

4.3.1 Rats treated with fluoxetine had serum levels within an appropriate therapeutic range.

The dose of fluoxetine or citalopram was based on the predicted weight of the rats at the end of the drug delivery period. Fluoxetine-treated rats received an average dose of 10.04±0.12mg/kg/day, and citalopram-treated rats received an average dose of 10.17±0.18mg/kg/day, which fell within the range of the target dose of 10mg/kg/day.

Cardiac blood samples taken at the end of the 8-week experimental period were analysed by mass spectroscopy by Sydney Southwest Pathology for plasma levels of fluoxetine and norfluoxetine. Plasma fluoxetine (0.38±0.03μmol/L) and plasma norfluoxetine (3.02±0.16μmol/L, Figure 4.2) levels fell within the same range as the validation study. There were no detectable levels of fluoxetine or norfluoxetine in vehicle-treated rats. Plasma citalopram levels were not analysed (see Section 2.4.7).

![Figure 4.2: Fluoxetine (A) and norfluoxetine (B) levels as measured by mass spectroscopy. Individual concentrations for each rat are represented in addition to the mean (line).](image)

4.3.2 Seizure threshold is reduced by kindling, but is not affected by chronic SSRI treatment

Seizure threshold was assessed prior to kindling to determine the seizure threshold for each rat, at which each rat would be stimulated twice daily. At the end of the kindling procedure, seizure threshold was assessed again to determine the effects of both kindling and SSRI treatment.
(Figure 4.3). As expected, repeated electrical stimulations resulted in a significantly reduced seizure threshold (fluoxetine cohort: $F_{(1,17)}=30.62$, $p<0.0001$; citalopram cohort: $F_{(1,33)}=91.84$, $p<0.0001$) however neither fluoxetine ($F_{(1,17)}=0.007$, $p=0.933$) nor citalopram ($F_{(1,33)}=3.321$, $p=0.078$) treatments significantly affected seizure threshold before or after kindling.

### 4.3.3 Chronic fluoxetine and citalopram treatments accelerate kindling epileptogenesis

During the kindling procedure, seizure severity was recorded for each stimulation, from class 1 (least severe) to class 5 (most severe). Significant differences were observed in the progression of kindling and in response to drug treatment (Figure 4.4). As expected, all treatment groups showed an increase in seizure severity with progressive stimulations (fluoxetine cohort: $F_{(1,17)}=46.71$, $p<0.0001$; citalopram cohort: $F_{(1,33)}=159.36$, $p<0.0001$). This effect was accelerated in both fluoxetine- and citalopram-treated rats, with rats both groups showing a more rapid progression through the different stages of kindling (fluoxetine: $F_{(1,17)}=5.23$, $p=0.040$; citalopram: $F_{(1,33)}=16.18$, $p=0.0004$). These results suggest that chronic treatment with fluoxetine and citalopram accelerates the rate of kindling epileptogenesis.

### 4.3.4 Chronic SSRI treatments accelerate the progression of increase in seizure duration early in epileptogenesis

Total seizure duration was recorded at each stimulation, from the end of the elicted electrographic seizure, to a maximum length of 300s (Figure 4.5). Usually during kindling, seizure duration increases and plateaus once maximal seizure duration is reached. In this study, in order to appropriately compare the differences in seizure duration as epileptogenesis progressed, data for seizure duration was analysed with t-tests at each subsequent group of stimulations, comparing the seizure duration between fluoxetine- or citalopram-treated groups with their respective vehicle-treated counterparts (as depicted in the x-axis of Figure 4.5A and B). Analysis showed that the increase in seizure duration was accelerated in the early stages of kindling, occurring significantly quicker in both fluoxetine- and citalopram-treated rats. This increase in seizure duration was significant at stimulations 1 to 3 (fluoxetine: $p=0.018$; citalopram: $p=0.001$), 4 to 6 (fluoxetine: $p=0.044$; citalopram: $p=0.004$) and 7 to 9 (fluoxetine: $p=0.018$; citalopram: $p=0.020$). From stimulation 10 onwards, seizure duration in all treatment groups reached a plateau, with no significant differences in seizure duration from stimulations 10 to 30 ($p>0.05$).
Figure 4.3: The effects of kindling and chronic fluoxetine (A) and citalopram (B) treatment on seizure threshold before and after kindling. Kindling significantly reduced seizure threshold (p<0.0001) but was not affected by chronic fluoxetine or citalopram treatments, **p<0.001.
Figure 4.4: The effect of chronic fluoxetine (A) and citalopram (B) treatments on kindling epileptogenesis. Both fluoxetine- and citalopram-treated rats required significantly less stimulations to reach a more severe seizure class, *p<0.05, **p<0.001.
Figure 4.5: The effect of chronic fluoxetine (A) and citalopram (B) treatments on seizure duration. Seizure duration was significantly greater in both fluoxetine- and citalopram-treated groups compared to vehicle treatment in the early stage of epileptogenesis, at stimulations 1-3, 4-6 and 7-9 (p<0.05). The graph represents data for seizure duration that was averaged over three stimulations (stimulations 1-3, 4-6 etc.) however, analysis was performed on individual points of data, *p<0.05.
4.3.5 Chronic fluoxetine and citalopram treatments differentially affect anxiety-like behaviours

Anxiety-like behaviours were assessed approximately three weeks after the end of the kindling procedures. Firstly, in the fluoxetine cohort, kindled rats made significantly fewer entries ($F(3, 40) = 5.19$, $p = 0.03$, Figure 4.6A) and spent significantly less time ($F(3, 40) = 4.58$, $p = 0.04$, Figure 4.6B) in the open arms of the plus maze, indicating an anxiogenic effect of kindling. However, in the citalopram cohort, there was no significant effect of kindling on anxiety-like behaviours, with no significant differences in the percentage of open arm entries ($F(3, 40) = 2.93$, $p = 0.09$, Figure 4.6D) or percentage of time spent in the open arms ($F(3, 40) = 1.77$, $p = 0.19$, Figure 4.6E) of the plus maze.

Analysis of the effect of drug treatment revealed no significant effect of either fluoxetine or citalopram treatments in the percentage of open arm entries (fluoxetine: $F(3, 40) = 2.60$, $p = 0.12$; citalopram: $F(3, 40) = 2.67$, $p = 0.11$) or percentage of time spent (fluoxetine: $F(3, 40) = 3.14$, $p = 0.08$; citalopram: $F(3, 40) = 2.35$, $p = 0.13$) in the open arms of the plus maze.

Locomotor activity was assessed by quantifying the total distance travelled during the testing session. For rats in the fluoxetine cohort, neither kindling ($F(3, 40) = 2.91$, $p = 0.09$, Figure 4.6C) nor drug treatment ($F(3, 40) = 0.01$, $p = 0.91$) affected locomotor activity, while kindling ($F(3, 40) = 5.46$, $p = 0.02$) but not SSRI treatment ($F(3, 40) = 0.51$, $p = 0.48$) significantly reduced locomotor activity in the citalopram cohort (Figure 4.6F).

4.3.6 Kindling, fluoxetine or citalopram treatments do not affect depressive-like behaviour

Depressive-like behaviours were assessed using the forced swim test. It was found that there was no effect of kindling, fluoxetine or citalopram treatments on time spent climbing (fluoxetine cohort: drug, $F(3, 40) = 0.0002$, $p = 0.98$; kindling, $F(3, 40) = 0.10$, $p = 0.75$; citalopram cohort: drug, $F(3, 40) = 1.52$, $p = 0.22$; kindling, $F(3, 40) = 0.82$, $p = 0.37$), time spent immobile (fluoxetine cohort: drug, $F(3, 40) = 0.94$, $p = 0.33$; kindling, $F(3, 40) = 0.33$, $p = 0.56$; citalopram cohort: drug, $F(3, 40) = 0.39$, $p = 0.54$; kindling, $F(3, 40) = 0.90$, $p = 0.35$) or in the latency to the first immobile activity (fluoxetine cohort: drug, $F(3, 40) = 0.33$, $p = 0.57$; kindling, $F(3, 40) = 1.58$, $p = 0.22$; citalopram cohort: drug, $F(3, 40) = 0.99$, $p = 0.33$; kindling, $F(3, 40) = 0.11$, $p = 0.74$) (Figure 4.7).
Figure 4.6: The effects of kindling and fluoxetine or citalopram treatment on anxiety-like behaviours. In the fluoxetine cohort (Figures A-C), kindled rats made significantly less entries (A) and spent significantly less time (B) in the open arms, both of which were not significantly affected by drug treatment. Distance travelled (C) was also not significantly affected by kindling or drug treatment in the fluoxetine cohort. In the citalopram cohort (Figures D-F), there was no effect of kindling or citalopram treatment on the percentage of open arm entries (D) or percentage of time spent in the open arms (E), while kindled rats of the citalopram cohort travelled less (F). Sample sizes are presented in the bars of graphs C and F. *p<0.05.
Figure 4.7: The effects of kindling, fluoxetine (A) and citalopram (B) treatments on depressive-like behaviours. There was no significant difference in any of the behavioural aspects analysed, p>0.05.
4.3.7 Chronic fluoxetine, but not citalopram, treatment reduces the corticosterone response to swim stress

Tail vein blood samples were taken before and after five minutes of swim stress and corticosterone levels were assessed. As there was no significant effect of drug (fluoxetine cohort: $F_{(3,40)}=2.24$, $p=0.14$; citalopram cohort: $F_{(3,47)}=0.26$, $p=0.61$) or kindling (fluoxetine cohort: $F_{(3,40)}=2.92$, $p=0.09$; citalopram cohort: $F_{(3,47)}=2.25$, $p=0.14$) in corticosterone levels before swim stress, data were analysed as the percentage change in corticosterone from pre-stress to post-stress levels (Figure 4.8). It was found that fluoxetine-treated rats had a significantly suppressed corticosterone response following a stressor, as evidenced by lower percentage change in corticosterone ($F_{(3,40)}=11.04$, $p=0.002$) compared to vehicle treatment (Figure 4.8A), which was not found in the citalopram cohort ($F_{(3,47)}=0.006$, $p=0.94$, Figure 4.8B). There was no significant effect of kindling on the percentage change in corticosterone levels in either the fluoxetine ($F_{(3,40)}=0.07$, $p=0.79$) or citalopram ($F_{(3,47)}=0.0008$, $p=0.98$) cohorts. This data indicates that fluoxetine, but not citalopram, is able to suppress the corticosterone response to stress, while kindling does not affect the corticosterone response to stress.

**Figure 4.8: The effects of kindling and fluoxetine (A) or citalopram (B) treatments on corticosterone response following swim stress.** Fluoxetine, but not citalopram, treatment significantly suppressed the corticosterone response following a stressor ($p=0.002$). Stress-induced corticosterone responses were not affected by kindling ($p>0.05$) in either cohort. Sample sizes are presented within the bars. Data are presented as percentage change from prestress levels, *$p<0.05$.**
4.3.8 Neither kindling nor chronic fluoxetine treatment affect dentate gyrus neurogenesis

BrdU-positive cells were quantified in the dentate gyrus and hilus (ectopic cells). As there was no significant difference in the total number of cells between the left and right dentate gyrus or hilus, cell counts were combined to give total dentate gyrus and hilar cell counts (Figure 4.9). There were no significant effects of fluoxetine treatment or kindling on the total number of BrdU-positive cells in the dentate gyrus (drug: $F_{(3,66)}=1.55$, $p=0.22$; kindling: $F_{(3,66)}=0.07$, $p=0.79$) or the hilus (drug: $F_{(3,66)}=0.34$, $p=0.56$; kindling: $F_{(3,66)}=0.56$, $p=0.46$).

![Figure 4.9: Total number of BrdU labelled cells in the subgranular zone of the dentate gyrus (A) and hilus (B). There were no significant effects of drug treatment or kindling ($p>0.05$) on the number of BrdU-positive cells.](image-url)
4.4. Discussion

In the majority of studies that report on the effects of antidepressant treatment in epilepsy, acute dosing paradigms and acute seizure models have been used, with few studies employing chronic treatment paradigms or performing any long-term follow up of epilepsy progression (Cardamone et al., 2012). Furthermore, reports of the proconvulsant effects of SSRIs mostly derive from animal studies in which acute seizure models were used or from patient reports of SSRIs used in overdose. This is the first study to report specifically on the effects of chronic SSRIs treatment at therapeutically relevant levels on progression of the epileptic disorder, by investigating the effect on the progression of kindling epileptogenesis. The key finding of this study was that rats chronically treated with SSRIs, either fluoxetine or citalopram, demonstrated an accelerated rate of kindling epileptogenesis, in which rats developed more severe seizures with less stimulations, compared to vehicle-treated rats. Seizure duration was also significantly greater in both fluoxetine- and citalopram-treated rats in the early stage of epileptogenesis, but not in the later stages and there was no significant effect of fluoxetine or citalopram treatment on seizure threshold. Anxiety-like behaviours were differentially affected by kindling and drug treatments but depressive-like behaviours were not significantly affected. Stress-induced corticosterone responses were significantly reduced by fluoxetine but not citalopram treatment and it was also found that neurogenesis was not significantly affected by fluoxetine treatment or kindling.

4.4.1 Kindling epileptogenesis is accelerated by chronic antidepressant treatment

The kindling model of epileptogenesis is a useful one to investigate epileptogenesis, as it allows for the close monitoring of the progressive increase in seizure susceptibility and the epileptic response with subsequent electrical stimulations, by assessing behavioural severity of seizures and quantifying electrographic seizure activity. In this study, chronic fluoxetine and citalopram treatments accelerated the progression of kindling epileptogenesis, with fluoxetine- and citalopram-treated rats displaying more severe seizures after less electrical stimulation. This indicates that both chronic fluoxetine and citalopram treatments have a proepileptogenic effect. This is neither consistent nor inconsistent with previous studies, as there have been none to investigate epileptogenesis during chronic antidepressant treatment, and the effect was not due to alterations in the acute excitability of the brain as seizure threshold was the same between SSRIs and vehicle treatments. However, this is an unexpected finding as much of the previous literature in humans and animals suggests that antidepressant treatment has no effect on seizures, or even an anticonvulsant effect.
In the human literature, many studies have focused on more patient-relevant outcomes that affect a person with epilepsy’s daily life, such as seizure frequency. Of these studies, it was found that chronic SSRI treatment (treatment periods of 1 to 15 months) in epilepsy had no overall effect on seizure frequency (Harmant et al., 1990, Gigli et al., 1994, Hovorka et al., 2000, Kanner et al., 2000, Kuhn et al., 2003, Thome-Souza et al., 2007, Okazaki et al., 2011), with some reporting an improvement in seizure frequency (Favale et al., 1995, Favale et al., 2003, Specchio et al., 2004). Specifically, studies with fluoxetine in patients with epilepsy have shown that treatment for three to fourteen months resulted in an overall reduction in seizure frequency when compared to baseline seizure frequency, with some patients even reporting complete seizure freedom. There were also reports of four patients having an increase in seizure frequency (Gigli et al., 1994, Favale et al., 1995). Other studies reported that there were no changes in seizure frequency with citalopram treatment (Hovorka et al., 2000, Kuhn et al., 2003) or overall reductions in seizure frequency (Hovorka et al., 2000, Favale et al., 2003, Specchio et al., 2004), while high doses of citalopram (in overdose) have been associated with increases in seizure occurrence (Favale et al., 2003, Isbister et al., 2004, Kelly et al., 2004).

While the reported improvements or no effects of SSRIs on seizure frequency are an important outcome, interpretation of the results of these studies is limited. This is because a majority of these studies used a relatively short time-frame of SSRI treatment and follow up, and no studies were performed with untreated or placebo-treated controls. Therefore, none of these studies made comparisons with appropriate controls of the changes in seizure outcomes, such as seizure frequency or severity, over time. This is an important outcome given that there is some evidence that epilepsy may be a progressive disorder (Pitkanen and Sutula, 2002, Nearing et al., 2007, Cascino, 2009), and therefore it is important to investigate the effects of SSRIs on longer term outcomes, rather than just the effects on seizures at discreet time points of investigation.

Of the few animal studies that have reported proconvulsant effects of SSRI treatment, a majority have only administered SSRIs acutely, with only two studies treating for up to 21 days. In the studies that found proconvulsant effects of fluoxetine, Zienowicz et al. (2005) found that acute treatment with 10mg/kg fluoxetine increased seizure severity and Ferrero et al. (2005) found that 21-day treatment with 10mg/kg/day fluoxetine reduced seizure threshold. Studies with citalopram that found proconvulsant effects have utilised only acute doses, finding a reduced seizure threshold (Payandemehr et al., 2012, Vermoesen et al., 2011) or an increase in seizure frequency (Arai et al., 2003). Overwhelmingly, animal studies investigating SSRI treatment in animal models of epilepsy find that there is an anticonvulsant, or no effect of SSRI treatment on seizures.
Overall, data from the previous studies would suggest that chronic treatment with fluoxetine or citalopram, at therapeutically relevant doses, should not adversely affect seizure frequency. The kindling model does not specifically investigate seizure frequency, as seizures are induced by electrical stimulation and rats do not experience spontaneous seizures. However, this model is advantageous in that it allows for the monitoring of changes in seizure susceptibility overtime, allowing for monitoring of the progression of the disorder, which in this study has shown that seizure susceptibility increases and consequently the rate of epileptogenesis is accelerated during chronic SSRI treatment. Furthermore, the results of this study were replicated across two separate cohorts using two different SSRIs, in experiments conducted independently of each other, adding support to the notion that the results were not merely a false-positive.

Another advantage of this study is the use of a chronic treatment paradigm. In the majority of animal studies of epilepsy, acute treatments are administered, and if SSRIs are administered chronically, the period of treatment, in a majority of studies, is relatively short (2-14 days) compared to the current study, with few studies treating for longer periods (21-28 days). SSRIs are known to have a delayed onset of action, in which at least 3 weeks of treatment are required for the therapeutic effect to manifest (Goodnick and Goldstein, 1998, Rosebook and Kalin, 2011). During this period, adaptive changes to biological systems that exert the effects of antidepressants are taking place, which can only occur with chronic treatment paradigms. As such, chronic treatment with these agents would display different effects than acute or even shorter-term (less than 3 weeks) chronic treatment regimes.

One example of the adaptive changes occurring during chronic treatment, which may be relevant to kindling, is alterations in serotonergic receptors (Eison and Mullins, 1996, Stahl, 1998, Keltner et al., 2002). Various studies have investigated the effects of serotonin receptor agonists and antagonists on kindling epileptogenesis. These studies are important to consider, given that one mechanism by which SSRIs exert their effects is by increasing serotonin levels, which has subsequent effects on serotonergic receptors. There has been only one study which investigated amygdala kindling epileptogenesis following administration of serotonin (5HT) agonists and antagonists (Wada et al., 1997). In this study, it was found that a 5HT\textsubscript{1A} receptor agonist, 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) inhibited kindling rate while a 5HT\textsubscript{2} receptor agonist, 2,5-dimethoxy-4-iodoamphetamine (DOI) facilitated kindling. The effect of DOI was blocked by the addition of a 5HT\textsubscript{2} receptor antagonist, ketanserin. Other studies have investigated the effects of serotonin receptor agonists and antagonists on seizure susceptibility, overall finding that 5HT\textsubscript{1A} receptor agonists have antiseizure effects (Wada et al., 1992, Wada et al., 1993, Gariboldi et al., 1996a, Lu and Gean, 1998, Pericic et al., 2005) while
agonists of 5HT$_2$ and 5HT$_3$ receptors have proconvulsant effects (Wada et al., 1992, Wada et al., 1999). However, Watanabe et al. (2000) found that antagonists of 5HT$_{2A}$, 5HT$_3$, 5HT$_{2B}$ and 5HT$_{2C}$ do not change seizure threshold and Watanabe et al. (1998) found that the administration of agonists of 5HT$_{1A}$, 5HT$_{2A}$, 5HT$_2$ and 5HT$_{3}$ receptors had no anticonvulsant effect in the kindling model. The inconsistencies between these studies may be explained by disparity in the routes of administration or by the seizure models employed.

Other studies have specifically examined which serotonergic receptors are involved in the effects of SSRIs on seizures. For example, Payandehmehr et al. (2012) found that citalopram dose dependently exerted anticonvulsant (0.5 and 1mg/kg) and proconvulsant (28 and 50mg/kg) effects on seizures in the pentylenetetrazol model of seizures. A dose dependant effect has also been shown by Clinckers et al. (2004a). Although the anticonvulsant actions of citalopram at low doses are not completely understood yet, it was verified that citalopram mediated its anticonvulsant effect through the 5HT$_3$ receptor, and its proconvulsant effect through a modulatory role of nitric oxide. The proconvulsant effect of nitric oxide has also been reported in previous studies (De Sarro et al., 1991, Osonoe et al., 1994, Gholipour et al., 2010, Bahremand et al., 2011). Overall, these studies indicate that the proconvulsant effects of SSRIs may be mediated through activation of 5HT$_2$ receptors and this may also be mediated through effects on nitric oxide. Whether such effects on these targets occurred in the present study cannot be confirmed, and furthermore, the effects of fluoxetine and citalopram are more wide-ranging than solely effects on specific serotonergic receptors, as discussed in Section 1.4.2. Investigating these alterations in future studies should be considered.

4.4.2 Chronic fluoxetine and citalopram treatments increase seizure duration in the early stages of epileptogenesis

In this study, it was found that both fluoxetine and citalopram treatments significantly increased seizure durations in the early stages of kindling epileptogenesis, from stimulations 1 to 9. In this period, seizure lengths increased differently between SSRI-treated and vehicle-treated rats, with fluoxetine- and citalopram-treated rats having longer seizures. Seizure duration reached a plateau in all treatment groups from stimulation 10 onwards: at this stage rats were beginning to experience class 3 seizures, which corresponds to later stages of epileptogenesis when seizures are beginning to generalise and become more severe, indicating a ceiling effect on seizure duration. As such, all treatment groups had similar seizure durations at the end of kindling however the fluoxetine- and citalopram-treated rats reached this stage faster.
This data indicates that, as expected, repeated electrical stimulation increase seizure duration, and in fluoxetine- and citalopram-treated rats, this increase in seizure duration progressed at a faster rate. This is consistent with a proepileptogenic effect of fluoxetine and citalopram treatments, in that the epileptic disorder is progressing to a more severe stage at a quicker rate. The results of this study conflict with other studies in which treatment with fluoxetine or citalopram reduced or had no effect on seizure duration. For example, it has been reported that 2mg/kg fluoxetine but not 6 or 20mg/kg reduced primary after discharge duration (a measure of seizure activity after a stimulation) (Watanabe et al., 1998), that four days of 15mg/kg citalopram treatment but not 5 or 10mg/kg reduced cumulative seizure duration (Vermoesen et al., 2012) while other studies found no effect of acute fluoxetine treatment on seizure duration at doses of 10-50mg/kg (Wada et al., 1999, Jin et al., 2009). These studies are not consistent with the current study, which may be explained by the use of different epilepsy models, as these studies used post-status epilepticus models, in which rats experience spontaneous seizures, as well as using a shorter period of drug administration. Furthermore, these studies only investigated seizure duration at a single time point, or for short periods of analysis (maximum of 3 days), and did not investigate the change in seizure duration as the epileptic disorder progressed. There have been no studies to investigate the changes in seizure duration over time during longer-term SSRI treatment.

4.4.3 Chronic fluoxetine and citalopram treatments do not affect seizure threshold

There was no effect of fluoxetine or citalopram treatments on seizure threshold before or after kindling. As already discussed, most other studies focused on seizure threshold, as it is the key indicator of neural excitability. Most such studies found that chronic SSRI treatment does not affect seizure threshold. For example, following 14-21 days of fluoxetine treatment, it was found that there was no difference in seizure threshold in models of maximal electroshock (Raju et al., 1999) and ear shock (Borowicz et al., 2007). Additionally, no difference in seizure threshold was found following chronic citalopram treatment in El mice, a genetic model of sensory-precipitated seizures (Kabuto et al., 1994a) or following lidocaine-induced seizures (Arai et al., 2003). Conversely, a number of studies found that chronic fluoxetine (Dailey et al., 1992, Wada et al., 1995, Richman and Heinrichs, 2007, Mazarati et al., 2008) and citalopram (Kabuto et al., 1994b) treatments increased seizure threshold while only one study found that chronic fluoxetine treatment reduced seizure threshold (Ferrero et al., 2005).
4.4.4 Interim conclusion of kindling data

Overall, this data indicate that chronic fluoxetine and citalopram treatments accelerate the progression of kindling epileptogenesis, as demonstrated by an accelerated kindling rate and greater seizure duration in the early stages of epileptogenesis, however chronic SSRI treatment did not affect seizure threshold and therefore local excitability within the brain. Potentially, these results could have implications for the treatment of depressive symptoms in epilepsy, which will be discussed in detail in Chapter 5.

4.4.5 Chronic fluoxetine and citalopram treatment and effects on anxiety- and depressive-like behaviours

Psychiatric disorders are highly prevalent in patients with epilepsy, particularly in TLE (Altshuler et al., 1999, Gaitatzis et al., 2004). Similarly, anxiety- and depressive-like behaviours have been reported in animal models of epilepsy (Adamec and Young, 2000, Groticke et al., 2007, Jones et al., 2008b). However when specifically investigating the kindling model, reports of the effects of kindling on anxiety-like behaviour are mixed. Studies have reported both anxiogenic (Adamec, 1990, Helfer et al., 1996) and anxiolytic (Adamec and Morgan, 1994, Jones et al., 2009) effects of kindling, the results of which depend on various factors such as kindled hemisphere (Adamec and Morgan, 1994), bipolar electrode location (Adamec et al., 2004) and baseline state of anxiety (Adamec et al., 2005). For depressive-like behaviours, other studies have reported no effect of kindling (Corcoran et al., 1992, Helfer et al., 1996, Adamec et al., 2004), which was also found in this study. This supports the notion that the behavioural alterations associated with kindling, in many studies, specifically affect anxiety-like behaviours.

In this study, kindled rats in the fluoxetine cohort displayed increased anxiety-like behaviours. This study involved kindling of the left basolateral amygdala, and therefore is contrary to previous studies that have found that left basolateral amygdala kindling has anxiolytic effects (Adamec and Morgan, 1994, Jones et al., 2009). However, this was only found in the fluoxetine but not citalopram cohort, the reasons for which are unclear. In the citalopram cohort, kindled rats moved less, demonstrated by a reduced distance travelled in the EPM, however there was no significant difference in mobility in the FST, with no significant differences between any treatment groups in any of the active behaviours such as swimming or climbing. This difference for distance travelled in the EPM may be related to the small sample size in the vehicle-sham group, and furthermore, determining effects on locomotor activity would need to be confirmed using a battery of tests for locomotion for which analysis of anxiety-like behaviours is not the main outcome.
Overall, SSRI treatment had no effect on anxiety-like behaviours. This is consistent with Borsini et al. (2002), in which 107 studies analysing the effects of antidepressants in animal model of anxiety were reviewed, finding that in a majority of studies, antidepressant treatment does not affect anxiety-like behaviours. Specifically, there were studies that reported no effect (Durand et al., 1999, File et al., 1999, Griebel et al., 1999, Silva and Brandao, 2000) of chronic fluoxetine or citalopram treatment on anxiety-like behaviours in the EPM, but this was contrary to others in which an anxiogenic-like effect was found, which has been reported only in studies using fluoxetine (Durand et al., 1999, File et al., 1999, Silva et al., 1999, Kurt et al., 2000, Oh et al., 2009, Kobayashi et al., 2011). Therefore, the results found in this study are consistent with literature, in that SSRI treatment had no effect on anxiety-like behaviours.

4.4.6 Chronic fluoxetine, but not citalopram, treatment reduces the corticosterone response to swim stress

It is widely reported that depression is associated with alterations in HPA axis functioning (Pariante and Lightman, 2008) and that antidepressant treatment normalises these alterations, returning cortisol (in humans; Holsboer, 2001, Himmerich et al., 2007) and corticosterone (in rodents; Jensen et al., 1999, Jongsma et al., 2005, Mazarati et al., 2008) levels back to normal. Studies have also investigated the role of corticosterone and HPA hyper-reactivity in epilepsy, with studies in rats suggesting that stress accelerates kindling rate (Kumar et al., 2007) which is associated with increased corticosterone levels (Jones et al., 2012) and maternal separation, a model of early life stress, augments the corticosterone response to a seizure, which is reversed by a corticosterone-synthesis antagonist, metyrapone (Koe, 2012). However, there have been no studies to determine the effects of chronic SSRI treatment on HPA functionality in epilepsy. This was explored in this study, in which it was found that kindling had no effect on stress-induced corticosterone levels, but there was an effect of fluoxetine treatment, in which fluoxetine-treated rats had a significantly reduced stress response compared to vehicle-treated rats, consistent with previous studies showing that fluoxetine is able to normalise the increases in corticosterone that occur after stress (Linkowski et al., 1987, Souetre et al., 1989, Steiger et al., 1989, Pariante, 2006) however inconsistent with the notion that corticosterone may be accelerating kindling rate in these rats during chronic SSRI treatment. Furthermore, there was no effect of citalopram on stress-induced corticosterone levels. There have been no previous studies to investigate the effects of chronic citalopram treatment on corticosterone levels post-swim stress, however using other stress paradigms, studies have showed that chronic citalopram treatment has no effect on corticosterone levels post-stress (Jensen et al., 1999, Moncek et al., 2003, Hesketh et al., 2005), consistent with this study.
While the lack of difference between vehicle- and citalopram-treated groups could be due to small sample size in the vehicle-treated group of the citalopram cohort, this factor also makes interpretation of this data difficult. Pharmacological differences between fluoxetine and citalopram may also explain the disparity of the results between the two SSRI treatments. While both drugs have similar mechanisms of action, there are differences in their affinity for blocking the serotonin transporter (Hyttel, 1994), thus having effects on serotonin concentrations and on potential subsequent downstream effects on the HPA axis. Furthermore, the dose of citalopram may not have been sufficient to induce alterations in the HPA axis. While doses of 10mg/kg/day, as used in this study, have been used in previous studies and shown to increase brain concentrations serotonin (Marsteller et al., 2007, Kanemaru et al., 2009) and to affect behaviour (Kugelberg et al., 2002), other studies commonly use doses of 20-60mg/kg/day (Kugelberg et al., 2001, Wegener et al., 2003, Payandemehr et al., 2012). However, due to solubility issue with using the osmotic pumps, a dose of 10mg/kg/day was chosen. While 10mg/kg/day maybe have been a dose sufficiently high enough to have effects on kindling, it may not have been effective on other physiological parameters such as the HPA axis. Comparatively, the dose of fluoxetine at 10mg/kg/day was sufficiently high enough to affect kindling rate and the corticosterone response to stress, the latter consistent with previous studies (Serra et al., 2001, Zhang et al., 2010, Pawluski et al., 2012, Pineda et al., 2012).

In both cohorts, there was no effect of kindling on the corticosterone response to swim stress. While previous studies implicate a role of corticosterone in epileptogenesis (Kumar et al., 2007, Jones et al., 2012, Koe, 2012), this was not specifically investigated in this study. One previous study found that kindling does not alter basal corticosterone levels (Adamec and McKay, 1993), suggesting that it is unlikely that corticosterone levels were elevated throughout kindling in the studies reported in this thesis. Therefore, it is unlikely that increases in corticosterone are a mechanism by which SSRIs accelerate kindling rate here. Furthermore, the results of this study suggest that fluoxetine was able to suppress the corticosterone response to stress four weeks after kindling which could indicate that there may also have been suppression of corticosterone throughout kindling.

One limitation of the analysis of the corticosterone response to stress in this study is the time point of blood sampling post-stress. Blood samples were taken five minutes after swim stress, which may not have been sufficient time for corticosterone levels to peak. Previous studies have shown that corticosterone levels take up to 30 minutes to reach a peak post-stress (de Kloet et al., 2008, Joels et al., 2007), and that at five minutes the difference between treatments, particularly in the kindled versus sham-kindled rats, may not be large enough to be determined.
Nevertheless, analysis of corticosterone levels at 5 minutes post-stress would still be able to give an initial indicator of any possible differences between treatment groups.

Overall, the results suggest that it is unlikely that corticosterone is mediating the accelerated kindling rate observed in the SSRI-treated rats in this study. Future studies could investigate the alterations in corticosterone receptors or other HPA intermediaries during kindling and SSRI treatment, which would give a better understanding the effects of chronic SSRI treatment on the HPA axis during kindling epileptogenesis.

4.4.7 Neurogenesis is not affected by kindling or chronic fluoxetine treatment

In this study, there were no differences in the number of BrdU-positive cells between any treatment groups of the fluoxetine cohort. This is opposite to what was hypothesised, as it was expected that there would be increases in neurogenesis in response to kindling and during fluoxetine treatment, which has been shown to occur in previous studies (Boldrini et al., 2009, Malberg et al, 2000, Parent et al, 1998). This may be because not all rats experienced the same number of class 5 seizures, which could potentially mean that neurogenesis would not have occurred to the same extent in all rats, creating greater variability in the data. However, there was no correlation between the number of class 5 seizures and the number of BrdU-positive cells (data not shown). Cell death may also account for the low levels of neurogenesis observed, as cells were labelled with BrdU immediately after kindling, however rats were not culled until 3 weeks later. Any cells counted at 3 weeks post-kindling would indicate cell survival after kindling and chronic SSRI administration, which may not be as pronounced as cell proliferation immediately after kindling. Future studies should investigate the differences in cell proliferation during kindling and chronic antidepressant treatment. Additionally, BrdU administration is only able to provide information on the number of cells that survive, and not of the integration and functionality of the surviving cells. Future experiments should also include an investigation of cell phenotype, with double labelling for markers of mature neuronal cells, as well as electrophysiological investigation of the functionality of BrdU-positive cells.

4.4.8 Final conclusions

In summary, this study demonstrated that chronic treatment with the SSRIs fluoxetine and citalopram both accelerate the progression of kindling epileptogenesis. While this may have implications for the way in which antidepressants are considered for use in people with epilepsy, this effect should also be established in another model of epilepsy, such as the post-status epilepticus model. The kindling model is advantageous as it provides comparatively
easily attainable data on disease progression however there is no development of spontaneous seizures and little associated epileptic pathology. As such, replicating this study in a more clinically-relevant epilepsy model is crucial. Furthermore, while biomarkers of kindling epileptogenesis were investigated in this study, such as the effects on behaviour, HPA functionality and neurogenesis, no clear associations were found, and mechanisms underlying the SSRI-induced increase in kindling vulnerability remain to be established.
CHAPTER 5: GENERAL DISCUSSION

5.1. Key findings

The high prevalence of psychiatric symptoms and syndromes in patients with epilepsy highlights the importance of treating these disorders within this patient group. Antidepressants, in particular SSRIs, are widely prescribed to patients with epilepsy and are administered chronically over months or years to alleviate depressive symptoms and improve quality of life. In recent times, studies have further emphasised the increasing need for the screening and treatment of mood disorders in epilepsy (Kanner, 2003, Cotterman-Hart, 2010). However, most studies, in both humans and animals, have limited their investigations to short-term seizure outcomes following short-term SSRI treatment periods; little data is available on the effects of chronic SSRI treatment on epileptogenesis. These types of studies are essential for a comprehensive investigation of the effects of antidepressants on epilepsy, as increasing evidence suggests that epilepsy is a progressive disorder (Pitkanen and Sutula, 2002, Nearing et al., 2007, Cascino, 2009) that evolves over several years (Ono and Galanopoulou, 2012). As such, while the symptoms of the disorder, in particular the seizures, are generally controlled, the underlying pathological mechanisms and the associated adverse consequences continue to progress. Whether disease progression is affected by chronic SSRI treatment has not been previously investigated. The studies in this thesis aimed to address this, by investigating the effects of chronic fluoxetine and citalopram treatments on the progression of the epileptic disorder in a rat kindling model of epileptogenesis.

The results presented in Chapter 3 showed that fluoxetine administered by osmotic pumps was an effective method of drug delivery for the experimental time period and that fluoxetine treatment had measurable effects in this model. Osmotic pumps were an advantageous method of drug delivery as they negated the need for repeated daily handling and injections during the kindling experiments, which in itself may have affected kindling epileptogenesis. These studies also confirmed that fluoxetine was delivered at the appropriate concentration at the end of the experimental time period and that appropriate plasma levels were attained. Contrary to expectations, fluoxetine treatment also significantly increased depressive-like behaviours, and there was a trend for an effect on anxiety-like behaviour and if sample sizes had been larger, likely would have also significantly suppressed the corticosterone response to stress and increased dentate gyrus neurogenesis. These results indicated that fluoxetine had biological effects in rodents that have also been previously reported.
The effects of chronic SSRI treatment on epileptogenesis, using fluoxetine, and a more clinically relevant SSRI, citalopram, were described in Chapter 4. The effects on some of the common neurobiological substrates of SSRI treatment and epileptogenesis were also investigated. The results of this study demonstrated that chronic treatment with fluoxetine or citalopram had a proepileptogenic effect in the kindling model: fluoxetine- and citalopram-treated rats displayed accelerated kindling rates, reaching a more severe stage of seizures earlier than those in the vehicle-treated group. This was associated with an accelerated increase in seizure duration. These results indicated that chronic fluoxetine and citalopram treatments accelerated the progression of epileptogenesis. This is inconsistent with the few previous studies that employed comparable paradigms to this study, with most studies reporting that antidepressants were anticonvulsant, or had no effect on seizures. However, as discussed in Section 4.4.1, a majority of these studies only administered antidepressants acutely. Importantly, seizure threshold (the point at which the brain becomes susceptible to seizures) was not significantly affected by chronic fluoxetine or citalopram treatments, which is consistent with previous studies as discussed in Section 4.4.3, suggesting a truly disease-modifying intervention (as opposed to changing excitability or being “proconvulsive” as has been reported for tricyclic antidepressants). Overall, this data indicates that while excitability within the brain is not affected by chronic SSRI treatment, the disorder itself progresses at a faster rate.

The original hypothesis of this study was that SSRIs would retard the rate of kindling epileptogenesis, and in doing so, would also limit the behavioural and neuroendocrinological consequences of kindling. After evaluating the proepileptogenic effects on kindling, one would anticipate that the other consequences of kindling would likewise be exacerbated. However, in investigating these associated alterations few conclusions could be drawn from this data to explain the accelerated kindling rate during SSRI treatment. None of the common alterations investigated showed an effect of kindling, likely due to the time point of analysis. These behavioural and neuroendocrinological alterations were investigated after the completion of kindling, and not during the kindling period. As such, it is only possible to speculate upon how each of these factors may be affected by SSRI treatment in epileptogenesis.

The behavioural outcomes of this study found that, kindling itself had some effect on anxiety-like behaviours but no effect on depressive-like behaviours. In the fluoxetine cohort, kindled rats displayed an increase in anxiety-like behaviour compared to sham-kindled rats, with no difference between kindled and sham-kindled rats in depressive-like behaviours. However, this was not replicated in the citalopram cohort. Neither fluoxetine nor citalopram treatments affected anxiety- or depressive-like behaviours. However, fluoxetine treatment was able to
suppress the corticosterone response to stress, with a trend towards such a suppression also seen after citalopram treatment, while there was no effect of kindling in any treatment group. Similar to the behavioural tests, these data were analysed after the completion of kindling, and as such cannot implicate corticosterone in the accelerated kindling rate in this study. These results suggest that even though kindling itself did not have long-term effects on corticosterone release following a stressor, the stress response was still successfully suppressed by fluoxetine treatment. Although an effect of fluoxetine was found in the corticosterone analysis, no effect of either SSRI treatment or kindling was found in BrdU cell counts. However, the lack of effect is likely due to methodological limitations, as discussed in Chapter 4.

In summary, the common neurobiological alterations of kindling and SSRI treatment investigated in this study were not significantly affected by kindling at the time point analysed, therefore their effects cannot be implicated as associative or causal factors to fluoxetine or citalopram accelerating kindling epileptogenesis in this study. However, this does not exclude the possibility that these alterations may still play a role in the effect of SSRIs on kindling epileptogenesis, and will need to be investigated with further studies, as described below (Section 5.5).

### 5.2. Potential mechanisms by which fluoxetine and citalopram accelerated kindling rate

A number of possible mechanisms could explain the proepileptogenic effect of fluoxetine and citalopram seen in this study. Particularly relevant to the SSRIs would be effects on serotonin levels, serotonergic neurotransmission and receptor alterations during kindling. However, increases in synaptic serotonin levels following SSRI administration are not the sole mechanism by which SSRIs exert their effects and other downstream alterations may also be involved. Furthermore, concentrations within the nanomolar range are required for blockade of the serotonin transporter by SSRIs (Owens et al., 1997) while in humans, therapeutic concentrations of SSRIs in the brain can range from 5-30uM (Karson et al., 1993, Bolo et al., 2000). Therefore, at these much higher concentrations, fluoxetine and indeed other SSRIs are unlikely to be specific for SERT and may have off-target effects (Bianchi and Botzolakis, 2010). As reviewed in Chapter 1 (section 1.5), there are a number of common neurobiological substrates between SSRI treatment and epileptogenesis, and any number of these may have been contributing to the proepileptogenic effects observed here. Potential mechanisms by
which each of these factors may be contributing to the accelerated kindling rate are discussed individually below however it is possible that the results found in this thesis are due to a combination of factors.

5.2.1 Serotonin receptor alterations

Since serotonergic alterations have been implicated in epileptogenesis (Bonnycastle et al., 1957, Lowenstein, 1996, Bagdy et al., 2007) and the primary pharmacological action of SSRIs is to increase synaptic serotonin levels, it is reasonable to suggest a role for serotonin in the proepileptogenic effects of SSRIs seen in this study. Previous studies have shown that 5HT₁A (Sarnyai et al., 2000), 5HT₂C (Przegalinski et al., 1994, Tecott et al., 1995, Brennan et al., 1997, Upton et al., 1998, Isaac, 2005) and 5HT₃ (Wada et al., 1997, Payandemehr et al., 2012) receptors are particularly relevant to epilepsy while these receptors are also affected by SSRI treatment.

Previous studies have shown that 5HT₁A receptor knockout mice have a reduced seizure threshold (Sarnyai et al., 2000) and display increased mortality following administration of the chemoconvulsant kainic acid (Parsons et al., 2001). Similarly, 5HT₁A receptor antagonists have been shown to increase seizure severity and duration (Watanabe et al., 2000; Pericic et al., 2005), while agonists are protective against induced seizures (Hagan et al., 1995, Gariboldi et al., 1996b). Autoradiographic analysis of serotonin receptors in the fully kindled rat brain showed a selective increase in 5HT₁A receptor binding in the dentate gyrus, indicating that 5HT₁A receptors may increase in order to inhibit seizure activity, particularly in the hippocampus (Clark et al., 1993). In addition to this, an established delayed effect of SSRIs is a desensitisation of the presynaptic 5HT₁A autoreceptors (Charney et al., 1981, Charney et al., 1991, Stahl, 1992, Stahl, 1994, Leonard, 1996, Stahl, 1998). Therefore, if kindling leads to an increase in 5HT₁A receptor levels as a compensatory mechanism to inhibit and protect against seizures, but chronic SSRI treatment is desensitising these 5HT₁A receptors, this desensitisation may be contributing to a proconvulsant effect of fluoxetine and citalopram.

Similar to 5HT₁A receptors, 5HT₂C receptor knockout mice are more susceptible to audiogenic seizures and are prone to spontaneous death from seizures (Tecott et al., 1995, Brennan et al., 1997, Applegate and Tecott, 1998). This suggests that 5HT₂C receptors are necessary to suppress neuronal excitability and seizure activity. Previous studies have also shown that fluoxetine is a potent antagonist of the 5HT₂C receptor, (Maj and Moryl, 1993, Kennett et al., 1994, Palvimaki et al., 1996, Stahl, 1996, Ni and Miledi, 1997) therefore if fluoxetine is acting to inhibit the 5HT₂C receptor, this may be contributing to its proconvulsant effect, as found in this
study. This action has not been reported with citalopram treatment, and as such cannot explain the proepileptogenic effect observed with citalopram. However, as discussed in Chapter 4, citalopram may mediate a proconvulsant effect through other mechanisms. This has been suggested by Payandemehr et al. (2012) where it was found that the proconvulsant activity of citalopram was mediated through a modulatory role on nitric oxide, also shown in other studies (De Sarro et al., 1991, Osonoe et al., 1994, Gholipour et al., 2010, Bahremand et al., 2011). Therefore, although only speculative, it may be that functional or expression alterations in 5HT\textsubscript{1A} and 5HT\textsubscript{2C} receptors, as well as modulation of nitric oxide, may be involved in accelerating kindling rate during chronic fluoxetine and citalopram treatments.

5.2.2 Alterations in other aspects of neuroplasticity

Although no alterations in neurogenesis were found in this study, it is possible that subtle alterations in other neuroplastic mechanisms may be involved in the actions of SSRIs on the kindled brain. There is considerable evidence implicating a role for aberrant hippocampal plasticity, which also includes neurogenesis, in acquired epilepsies and epileptogenesis (Bengzon et al., 1997, Parent et al., 1997, Hattiangady et al., 2004, Jakubs et al., 2006, Parent, 2007, Scharfman et al., 2007, Hattiangady and Shetty, 2008, Kron et al., 2010, Murphy et al., 2011) however the results of this study do not suggest aberrant hippocampal plasticity occurred following kindling. Furthermore, there was no effect of fluoxetine on neurogenesis, which is contrary to reports from previous studies (Malberg et al., 2000, Santarelli et al., 2003, Encinas et al., 2006, Boldrini et al., 2009, Kobayashi et al., 2010, Karpova et al., 2011). This indicates that either there were methodological limitations in detection of BrdU-positive cells (see Section 4.4.7 in Chapter 4), or that other, more subtle, alterations occurred that were not detected in the experiments presented here.

Previous studies have found that SSRIs have effects on the functional characteristics of newborn cells, rather than solely affecting newborn cell number. There have been two recent studies that have shown SSRI treatment reversed the maturation of a large proportion of dentate granule cells into cells with immature neuronal properties (Kobayashi et al., 2010; Karpova et al., 2011). Reversing the maturational stage of dentate granule cells may result in reduced synaptic facilitation from the dentate gyrus to the CA3 region. While this may potentially reduce seizures, it was not specifically investigated in these studies and the function of these immature cells is still speculative. It is possible that these immature cells may be rendered hyperexcitable, and as such contribute to seizures rather than suppress them. While this was not specifically investigated in the studies presented in this thesis, it may be possible that the functionality of newborn cells following kindling and SSRI treatment was altered. However, in order to confirm
this, newborn cell function using electrophysiological techniques would need to be employed to determine such an effect.

5.2.3 Modulation of excitatory and inhibitory neurotransmission

Alterations in excitatory and inhibitory neurotransmission are also potential candidates to explain the effects of SSRIs on epileptogenesis. There are a small number of studies that suggest antidepressants may influence the gamma-aminobutyric acid (GABA) system (Krystal et al., 2002) and enhance GABA activity (Robinson et al., 2003, Ugale et al., 2004). Other studies have also shown that SSRIs can increase (Sanacora et al., 2002) or decrease (Cuenca et al., 2004, Isbister et al., 2004) the concentration of GABA in the brain, which could have potential effects on synaptic transmission during a seizure. However, most studies investigating the effects of SSRIs on GABAergic neurotransmission generally use fluoxetine and hence it is unclear whether such results are common to all SSRIs.

A study by Choi et al. (2010) specifically investigated the effects of various SSRIs, including fluoxetine and citalopram, on vesicular GABA transporter (VGAT) immunoreactivity in normal and epileptic rats. It was found that overall VGAT immunoreactivity was increased in the hippocampus of epileptic but not normal rats, indicating a compensatory mechanism in the epileptic hippocampus to increase inhibitory neurotransmission. Then, following SSRI treatment, VGAT immunoreactivity was decreased in both normal and epileptic hippocampi, which suggests that chronic SSRI treatment reduces GABAergic inhibitory neurotransmission, which could potentially increase seizure susceptibility in normal brains, and increase seizure frequency in epileptic brains.

Other studies have shown that high levels of monoamines, in particular dopamine and serotonin, promoted glutamate release which may potentially lead to an increase in neuronal excitability and proconvulsant effects (Clinckers et al., 2004b, 2005). However, the same studies have also shown that the sustained increase in monoamine levels also contributed to an anticonvulsant effect. These studies suggest that other factors, besides the effects on monoamine levels, also contribute to the effects that monoamines are having on neurotransmission.

5.2.4 Overall conclusion of potential mechanisms

As previously stated, currently we can only speculate on possible mechanisms for the accelerated kindling rate found in SSRI-treated rats in this study. The studies supporting a proepileptogenic effect of various mechanisms are few and it is likely that a combination of
factors is involved. Further studies will be required to elucidate the mechanisms by which chronic SSRI treatment accelerates kindling epileptogenesis.

5.3. The potential clinical implications of an accelerated rate of epileptogenesis

An accelerated rate of epileptogenesis during SSRI treatment potentially has significant implications for the treatment of patients with epilepsy. While these outcomes discussed below are speculative, they are outcomes that have been reported to manifest in patients, and as such are extrapolated from these data. The results of this study suggest that although the threshold at which a seizure occurs may not be affected in a patient taking SSRIs, the underlying disorder itself may develop or progress more rapidly. If this is the case, it is possible that the adverse alterations that occur during epileptogenesis may manifest at an earlier stage as compared to patients with epilepsy who are not taking SSRIs. This is discussed in more detail below.

In animal studies, the alterations that occur in association with epileptogenesis, as discussed in Section 1.2.4, are changes which have been shown to develop in the months after the initial insult (Tanaka et al., 1992, Hellier et al., 1998, Kharatishvili et al., 2006) while in humans this can take years (French et al., 1993, Mathern et al., 1995). Current antiepileptic drugs (AED) are only able to suppress the symptoms of the disorder, i.e. the seizures, and no medications are currently able to prevent epileptogenesis in humans. While several studies in animals have shown that AEDs can delay the process of kindling (Loscher 1998a, b, 1999), this has not been shown to occur in people with epilepsy, whereby AEDs could slow the progression of epileptogenesis. In fact, in many patients with epilepsy, while the seizures are being managed, the underlying disorder itself may continue to progress. That epilepsy is a progressive disorder is becoming more accepted, particularly for TLE (O’Brien et al., 1999, Tasch et al., 1999, Bouilleret et al., 2000, Cole, 2000, Pitkanen and Sutula, 2002, Nearing et al., 2007, Cascino, 2009, Yang et al., 2010) and various alterations have been shown to occur in association with epileptogenesis, which are discussed below. While there is some data to support the association of these changes with epileptogenesis, the way in which the underlying neurobiology is altered during epileptogenesis is undiscovered, and therefore such alterations may be affected by antidepressants in ways that are yet unclear. Additionally, it is unclear whether these changes occur in all patients, and whether the severity of these alterations is increasing in patients. Hence, the way in which SSRIs may affect these alterations is speculative and requires more thorough investigation.
There are a number of potential clinical implications of an accelerated rate of epileptogenesis. Firstly, treatment with SSRIs for a comorbid mood disorder may increase the risk that a person develops epilepsy after a brain insult, such as traumatic brain injury (TBI). Secondly, an increased severity and duration, as well as frequency, of seizures associated with accelerated disease progression (Sillanpaa et al., 1998, Kwan and Sander, 2004, Shorvon and Luciano, 2007) may increase the chance of physical injury from a seizure, while also leading to decreased quality of life from a loss of independence, both of which can affect employment and family life. In addition to this, as seizures become more frequent and severe, AEDs may need to be altered or increased, which in themselves have side effects. With progression of the disorder, there may also be the possibility of drug-resistance (Kwan and Brodie, 2000, Arts et al., 2004, Mohanraj and Brodie, 2006, Berg et al., 2009, Kwan et al., 2010). Drug-resistance is also of great concern to patients, as seizures cannot be managed with conventional medication. While there are other options for seizure management such as dietary manipulations (Bergqvist et al., 2005, Neal et al., 2008), more invasive measures may also need to be considered, such as surgical removal of the epileptogenic region (Zentner et al., 1995). On the other hand, increased rates of anxiety and a history of depression have been found to be associated with an increased risk of developing seizure recurrence in newly treated epilepsy (Hitiris et al., 2007, Petrovski et al., 2010) and following epilepsy surgery (Harden et al., 2007, Kanner, 2007). Therefore, it is possible that in patients with mood disorders the pharmacological effects of SSRIs to enhance epileptogenesis may be balanced by reducing the proepileptogenic effect of mood disorders.

Other potential consequences an accelerated progression of epileptogenesis includes an increased severity of psychiatric comorbidities (Hermann et al., 2008), or the emergence of comorbidities that previously were not present. This can include anxiety and depression, as well as psychoses, memory impairments or cognitive decline (Kwan and Brodie, 2001, Motamedi and Meador, 2003, Andersson-Roswall et al., 2010). In fact, previous studies have shown that cognitive impairment correlated with seizure severity. Therefore if seizures become progressively worse as epileptogenesis progresses this may greatly impact upon cognitive functioning (Beume and Steinhoff, 2010, Ono and Galanopoulou, 2012). While treatment with SSRIs may at the same time treat these psychiatric and cognitive symptoms, management of these symptoms may also become more difficult as epileptogenesis progresses.

There may also be a progressive increase in neuropathological alterations, changes that have been consistently observed in the brain tissue of patients with epilepsy post-mortem, and in animal models of epilepsy. This can include neuronal cell loss (Armstrong, 1993, de Lanerolle et al., 2003, Sharma et al., 2007, Bae et al., 2010, Zheng et al., 2011) which may result from
neuronal damage occurring due to excessive glutamate release during prolonged seizures (Naegele, 2007); gliosis (Feindel et al., 1952, Feindel and Penfield, 1954, Pitkanen and Sutula, 2002, Sharma et al., 2007, Shapiro et al., 2008) which has been shown to affect neuronal excitability (Pitkanen and Sutula, 2002, Vezzani et al., 2008) and potentially contribute to the development of epilepsy; synaptic remodelling and formation of aberrant circuitry, possibly as a result of mossy fibre sprouting which can lead to a hyperexcitable hippocampus (Houser et al., 1990, Mathern et al., 1995, Buckmaster and Dudek, 1999, Gorter et al., 2001, Sutula, 2002). It is possible that these neuropathological changes may also be affected by SSRI treatment, which themselves have been shown to affect neurogenesis and synaptic plasticity (Malberg et al., 2000, Kobayashi et al., 2010, Kron et al., 2010, Murphy et al., 2011). However, how these neuropathological alterations are affected by SSRI treatment in epileptogenesis has not been investigated.

The alterations discussed and the ways in which they progress with SSRI treatment in epilepsy remain speculative. However, as the literature suggests that such candidate mechanisms that occur in epileptogenesis (for example, neuroplasticity) may be affected by antidepressants, it is reasonable to speculate upon how such changes may progress during epileptogenesis and antidepressant treatment. It is possible that the benefits that SSRIs are providing to patients, in terms of managing their depressive symptoms, may outweigh any longer-term adverse effects of SSRIs on epileptogenesis. Epileptogenesis and the alterations associated with it may not manifest in all patients and therefore treatment of the current depressive symptoms, along with management of seizures, is currently the most important aspect to consider.

Future studies should still investigate the effects of SSRIs on epileptogenesis in epilepsy patient populations, which would not only include monitoring of the depressive symptoms, but also careful monitoring of changes in seizures over time. In addition to this, changes in various biomarkers of disease progression could also be assessed and followed over time. The search for biomarkers of epileptogenesis is an active field of research (Pitkanen, 2010, Pitkanen and Lukasiuk, 2011, Galanopoulou et al., 2012a, Galanopoulou et al., 2012b), and ultimately, the goal is to target these alterations and produce truly antiepileptogenic medications, rather than antiseizure medications (Galanopoulou et al., 2012a). Various biomarkers of epileptogenesis have been reported, and can be followed up over time in a patient with epilepsy taking SSRIs. One such example is the alteration in brain structures identified with neuroimaging techniques. In rat models of TBI, in which a percentage of animals develop seizures post-injury, or post-status epilepticus, MRI scans have shown that increases in T2 signal (a marker of tissue abnormality) in the piriform and entorhinal cortices can predict the development of seizures.
In humans, sporadic reports suggest that early changes in MRI that are indicative of hippocampal sclerosis occur after prolonged seizures or even prior to seizure onset (Wieshmann et al., 1997, VanLandingham et al., 1998, Kuster et al., 2007). Therefore, monitoring alterations in epileptogenic structures such as the hippocampus with MRI scans over time may provide insight into disease progression. Other more sensitive and advanced neuroimaging techniques that can detect functional alterations may also be employed, such as magnetic resonance spectroscopy, diffusion tensor imaging or positron emission tomography (Tokumitsu et al., 1997, Rugg-Gunn et al., 2001, Mirrione et al., 2006). Additionally, assessment of memory, cognitive and social changes, as well as changes in neuropsychiatric symptoms over time could also be monitored (Motamedi and Meador, 2003, Pitkanen and Lukasiuk, 2011).

5.4. Methodological considerations and limitations

The findings of this thesis suggest that epileptogenesis and the associated consequences, as discussed above, may become more pronounced or manifest more severely in patients who are also taking SSRIs. However, as this was investigated in an animal model of epileptogenesis, caution must be exercised when applying these results to the human epileptic condition. This section outlines the methodological limitations of this study that should be accounted for when translating the findings to human disease.

5.4.1 Validity of the kindling model for comparison to epileptogenesis in humans

As outlined in Chapter 1 (Section 1.2.5), animal models need to be able to reliably and measurably model the human condition in order for comparisons and conclusions to be drawn. However, it should always be acknowledged that animal models do not replicate the human disorders and should only be considered as experimental systems by which certain aspects of disorders can be investigated where it is not possible to do so in humans. Nevertheless, animal models of epilepsy have shown to be valuable tools to investigate epilepsy. In particular, the kindling model shows various elements of a good animal model for the human epileptic condition: there is the potential for the development of spontaneous seizures (Pinel and Rovner, 1978, Michalakis et al., 1998, Coulter et al., 2002, Sayin et al, 2003); similar circuitry to the human condition is involved; similar behavioural and neuropathological alterations are observed (Peele and Gilbert, 1992, Ebert and Loscher, 1995, Helfer et al., 1996, Bengzon et al., 1997, Adamec, 1998, Bertram, 2007); it is responsive to AEDs (Honack and Loscher, 1989,
Becker et al., 1995, Amano et al., 1998, Gu et al., 2004); and importantly for this study, the model shows elements of disease progression which can be easily monitored during controlled stimulations.

However, the kindling model does not always appropriately model the human epileptic condition. As summarised in Bertram (2007), there are two common concepts of epileptogenesis in humans. Firstly, there is the process whereby following an initial insult, a sequence of events occurs during the seizure-free latent period that increases neuronal excitability to the point where seizures begin to spontaneously occur (as shown in Figure 1.4). The other concept of epileptogenesis is more in parallel with the kindling model. This process is similar to the first, in that following the first spontaneous seizure (not occurring due to a known precipitating event), there is an enhancement of seizure strength and an engagement of wider-ranging neuronal networks. As this occurs, seizures begin to spread and become more severe (in terms of frequency and duration). This is comparable to the kindling model, whereby repeated stimulations, and consequently seizures, induce an increase in the epileptic response, beginning with focal seizures that spread and engage ipsilateral and eventually bilateral neuronal circuits to generate seizures. However, epilepsy does not always occur via this pathway in humans, and therefore the kindling model does not always accurately model the human condition. Whether it is that “seizures beget seizures” (Gowers, 1881) or that underlying changes continue to develop which lead to more seizures, is not always clear.

Additionally, although aspects of disease progression are apparent in the kindling model, it is limited in some areas. Seizures must be induced, and do not normally arise spontaneously (unless “overkindled”), as in post-status epilepticus models or in people with epilepsy. Furthermore, kindled rats only model certain aspects of TLE and the associated emotional alterations. The neuropathology associated with kindling is not as extensive or defined as that which occurs in models of spontaneous seizures and in the human condition however these were not investigated in this thesis. Furthermore, behavioural alterations do not always consistently manifest in kindling models, and in most studies, specifically affect anxiety-like behaviours, similar to what was found in this study. Additionally, as kindling requires the long-term implantation of an electrode into the temporal lobe, damage from this electrode may contribute to the epileptogenic process. Previous studies have shown that merely having an electrode implanted in the amygdala can produce proepileptogenic effects (Loscher et al., 1995, Niespodziany et al., 1999).
5.4.2 Normal rats versus the pathological state

In this study, rats did not display aspects of depressive-like behaviours prior to or after kindling. Therefore, the effects of SSRIs in this setting may not be entirely applicable to a person with both epilepsy and depression. Divergence in the effects of SSRIs in normal rats versus those displaying aspects of depressive-like behaviours has been shown. For example, Ferrero et al. (2005) found that naïve rats chronically treated with fluoxetine had a reduced seizure threshold following administration of the chemoconvulsant, pentylenetetrazol (PTZ), while there was no effect of fluoxetine on seizure threshold observed in rats exposed to the learned helplessness paradigm, a model of depression, when treated with PTZ. Furthermore, previous studies have shown that SSRIs have little effect on elevating mood in normal animals or healthy controls (Gelfin et al., 1998, Barr et al., 2002, Geyer and Markou, 2002). Therefore, in this study, SSRIs were shown to accelerate epileptogenesis in rats that were not currently depressed. Whether the effect on kindling epileptogenesis would remain the same or differ in a rat model displaying depressive-like behaviours has not been investigated.

A further methodological limitation of this study was the timing of drug administration. Fluoxetine and citalopram were administered prior to kindling, to rats that were normal and subsequently had seizures induced. While it is important to consider that a small number of patients may be depressed prior to epilepsy onset and therefore already taking SSRIs, the more common clinical scenario is that patients with epilepsy are administered SSRIs after epilepsy onset to treat depressive symptoms. While it may not be common for patients to be taking SSRIs prior to the development of epilepsy, and as such difficult to investigate in a similar fashion to the rat studies in this thesis, it would still be an important group of patients to follow. In addition, there are studies to suggest that there may be a bidirectional relationship between epilepsy and depression (Tellez-Zenteno et al., 2007, Forsgren and Nystrom, 1990, Hesdorffer et al., 2000, Hesdorffer et al., 2006, Morgan et al., 2012) and the study by Alper et al (2007) found that antidepressants significantly reduced the rate of spontaneous seizures in depressed (non-epileptic) patients. As antidepressants have been shown to reduce seizure occurrence, future studies following the potential development of seizures in patients with depression treated with SSRIs could be investigated. In addition, whether epileptogenesis would still be accelerated when SSRIs are first administered after seizures emerge, or during the epileptic period, should also be investigated.
5.4.3 Time point of analysis of common neurobiological substrates

As discussed above, kindling did not appear to affect outcomes of the behavioural tests, the corticosterone response to stress or neurogenesis. While it is feasible that such changes may not have been detected at the time point investigated (four weeks after kindling), previous studies do suggest that kindling is a permanent state (Racine, 1972b, Goddard, 1969) and if any alterations in the factors investigated did occur, that they would persist four weeks after kindling. In particular, behavioural and HPA axis changes would likely still be present four weeks after kindling if they were also present immediately after kindling. However, alterations in cell proliferation may have occurred immediately after kindling (which was not specifically investigated), while cell survival, which was investigated in this study, was not altered. In fact, previous studies have shown that some changes occurring soon after kindling (24-72h) are not present in the weeks after kindling, including effects on CRH mRNA, neurogenesis and gene and protein expression (Smith et al., 1991, Nakagawa et al., 2000, Sohl et al., 2000, Beheshti et al., 2010). As such, it cannot be ruled out that such changes may have occurred immediately after kindling.

5.4.4 Behavioural testing and comparison to human behaviours

Depression and anxiety are heterogeneous disorders with symptoms that occur not only as behavioural manifestations, but also as psychological, emotional, cognitive and physiological alterations. Clinically, these disorders are assessed with various tools, including assessment and rating scales of depression, as well as self-reporting. Mimicking these in animals is difficult, and many aspects of the disorder do not arise in animals, such as guilt or thoughts of suicide, and only measurable aspects of the disorder can be investigated. This is achieved with the use of various behavioural tests, such as those used here. However despite being validated for their ability to assess certain aspects of behaviour, the behavioural tests used in this study also have various limitations.

Firstly, the FST was initially designed to assess the effectiveness of antidepressant medications, rather than the test itself being able to assess depressive-like behaviours, although it is commonly used for this purpose (Bilge et al., 2008, Mazarati et al., 2008, Homberg et al., 2011, Xing et al., 2011). Additionally, the FST only investigates behavioural despair, a single aspect of the depressive disorder, and therefore other aspects associated with the depressive disorder, such as social behaviours, cognition, memory, attention and anhedonia, should also be assessed. These are all dysfunctions that have been shown to occur as part of depressive disorders and
are endophenotypes that can be easily assessed in rodents and are comparable to humans (Cryan and Mombereau, 2004).

Secondly, the EPM was first designed to assess the effectiveness of benzodiazepine anxiolytic drugs, rather than specifically to assess anxiety-like behaviours in other paradigms. Similar to the FST, only certain aspects of the anxiety disorder are able to be determined with the EPM: anxiety induced by exposure to a novel environment. The full spectrum of anxiety traits that are present in an anxiety disorder are not assessed by the EPM, and a battery of tests that probe different aspects should also be considered including, fear-conditioning, social interactions, sustained attention and fear-associated memory (Haller and Alicki, 2012).

It is important to study as many aspects of the disorder as possible in animal models to gain a more comprehensive understanding of behavioural changes in relation to epilepsy. However, this was not performed in this study as the exposure of animals to multiple procedures, some of which may act as stressors, was considered to be a potential confounder to the main investigations of the study.

5.5. Future directions

5.5.1 Investigating the mechanisms involved in accelerated kindling epileptogenesis during chronic SSRI treatment

The mechanisms by which SSRIs are accelerating kindling rate need to be further investigated. Specifically, future studies should investigate the effects on synaptic serotonin levels as well as alterations in serotonin receptors, particularly those implicated in epileptogenesis and antidepressant action, such as the 5HT$_{1A}$ and 5HT$_{2C}$ receptors, with a focus on expression and function.

Various neuroplastic endpoints, besides BrdU labelling, could also be investigated. While quantifying cell numbers is an important first step, this does not give an indication of newborn cell functionality or connectivity. Immunohistochemistry could be carried out to determine cell phenotype, such as staining with NeuN for mature neurons. In addition to this, BrdU and other thymidine analogues could be administered at different time points throughout kindling for analysis by immunohistochemistry, with each thymidine analogue giving an indication of neurogenesis at different time points.
The functionality and connectivity of newborn cells could also be investigated using electrophysiological techniques. This will allow for determination of whether the cells are functionally integrated or aberrantly connected, and whether this is contributing to the proepileptogenic effect observed. This should also be investigated throughout kindling to determine the changes occurring throughout epileptogenesis, in addition to the same time point investigated in this study (four weeks after kindling) in order to give an indication of cell survival. Investigating each of these intermediaries at the appropriate time points will give some indication as to their effects and/or roles in epileptogenesis.

5.5.2. Investigating the effects of SSRIs in a chronic epilepsy model

The post-status epilepticus model could be used to confirm the result found in this study. This model is advantageous as there is development of spontaneous seizures, which can be monitored over a period of months to determine the effects of chronic SSRI treatment over time. SSRIs could be administered immediately after status epilepticus, and treatment would need to continue throughout the latent period, prior to the emergence of spontaneous seizures. In this model, the latency to spontaneous seizure occurrence, as well as seizure severity and frequency could be monitored. Another option may be to administer SSRIs at other time points that are clinically relevant, such as after the emergence of spontaneous seizures, to investigate the effects of chronic SSRI treatment on the seizure severity and the progression of epileptogenesis in the established disorder. Behavioural alterations could also be investigated, both prior to the emergence of spontaneous seizures and once seizures emerge, to better determine the effects of SSRI treatment upon epilepsy-related behaviours.

5.5.3 Investigating the effects of SSRIs in a model of epilepsy and depression

As discussed above, the effects of SSRIs on a normal brain as compared to one in a disease state can differ. Therefore, investigating the effects of SSRIs on epileptogenesis in a model of depression would be more relevant. A model of social defeat or unpredictable chronic mild stress, where the socio-environmental factors that contribute to depression will induce the depressive-like behaviours, could be used. In these models, SSRI treatment and kindling could also be completed in a similar fashion to what was done in this study.

Furthermore, as most previous clinical studies are limited to investigating the effects of SSRI on seizures in patients with epilepsy already taking AED medication (which, ethically, cannot be stopped) investigating the effects in an animal model in which AEDs are also administered
should also be considered. The rats in this study were not being administered AEDs and the AEDs themselves may be interacting with SSRIs. This in itself may be having effects on epileptogenesis. Whether the effect is the same in an animal model in which AEDs are concurrently administered is unknown.

5.5.4 Clinical directions

Future clinical studies could follow patients with epilepsy being treated with SSRIs over time, and monitor the progression of the epileptic symptoms. One possible study could be to investigate this in a population of patients that are susceptible to developing epilepsy, such as in patients following TBI. Studies have shown that up to 86% of patients may develop epilepsy in the days or months after TBI (Haltiner et al., 1997, Annegers et al., 1998). If these patients could be stratified based on treatment with SSRIs and compared to an untreated group, the delay to which seizures manifest could be investigated. However, this may not be ethically acceptable based on the results of this study, which suggested that SSRIs accelerated epileptogenesis. Therefore, this could be investigated retrospectively, taking data from TBI patients who were administered SSRIs and those who were not and comparing the development of epilepsy in these patients. However, patient follow up, as discussed above in section 5.3, should be monitored closely to determine disease progression. Similarly, retrospective analysis of data indicative of disease progression could be performed, comparing SSRI and non-SSRI treated patients with epilepsy, rather than initiating a new study which would not only be costly and time consuming, but also raise various ethical concerns.

5.6. Final conclusions

The findings of this thesis challenge the belief that SSRIs can be safely used in patients with epilepsy. It was found here that although SSRI treatments were not proconvulsant, chronic SSRI treatment was associated with acceleration in the progression of epileptogenesis. This thesis highlights the need to investigate the progression of the epileptic disorder over time, rather than just examine symptomatic endpoints. In addition, chronic epilepsy models should be used to determine the effects of SSRIs in epilepsy, rather than solely using acute seizure tests, given that acute models do not exhibit the underlying alterations associated with epileptogenesis. Although a chronic model of epileptogenesis was used in this study, none of the common alterations investigated could account for the acceleration of kindling rate seen with fluoxetine and citalopram treatment. This is possibly due to the time points chosen for analysis and
therefore investigation of these alterations at appropriate time points may give a better indication of whether or not these factors (either alone, or in combination with each other) are contributing to the effects of SSRIs in epileptogenesis. Clearly, it is essential to treat the depressive symptoms that manifest in people with epilepsy; however whether these medications affect the course of epilepsy and how they may do so should become priority areas for future research.
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