The Anticonvulsant Effects of 5α-Dihydroprogesterone in the Rat Amygdala-Kindled Model

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
Department of Pharmacology and Toxicology
University of Toronto

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2017

Abstract

The present study investigated the anti-seizure effects of 5α-dihydroprogesterone (5α-DHP) in an animal model of human drug-resistant complex partial seizures - the rat amygdala-kindling model - using different routes of administration and solvents.

5α-DHP in β-cyclodextrin administered via the subcutaneous (SQ) route was weakly effective (Rmax=30%). 5α-DHP via the intraperitoneal (IP) route in the "benzyl" vehicle, however, suppressed both generalized and focal seizures with ataxia shortly after injection; and generalized seizures around 130 minutes post-injection. The "benzyl" vehicle itself, however, was active at the earlier time, and benzyl alcohol was found to be anticonvulsant. 5α-DHP in β-cyclodextrin via the intravenous (IV) route suppressed both generalized seizures and focal seizures, with ataxia. A time-response IV study at 5mg/kg showed immediate onset of anticonvulsant action that lasted for about 60 minutes post-injection.

In conclusion, 5α-DHP demonstrated efficacy in the present study, and 5α-DHP analogs might be developed as a new anti-seizure therapy.
Acknowledgements

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Many thanks to Muyuan Zhong, Renjie Zhang, Ziying Chen and Junhan Liu for working alongside me on this project, and for sharing their enthusiasm for research. Thanks for being such good lab mates and friends.

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Lastly, to my dearest friends and family: thanks for your support and encouragement over the years.
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<th>Full Form</th>
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<tr>
<td>5α-DHP</td>
<td>5α-dihydroprogesterone</td>
</tr>
<tr>
<td>AD</td>
<td>afterdischarge</td>
</tr>
<tr>
<td>ADD</td>
<td>afterdischarge duration</td>
</tr>
<tr>
<td>ADT</td>
<td>afterdischarge threshold</td>
</tr>
<tr>
<td>AED</td>
<td>anti-epileptic drugs</td>
</tr>
<tr>
<td>ALLO</td>
<td>allopregnanolone; 3α, 5α- tetrahydroprogesterone</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CYP</td>
<td>cytochrome P450</td>
</tr>
<tr>
<td>ED50</td>
<td>median effective dose (50%)</td>
</tr>
<tr>
<td>ED75</td>
<td>75% effective dose</td>
</tr>
<tr>
<td>EEG</td>
<td>electroencephalograph</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>GABA</td>
<td>gamma-aminobutyric acid</td>
</tr>
<tr>
<td>ILAE</td>
<td>International League Against Epilepsy</td>
</tr>
<tr>
<td>IP</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>IV</td>
<td>intravenous</td>
</tr>
<tr>
<td>mL</td>
<td>milliliter</td>
</tr>
<tr>
<td>mPR</td>
<td>membrane progesterone receptor</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>nPR</td>
<td>nuclear progesterone receptor</td>
</tr>
<tr>
<td>PGRMC</td>
<td>progesterone receptor membrane component</td>
</tr>
<tr>
<td>PTZ</td>
<td>pentylenetetrazole</td>
</tr>
<tr>
<td>Rmax</td>
<td>maximal response</td>
</tr>
<tr>
<td>SQ</td>
<td>subcutaneous</td>
</tr>
<tr>
<td>StAR</td>
<td>steroidogenic acute regulatory protein</td>
</tr>
<tr>
<td>SUDEP</td>
<td>sudden unexplained death in epilepsy</td>
</tr>
<tr>
<td>TD50</td>
<td>median toxic dose (50%)</td>
</tr>
<tr>
<td>THP</td>
<td>$3\alpha, 5\alpha$-tetrahydroprogesterone (a.k.a allopregnanolone)</td>
</tr>
<tr>
<td>µA</td>
<td>microampere</td>
</tr>
<tr>
<td>µg</td>
<td>microgram</td>
</tr>
<tr>
<td>µL</td>
<td>microliter</td>
</tr>
<tr>
<td>v/v</td>
<td>volume by volume</td>
</tr>
<tr>
<td>w/v</td>
<td>weight by volume</td>
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Chapter 1. Introduction

1.1 Epilepsy

Epilepsy is a heterogeneous neurological disorder, characterized by spontaneous and recurrent seizures. Seizures themselves are periods of neural hyper-excitation, during which the neurons in the brain fire in excessive, synchronized bursts (Burnham, 2006).

1.1.1 Prevalence, Incidence, Age of Onset

According to the World Health Organization (WHO), epilepsy is the most common neurological disorder in the world, affecting 50 million people (World Health Organization, 2015). It affects about 1% of the world population at any given time, and around 4% of the population at some time during their lives (Banerjee & Hauser, 2008).

Epilepsy may have its onset at any time during life, but onset is more likely at some ages than others. The typical age of onset differs in the “developed” countries and the “developing” countries, reflecting a difference in risk factors (Banerjee & Hauser, 2008).

In “developed” countries, the age of onset is typically at the extremes of life – early or late. Incidence is highest during the first few months of life, and then decreases during the first year. Epilepsies in this early onset group are often due to genetic defects. The rate of onset is lower during childhood, and lowest during normal adulthood. It rises dramatically, however, in the elderly. The age-related onset of cerebrovascular diseases may contribute to the high incidence in the elderly (Banerjee & Hauser, 2008).

In contrast, in “developing” countries, the incidence of epilepsy is the highest among young adults. A lack of treatment for certain diseases (e.g. head injury, infections), along with
other sociocultural factors, may contribute to this high incidence in young adults. There is no identifiable increase in incidence in the elderly in developing countries (Banerjee & Hauser, 2008).

1.1.2 Etiology

Epilepsy and seizures can be classified based on etiology, which is to say based on causality (Beghi & Sander, 2008). A 1998 cohort study involving 2200 patients suggested that the underlying cause of epilepsy is a major prognostic factor for recurrence, so causality is important (Semah et al, 1998).

Classifying epilepsy by cause has become hard to discuss, however, because the International League Against Epilepsy (ILAE) is updating and redefining its system of classification.

In the following section, I will describe two types of classification, older and newer, and both still commonly used in clinical practice. The first is the 1989 classification, and the second is the 2010 version, somewhat updated in 2017.

1.1.2.1 1989 ILAE Classification

The 1989 classification divided the etiology of epilepsy into three categories: 1) idiopathic, 2) symptomatic, and 3) cryptogenic (or “probably symptomatic”).

The word “idiopathic” is commonly used to describe diseases that arise spontaneously, from an unknown cause. In the field of epilepsy, the term has been used to describe those epilepsies that are not associated with brain lesions or obvious neurological abnormalities. This type of epilepsy is usually drug-responsive and patients may achieve full remission (Berg et al, 2010).
The term “symptomatic” has been used to describe seizures related to identifiable brain lesions and/or other physical or metabolic abnormalities. Symptomatic epilepsy is less drug-responsive, resulting in a poorer prognosis (Berg et al, 2010).

The term “cryptogenic” describes epilepsies where there is no clear brain abnormality, but which resemble the symptomatic epilepsies. Some researchers argue that these epilepsies might be better called “probably symptomatic”, yet many formerly cryptogenic seizures - such as Dravet’s syndrome - have a genetic cause (Berg et al, 2010).

1.1.2.2 2010 ILAE Classification

Due to recent advances in epilepsy research, the ILAE has proposed a new classification – based on causality. It reclassifies epilepsy as: 1) genetic, 2) structural and/or metabolic, and 3) unknown (Berg et al, 2010).

As the name suggests, “genetic” refers to those epilepsies that are due to a known or presumed genetic defect(s), such as mutation in an ion channel. “Genetic” replaces the term “idiopathic”.

“Structural and/or metabolic” epilepsies are epilepsies related to clear-cut brain abnormalities, such as those caused by stroke, trauma, and brain infection.

“Structural/metabolic” replaces the term “symptomatic”. Some structural/metabolic abnormalities may also have a genetic cause, such as tuberous sclerosis. However, the abnormality in brain structure is thought to be the direct cause of the epilepsy.

Finally, those epilepsies that are neither clearly genetic nor structural-metabolic are classified as “unknown”. Those lesions, are possibly due to one or multiple unknown genetic defects, or are a consequence of a separate disorder that has not yet been recognized (Berg et al, 2010).
This classification, based on etiology, is largely unchanged in the newly published 2017 ILAE guidelines (Fisher et al, 2017).

1.1.3 Comorbidities

The term “comorbidity” is used to describe a disorder that often co-occurs with another disease or disorder. Epileptic patients often show comorbidities. In addition to epilepsy, they often have cognitive or psychiatric disorders - such as depression, schizophrenia, and substance abuse - as well as somatic disorders, such as migraine and asthma (Fazel et al, 2013; Hamid et al, 2014).

The comorbidities of epilepsy may relate, in part, to the effects of seizures on the brain. It is thought that repeated seizures may produce changes in the brain which can negatively affect the inter-ictal function (Richardson, 2012). The comorbidities may reflect these brain changes. In fact, seizures and emotional disorders both result in brain changes and it is thought that they may have a bidirectional relationship in which they exacerbate each other (Hesdorffer et al, 2012).

In addition to the comorbidities, epileptic patients are often stigmatized, not able to hold driver’s licenses and under-employed (Burnham, 2006). The comorbidities of epilepsy, combined with these factors, lead to a poor quality of life for people with uncontrolled epilepsy.

1.2 Types of Seizures

As mentioned above, seizures are periods of sustained, spontaneous neuronal hyperexcitation (Burnham, 2006). The symptoms of this hyperactivity vary, however, depending on the parts of the brain involved in seizure discharge. Thus, there are many types of seizures.
Once again, the ILAE is re-working its classifications, leading to old and new names for the different types of seizures. In addition to the 1989 terminology, there is a 2010 terminology, and further updating in the 2017 guidelines.

1.2.1 1989 ILAE Definition

In the older terminology, seizures are classified into “generalized seizures” and “partial seizures”, based on the extent of epileptic discharge in the brain (Burnham, 2006).

Generalized seizures are characterized by neuronal hyper-excitation occurring in both hemispheres. There are many subtypes of generalized seizures, but the two most common subtypes are “absence” and “tonic-clonic” seizures (Burnham, 2006).

Tonic-clonic seizures, once called “grand-mal”, are characterized by unconsciousness and dramatic convulsions. They are associated with a pattern of constant “spiking” in the EEG record. They usually last for less than 5 minutes. Following the seizure, the patient has no memory for the period of the attack (Burnham, 2006).

Absence seizures, once called “petit mal”, involve a brief period of unconsciousness with blank staring. They last for less than 30 seconds and are associated with a characteristic 3/sec spike and wave pattern in the EEG. As with tonic-clonic seizures, the patient has no memory for the period of attack (Burnham, 2006).

“Partial seizures” involve only a part of the brain, perhaps being limited to a specific neocortical and/or limbic area. In the 1989 terminology, they were further classified as “simple partial” seizures and “complex partial seizures” (Burnham, 2006).

During “simple partial seizures”, consciousness and memory are preserved. The duration of simple partial seizures varies, and sensory, motor or emotional signs are observed, depending
on the region involved in epileptic discharge. The study of simple partial seizures has helped to reveal the functions of different areas of the neocortex and contributed to our understanding of cortical organization (Burnham, 2006).

During “complex partial seizure”, it seems that the patient’s consciousness is preserved, but the patient is out of contact with the surrounding environment. Consciousness is said to be “impaired”. Following the attack, the patient has no memory of the period of the seizure. As with simple partial seizures, the duration of the complex partial seizure varies. The seizures often start as simple partial seizures, usually of limbic origin, before progressing to the state where awareness is lost. This form of non-convulsive attack is the most common form of seizure in the adult population (Burnham, 2006). Like simple partial seizures of temporal lobe origin, complex partial seizures are often drug-resistant. Thus, the control of complex partial seizures is recognized as one of the most important challenges in the modern field of epilepsy research (Burnham, 2006).

Following either simple or complex partial seizures, it is possible for discharge to spread throughout the brain, eventually recruiting the motor structures and producing a tonic-clonic seizure. These motor seizures used to be called “secondarily generalized” (Burnham, 2006).

1.2.2 2010 and 2017 ILAE Definition

While the terminology of generalized seizures remains fairly constant, an intensive discussion has taken place in renaming partial seizures.

“Partial seizure” is now called “focal seizure” under the new terminology, and degree of impairment is an important aspect in defining focal seizures.
Berg et al. (2010) suggested instead of “simple partial seizures”, one should use the term “focal seizures without impairment of consciousness or awareness”. “With observable motor or autonomic components” were suggested to describe the seizures with motor components, and “focal seizures without impairment of consciousness or awareness involving subjective sensory or psychic phenomena only” to describe seizures without motor components (Berg et al, 2010). In the 2017 terminology, the terminology is shortened to “focal aware seizure” (Fisher et al, 2017).

For “complex partial seizures”, Berg et al. (2010) have suggested that these seizures be called “focal seizures with impairment of consciousness or awareness”. The 2017 guidelines suggest “focal impaired awareness seizures” (Fisher et al., 2017).

For “secondarily generalized motor seizures”, Berg et al. (2010) suggest “focal seizures evolving to bilateral, convulsive seizures” to describe these attacks. In the 2017 terminology, the term “focal to bilateral tonic-clonic” is used to describe secondary generalization (Fisher et al, 2017).

1.2.3 Psychogenic Non-Epileptic Seizures

Psychogenic non-epileptic seizures, formerly called “pseudo-seizures”, are not, strictly speaking, seizures at all - despite the fact that patients display seizure-like behaviors, similar to convulsions. During these apparent seizures, no epileptic discharge is detected in the EEG recordings (Brigo et al, 2015). The patients do not respond to anti-epileptic drugs (AEDs), although they do suffer from the side effects of AEDs. The golden standard of diagnosis for these patients should be video-EEG. Clear communication of the non-epileptic nature of these events and psychotherapy are helpful for these patients (LaFrance et al, 2012).
1.2.4 Status Epilepticus

Although seizures are usually self-limiting, there are exceptions. In some cases, patients display very long seizures with continuous behavioral and EEG discharges, or seizures that repeat with no conscious periods in between. These episodes are described as “status epilepticus” (Burnham, 2006) (Brophy et al, 2012).

Status with convulsions is a life-threatening condition, and requires immediate medical attention. It is recommended to call 911 when any seizure lasts for more than 5 minutes. The primary goal of the treatment for status is to stop the clinical and EEG seizures as soon as possible. Screening for the underlying cause is also helpful (Brophy et al, 2012).

1.3 Treatment for Epilepsy

Even though most seizures are relatively brief and harmless, people with uncontrolled seizures generally have a low quality of life. As mentioned above, stigma, economic and social problems combine with the epilepsy-related psychiatric and somatic comorbidities (Fazel et al, 2013; Hamid et al, 2014) to make life difficult for people with seizures that are not controlled. In extreme cases, uncontrolled seizures can even lead to sudden unexpected death in epilepsy (SUDEP) (Terra et al, 2013). Therefore, therapy is very important for people with epilepsy.

Before starting therapy, it is important to rule out psychogenic non-epileptic seizures and active pathology. If active pathology (e.g. brain tumor, infection) is present, the pathology, not the seizures, should be treated (Burnham, 2006).

If there is no active pathology, and the seizures are genuine, therapy is initiated. The first step is to make an accurate diagnosis. The diagnosis must be accurate to achieve maximum
therapeutic effectiveness. The physician must answer three questions, if possible: 1) What is the etiology of epilepsy? This could range from brain structural abnormalities to genetic causation; 2) What is the seizure type? Some anti-epileptic drugs target specific types of seizures, and using the wrong drug can exacerbate seizures; and 3) Is there an epileptic syndrome present? Seizures that are part of epileptic syndromes may require special attention (Chadwick et al, 2008).

The most common treatment for epilepsy is anti-epileptic drug (AED) therapy. If drug therapy fails, surgery and diet therapy are the most common second lines of treatment (Burnham, 2006).

1.3.1 Anti-Epileptic Drugs (AEDs)

Since the 19th century, AEDs have been the first line of therapy for epilepsy. They successfully suppress seizures in 60-70% of cases. They do not cure epilepsy, however; they only suppress seizures on a temporary basis, and patients must take AEDs chronically – at least once a day - to suppress their seizures. (Beghi & Sander, 2008).

AEDs are generally safe, but sometimes may cause rare idiosyncratic side effects. Less serious, but unpleasant side effects are common. Due to those adverse effects, compliance remains an issue in epilepsy treatment (Burnham, 2006).

Most of the older (first generation) AEDs (phenobarbital, phenytoin, ethosuximide, carbamazepine) only suppress specific types of seizures, and may exacerbate other seizure types. The newer (second generation) AEDs (lamotrigine, topiramate, levetiracetam, zonisamide) are broad-spectrum drugs useful against most types of seizures (Burnham, 2006).
Most of the older AEDs act via three major mechanisms: 1) partial inhibition of voltage-gated sodium channels, 2) enhancement of the GABA-A system, and 3) inhibition of t-type calcium channels. Some of the newer AEDs claim to have novel mechanisms, such as inhibiting transmitter release or AMPA receptor channels (Burnham, 2006) (Meldrum, 2008) (MacDonald & Rogawski, 2008). A number of the common AEDs, with their side effects and indications, appear in Table 1.1.

Unfortunately, about a third of patients fail to achieve seizure freedom with AED therapy. This is true even with the newer AEDs. Despite the fact that the newer AEDs have demonstrated superiority in safety, long-term tolerability and favorable pharmacokinetic profiles, they have not demonstrated superior efficacy, as compared to the older drugs, in head-to-head clinical trials in patients with newly diagnosed epilepsy (French & Ben-Menachem, 2008).

Epilepsy is defined as “intractable” (drug resistant) after a patient fails to response to two appropriate AED therapies. Many of the patients who are drug-resistant have complex partial seizures. Intractability is common in people with complex partial seizures (Burnham, 2006). Drug control of complex partial seizures is a major goal of epilepsy research.

1.3.2 Surgery

Patients who have failed to find seizure freedom with AEDs may be referred for surgical therapy if they have focal onset epilepsy. The recent increase in the use of surgery in epilepsy treatment is attributed to four factors: 1) technological advances in neuroimaging and EEG techniques which allow better localization of the epileptic focus, 2) the recognition of the socioeconomic cost of intractable, recurrent seizures, 3) the identification of surgically
<table>
<thead>
<tr>
<th>Seizure type</th>
<th>Drug</th>
<th>Side effects</th>
</tr>
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<tbody>
<tr>
<td>Absence</td>
<td>Ethosuximide</td>
<td>Gastrointestinal upsets, sedation, photophobia</td>
</tr>
<tr>
<td>Tonic/Clonic</td>
<td>Carbamazepine</td>
<td>Diplopia, dizziness, gastrointestinal upsets, sedation, decreased white blood cell count</td>
</tr>
<tr>
<td>Partial</td>
<td>Gabapentin</td>
<td>Headache, ataxia, fatigue, nausea, sedation, somnolence</td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>Sedation</td>
<td>(excitement in children), withdrawal problems</td>
</tr>
<tr>
<td>Phenytoin</td>
<td>Gastrointestinal upsets, hirsutism, gingival hyperplasia, acne, sedation</td>
<td></td>
</tr>
<tr>
<td>Primidone</td>
<td>Sedation</td>
<td>Psychiatric disturbances, decreased libido</td>
</tr>
<tr>
<td>Tiagabine</td>
<td>Somnolence</td>
<td>Dizziness, nervousness, gastrointestinal upsets</td>
</tr>
<tr>
<td>Vigabatrin</td>
<td>Gastrointestinal upsets, sedation, transient psychosis</td>
<td></td>
</tr>
<tr>
<td>Broad spectrum</td>
<td>Clonazepam</td>
<td>Sedation, personality changes, withdrawal problems</td>
</tr>
<tr>
<td>Lamotrigine</td>
<td>Headache</td>
<td>Nausea, dizziness, gastrointestinal upsets, sedation, rash</td>
</tr>
<tr>
<td>Topiramate</td>
<td>Fatigue, somnolence, dizziness, weight loss, gastrointestinal upsets</td>
<td></td>
</tr>
<tr>
<td>Valproate</td>
<td>Weight gain</td>
<td>Hair loss, gastrointestinal upsets</td>
</tr>
<tr>
<td>Zonisamide</td>
<td>Somnolence</td>
<td>Dizziness, gastrointestinal upsets, rash</td>
</tr>
</tbody>
</table>
remediable epilepsy syndromes, and 4) the long-term benefits of early surgical intervention on quality of life (Duchowny et al, 2008).

There is curative surgery which eradicates all seizures, and palliative surgery which decreases seizure frequency and severity. Palliative surgery may prevent certain types of seizures (e.g. drop attacks) without preventing them all (Duchowny et al, 2008).

Surgical procedures can be classified into 2 categories: 1) resection surgery that removes the patient’s epileptic foci (e.g. temporal lobectomy, frontal lobectomy), 2) disconnection surgery that interrupts the neuronal pathways responsible for seizure spreading (e.g. multiple subpial transections, corpus callosotomy). Disconnection is used when the epileptic focus is too functionally important to lesion it, or when the epileptic focus is too big, so that entire removal would leave a huge empty space in the cranium, and make the patient prone to concussion (Duchowny et al, 2008).

Surgery candidates are usually patients with: 1) drug-resistant epilepsy with focal onset, 2) surgical remediable epileptic syndromes (tuberous sclerosis, focal cortical dysplasia, Rasmussen syndrome), and 3) a loss of quality of life caused by epilepsy. Among all surgeries performed, temporal lobe resections account for 2/3 of all the procedures performed in adults (Duchowny et al, 2008).

1.3.3 Ketogenic Diet

People with intractable epilepsy, but no identifiable focus, may be referred for diet therapy. Historically, people had used prayers and fasting to control seizures – with some success. Wilder in 1921 proposed a ketogenic diet - high in fat and low in carbohydrates – that, like fasting, would raise blood ketone levels (Levy et al, 2015). One theory proposed is that the
high blood acetone level caused by the diet is responsible for seizure suppression (Likhodii et al, 2003).

The ketogenic diet is mainly used in children with drug-resistant epilepsy. The diet is effective, but very strict. Sometimes even a little increase in carbohydrate intake (e.g. a cookie) can make the diet fail. Due to the unpalatable nature of the diet and its gastrointestinal side effects (diarrhea), compliance remains an issue (Levy et al, 2015).

The classic ketogenic diet involves a 4:1 ratio of fat to carbohydrates and protein. A less restrictive diet, the “modified Atkins diet” has a ratio of 2:1. El-Rashidy et al. (2013) have showed that both are effective but, that the classical ketogenic formula is superior. These researchers have hypothesized that the classical ketogenic diet is more effective at creating ketosis (El-Rashidy et al, 2013).

1.4 Animal Models of Epilepsy and Seizures

Due to ethical, technical and cost constraints, using human subjects in drug development is only done after animal trials have been completed. Animal models of epilepsy have been used for many years to study disease mechanisms, and animal seizure models have been used to predict human response to potential AEDs (Kandratavicius et al, 2014) (Baraban & Loscher, 2014) (Wegener & Rujescu, 2013).

Different animal models represent different types of epilepsy and seizures. Therefore, choosing the right animal model for the study of potential AEDs is extremely important. Two animal seizure models were used in the present study, the kindling model and the maximal pentylenetetrazol (PTZ) model.
1.4.1 The Electrical Kindling Model

The electrical kindling model is used as a model of limbic (complex partial) epilepsy with secondary generalization. The kindling phenomenon was first described by Goddard et al. in 1969. When experimental animals are given an electrical stimulus repeatedly via a chronically implanted electrode, they show focal electrographic seizures that gradually develop into secondarily generalized motor seizures. The focal seizures are detected by EEG, while the generalized tonic-clonic seizures are scored using Racine’s behavior seizure scale (Racine, 1972): Stage I mouth and facial clonus, Stage II head clonus, Stage III forelimb clonus, Stage IV rearing, and Stage V rearing and falling.

The kindling electrode is usually implanted into a limbic region, usually in rats. Common sites of electrode implantation include the amygdala, the perforant path, the hippocampus, and the perirhinal cortex. Thus, the kindling model is often used to model partial epilepsy with limbic origins (Coppola & Mose, 2012). The neocortex can also be kindled; however this kindling occurs at a slower rate (Racine et al, 1975).

1.4.1.2 Advantages of the Kindling Model for Drug Development

Even though the kindling model is time- and labor-intensive, it has several advantages for drug testing. 1) There is no life-threatening physical insult (as in the status models) and the mortality is low; 2) The model is easily replicated and reliable; it produces a viable model of focal seizures with secondarily generalization (Coppola & Mose, 2012); 3) Seizures can be triggered on demand at specific times after drug administration; and 4), the animals can be reused, so the effects of different doses can be measured in the same animal.

Most important, the amygdala-kindling model has been drug validated and has showed a drug-resistant profile similar to that of complex partial seizures in humans. The amygdala-
kindled focus models the human complex partial seizure, and the generalized convulsion models secondarily generalized human tonic-clonic seizures (Albright & Burnham, 1980).

It is hoped that compounds that can effectively suppress limbic focal seizures in animals will also suppress complex partial seizures in human.

1.4.2 Pentylenetetrazol Model

The pentylenetetrazol (PTZ) model is used as a model of generalized seizures. PTZ is a non-competitive antagonist for the GABA A system, where it mainly acts by binding to the t-butyl-bicyclo-phosphorothionial (TBPS) site on the GABA-A related chloride channel and decreases chloride flux (Olsen, 1981). To produce seizures, one may inject the compound subcutaneously, intraperitoneally or intravenously (Akula et al, 2009).

The injection of PTZ induces several types of generalized seizures in a dose-dependent manner. When moderate or high doses are injected, the following seizures are observed: 1) myoclonic jerking, 2) generalized clonus (forelimb clonus and/or whole body clonus), and 3) forelimb and hindlimb extension (Löschter et al, 1991; Akula et al, 2009). For scoring, one observes the latency to the different seizure onsets, and scores the different seizure types as either “present” or “absent”. This model most often is done in mice.

The PTZ model, in one form or another, has been one of the most important models in pre-clinical drug development (Krall, 1978; Löschter et al, 1991; Löschter, 2011). The model is popular because it is fast and not labor intensive. Pretreating the animal with an anticonvulsant delays seizure onset and increases seizure threshold, and may entirely suppress seizures. Properly used, the model can be used to screen for drugs effective against clonic seizures, myoclonic seizures, tonic-clonic seizures and generalized absence seizures (White, 1997).
1.5 Epilepsy and Steroid Hormones

The present study was designed to investigate the anti-seizure effects of dihydroprogesterone, a metabolite of the hormone progesterone. Progesterone and estrogen are steroid hormones produced in male and female reproductive organs, as well as in the central nervous system by both neurons and glia. Peripherally produced progesterone and estrogen play a major role in the female reproductive cycle, and also have important effects on seizure thresholds in females (Deutsch et al, 2013).

It has been known for centuries that there is a link between the female fertility cycle and seizure occurrence. In general, seizure thresholds are high when the blood progesterone levels are high, and low when the blood estrogen level are high (Edwards et al, 1999).

Females with epilepsy, therefore, often experience seizure clusters that are tightly or loosely correlated with their endogenous steroid hormone levels. A woman may have seizures clusters tightly linked to certain phases of her menstrual cycles, or she may have seizures throughout her cycles but more seizures at certain crucial phases. Depending on how strict the criteria are in epidemiology studies, the incidence of catamenial (hormone-related) epilepsy varies between 31% and 60%. Catamenial epilepsy is often drug-resistant (Herzog et al, 1997).

Catamenial seizures occur in both ovulatory and anovulatory cycles. In patients with ovulatory catamenial seizures, clusters of seizures often occur during the perimenstrual (C1) and/or periovulatory (C2) phases. The perimenstrual phase is characterized by low estrogen and low progesterone, whereas the periovulatory phrase is characterized by low progesterone level and a sudden surge of estrogen. In both cases, progesterone is low or falling.
In patients with anovulatory catamenial epilepsy, clusters of seizures often occur in the inadequate luteal (C3) phase. In normal subjects, the luteal phase involves a high level of progesterone. However, in those anovulatory patients, progesterone level remains low during luteal phases (Herzog et al., 1997).

1.6 Progesterone in Epilepsy

Progesterone and its metabolites have a demonstrated therapeutic efficacy in many models of neurological pathologies (ischemia, motoneuron disease, peripheral nerve injury, traumatic brain injury, spinal cord injury, Alzheimer’s disease, demyelinating disease, seizures) etc. (Deutsch et al, 2013).

Because of its effects on seizure threshold, progesterone and its metabolites have also been considered as potential agents for use in the therapy of epilepsy, as will be discussed in this thesis. The present section will discuss the synthesis and metabolites of progesterone. A subsequent section will discuss tests of progesterone and its metabolites in humans and in animal seizure models.

1.6.1 Progesterone Synthetic and Metabolic Pathway

Figure 1.1 presents the basic progesterone synthesis pathway. Cholesterol is trafficked by StAR (steroidogenic acute regulatory protein) and TSPO (translocator protein) to the mitochondrial membrane. Cholesterol’s sidechain at C20 is then cleaved, and it is converted into pregnenolone by CYP11A1. Pregnenolone is further metabolized by 3β-hydroxysteroid dehydrogenase to progesterone by substituting the alcohol (–OH) group on C3 with a ketone =O group.
Progesterone has a number of degradative pathways. Progesterone can be hydroxylated at the $5\alpha$, $5\beta$, $17\alpha$ (Hill et al, 2011), and 21C positions (Edwards et al, 2005). Among those metabolites, the $5\alpha$-hydroxylated metabolites (Lonsdale & Burnham, 2003; Lonsdale et al, 2006; Lonsdale & Burnham, 2007) and 21C-hydroxylated metabolite (a.k.a deoxycorticosterone) (Edwards et al, 2005) have demonstrated strong anticonvulsant activity.

1.6.2 Progesterone Synthesis and Metabolism in the Brain

Progesterone is synthesized de novo both in the body and in the brain, as shown by Corpéchot’s group in gonadectomised and adrenatectomized male and female rats (1993).

Progesterone’s major metabolic pathway in the brain is $5\alpha$ reduction, both in rats (Karvolas et al, 1976; Hanukoglu et al, 1977), monkeys (Billiar et al, 1975) and humans (Bixo, 1997). This pathway is illustrated in Figure 1.2. In this pathway, progesterone is converted to $5\alpha$-dihydroprogesterone ($5\alpha$-DHP) by $5\alpha$-reductase, which reduces the double bond between C4-C5. This step of conversion is not reversible (Karavolas et al, 1976). $5\alpha$-dihydroprogesterone is then converted by $3\alpha$-hydroxysteroid dehydrogenase to allopregnanolone ($3\alpha$, $5\alpha$-tetrahydroprogesterone, ALLO) by changing the ketone group on C3 to an alcohol group. This conversion is reversible (Li et al, 1997).

Progesterone and its metabolites have region-specific distributions in the brain. When injected i.v. in the $\mu$g/kg range in rats, both progesterone and $5\alpha$-DHP preferentially accumulate in the hypothalamus and anterior pituitary, but not in the cerebral cortex. In those regions in rats, most progesterone is metabolized to $5\alpha$-DHP within 10 minutes, $5\alpha$-DHP is then metabolized to allopregnanolone within 30 minutes (Karavolas et al, 1976).
Figure 1.2 Progesterone 5α reduction pathway. Adapted from Zhong (2015).
1.7 Past Studies of the Anti-seizure effects of Progesterone and its Metabolites

A number of past studies have looked at the anticonvulsant effects of progesterone, 5α-DHP and ALLO. In this section, clinical studies will be reviewed first and then animal studies will be discussed.

1.7.1 Clinical Studies

1.7.1.1 Progesterone

As an established contraceptive, progesterone has many commercially available analogs and formulations. Each of those has a slightly different spectrum of activity and potency. This has greatly facilitated the study of progesterone.

One of the first attempts to treat drug intractable epilepsy with a form of progesterone occurred in an 8 years old girl, who has frequent seizures prior to her menstrual period (Zimmerman et al, 1973). Zimmerman et al. believed that it would be rational to stop her menstrual cycle and thus to stop her seizures. Medroxyprogesterone acetate (MPA), a potent contraceptive with the brand name Provera, was prescribed in both oral form and intramuscular injection. The girl remained seizure free during her 4-month treatment period (Zimmerman et al, 1973).

In the years after Zimmerman’s first case report, many other open-label add-on clinical trials was conducted in women with seizures. These are summarized in Table 1.2. Progesterone-like compounds were given in the luteal phase of the menstrual cycle to mimic the normal physiological surge in the hormone. Most clinical trial reported a reduction in seizure frequency with one notable exception, which was a clinical trial conducted by Dana-Haeri & Richens (1983). In the Dana-Haeri & Richens study, noresthisterone was prescribed as the progesterone-
like compound. Noresthisterone is more potent than progesterone itself in terms of activating the progesterone nuclear receptors. Noresthisterone, however, was not effective. This failure suggests the potential lack of the role of progesterone nuclear receptor in producing progesterone’s anticonvulsant effects.

A full-scale double-blind, placebo-controlled randomized clinical trial (RCT) – a “gold standard” trial – was not done until recently. Herzog et al.’s recent trial recruited 294 patients with intractable partial seizures, with or without catamenial exacerbation (Herzog et al., 2012). In this well controlled trial, they failed to observe significant differences in responding between the treatment and control groups. A post hoc analysis, however, revealed that those with severe perimenstrual exacerbations (C1) did receive benefits from progesterone therapy.

Subsequently, a smaller double-blind, placebo-controlled clinical study conducted by Najafi’s at al. (2013) group showed a statistically significant reduction in seizure frequency in the progesterone treated group.

1.7.1.2 Allopregnanolone and Ganaxolone

Allopregnanolone has a commercially available analog, ganaxolone, whose chemical structure differs from allopregnanolone by one methyl group on C3 (Hogenkamp et al, 1997). Since ganaxolone could be patented, it is being developed as a new anti-seizure drug. Clinical trials have therefore been conducted, but with ganaxolone instead of allopregnanolone.

Table 1.3 presents a summary of the clinical trials using ganaxolone. All of the findings have been positive, showing that subjects achieve better seizure control while on ganaxolone. Since both ganaxolone and allopregnanolone are potent GABA-A receptor agonists (Hogenkamp et al, 1997), it is not surprising that their common side effects have included fatigue and
Table 1.2 Progesterone and its analogs as anticonvulsants in clinical studies (modified and adapted from Zhong, 2015). RCT: randomized clinical trial.

<table>
<thead>
<tr>
<th>Author</th>
<th>Patient</th>
<th>Seizure Type(s)</th>
<th>Medications</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dana-Haeri &amp; Richens (1983)</td>
<td>Double-blind, placebo-controlled RCT N=9, 20-30 y/o</td>
<td>Tonic-clonic seizures and/or partial seizures with catamenial exacerbation</td>
<td>On day 5-21 of each menstrual cycle, the patient will receive either 1. 5mg Noresthisterone, tid 2. 350ug Noresthisterone, tid 3. Placebo</td>
<td>No decrease in seizure frequency</td>
</tr>
<tr>
<td>Mattson et al (1984)</td>
<td>Open clinical trial N=14, adult women</td>
<td>13 patients with complex partial seizures 1 patient with absence seizures</td>
<td>Prior medications plus 1. Provera® (in 8 patients, 10 mg/q2d, p.o.) 2. Depo-Provera (in 6 patients, 120-150 mg i.m.)</td>
<td>39% reduction in seizure frequency (3 patients withdrew from study) Adverse effects: amenorrhea, spotting</td>
</tr>
<tr>
<td>Backstrom. et al (1984)</td>
<td>Open clinical study N=7, women, 22-43 y/o</td>
<td>Complex partial seizures with one distinct focus, selected for greater than 1 epileptic discharge per 5 min of EEG recording</td>
<td>Prior medications plus Progesterone, iv 0.5-3.0 mg bolus plus 4-12 mg/hr infusion to achieve luteal phase progesterone levels (29 ng/ml)</td>
<td>Significant reduction in frequency of epileptic discharges in 4 out of 7 patients</td>
</tr>
<tr>
<td>Herzog (1986)</td>
<td>Open clinical trial N=8, women, 16-41 y/o</td>
<td>Complex partial seizures (catamenial pattern)</td>
<td>Prior medications plus 50-400 mg progesterone bid during periods of high seizure frequency - achieved luteal phase levels of 5-25 ng/ml</td>
<td>68% reduction in seizure frequency Adverse effects: transient fatigue, depression in 50% of patients</td>
</tr>
<tr>
<td>Herzog (1995)</td>
<td>Open clinical trial N=25, women, 18-40 y/o</td>
<td>Complex partial seizures 13/25 patients also experienced secondarily generalized seizures (catamenial pattern)</td>
<td>Prior medications plus 200 mg progesterone lozenges tid during periods of high seizure frequency - achieved luteal phase levels of 5-25 ng/ml</td>
<td>55% reduction in seizure frequency Adverse effects: asthenia, depression (2/25 patients)</td>
</tr>
<tr>
<td>Herzog (1999)</td>
<td>Open clinical trial N=15, women from 1995 trial who remained on cyclic progesterone therapy</td>
<td>Complex partial seizures (catamenial pattern)</td>
<td>Prior medications plus 200 mg progesterone lozenges tid during periods of high seizure frequency - achieved luteal phase levels of 5-25 ng/ml</td>
<td>62% reduction in seizure frequency</td>
</tr>
<tr>
<td>Herzog et al (2012)</td>
<td>Double-blind, placebo-controlled, phase III, multicenter RCT, N= 294, women, 13-45 y/o</td>
<td>Intractable partial seizures (catamenial and non-catamenial pattern)</td>
<td>Prior medication plus 200mg progesterone lozenges tid on day 14-21 of menstrual cycle</td>
<td>No significant difference in responding rates between treatment and control groups, post hoc analysis revealed that the level of perimenstrual exacerbation is a significant predictor of responding rate</td>
</tr>
<tr>
<td>Najafi et al. (2013)</td>
<td>Double-blind RCT, N=38 women, 18-45 y/o</td>
<td>Complex partial seizures, secondarily generalized seizures, or primary generalized seizures (catamenial pattern)</td>
<td>Prior medications plus 40mg progesterone tablets (Mejestro) BID on day 15-25 of menstrual cycle</td>
<td>81% reduction in seizure frequency</td>
</tr>
<tr>
<td>Motta et al (2013)</td>
<td>RCT, 36 women, 20-40 y/o</td>
<td>Primary generalized tonic-clonic seizures, complex/simple partial seizures with and without generalized seizures, myoclonic seizures with primary generalized tonic-clonic seizures (catamenial pattern)</td>
<td>Prior medications plus 25mg progesterone sublingual tablets BID on day 16-25 of menstrual cycle</td>
<td>26/36 patients experienced a reduction of seizures</td>
</tr>
</tbody>
</table>
somnolence. It is worth noting that, in ganaxolone trials, both male and female subjects have been recruited - whereas the progesterone trials have been female only. This difference may reflect the lack of hormonal effects of allopregnanolone (Rapkin et al, 1997).

1.7.2 Animal Studies

1.7.2.1 Progesterone

Animal studies have demonstrated the anticonvulsant effects of progesterone in a variety of different animal seizure models. These studies are summarized in Table 1.4.

Selye (1942) was perhaps the first to report the anticonvulsant effects of progesterone in a study using the PTZ model in immature male rats. Later, these anticonvulsant effects were confirmed by other researchers, using both male and female animals, and in several different animal models, including the amygdala-kindling model (Mohammad et al, 1998; Lonsdale & Burnham, 2003; Lonsdale et al, 2006), the hippocampal-kindling model (Jeffrey et al, 2014), the PTZ model (Craig, 1966; Kokate et al, 1999; Reddy et al, 2004; Akula et al, 2009), and the kainic acid model (Frye & Scalise, 2000). Most studies have reported an ED50 higher than 50mg/kg, and have also reported sedation as a common side effect.

Progesterone has also showed pro-seizure effects in one animal model, the WAG/Rij model of general absence seizures. In this model, 20mg/kg of progesterone increased the number and duration of the spike-wave discharges (Van Luijtelaar et al, 2001). This pro-seizure action was probably due to a progesterone metabolite, not to progesterone itself; since the effect was attenuated by blocking the 5α hydroxylation pathway with finasteride (Van Luijtelaar et al, 2003). As noted above, ALLO enhances GABA-A related chloride flux (Hogenkamp et al, 1997), and GABA agonists are known to exacerbate absence seizures (Burnham, 2006).
### Table 1.3 Ganaxolone, an allopregnanolone analog, as an anticonvulsant in clinical studies.

<table>
<thead>
<tr>
<th>Author</th>
<th>Patient</th>
<th>Seizure Type(s)</th>
<th>Medications</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laxer et al. (2000)</td>
<td>Double blind</td>
<td>Complex partial seizures with and without secondary generalization</td>
<td>Ganaxolone 500mg tid on day 1 with food 625mg tid on day 2-8 with food • Medication given with other AEDs blood concentration below 25% therapeutic level</td>
<td>62.5% subjects in treatment group experienced seizure reduction compared to 29.2% in control group</td>
</tr>
<tr>
<td>Kerrigan et al. (2000)</td>
<td>Open label, add-on trial</td>
<td>Infantile spasms</td>
<td>Ganaxolone, titrated to 36mg/kg/d or max. tolerated dose over 4 weeks, Maintained for 8 weeks, then discontinued by tapering</td>
<td>Reduced spasm frequency by 50-33%</td>
</tr>
<tr>
<td>Pieribone et al. (2007)</td>
<td>Pilot, open label, dose escalation study</td>
<td>Seizures not controlled by at least 2 conventional AEDs</td>
<td>Ganaxolone oral suspension, 1mg/kg BID to 12mg/kg TID, maintained over 8 weeks</td>
<td>25% subjects experienced &gt;50% seizure reduction, 13% subjects experienced moderate (&gt;25%, but &lt;50%) seizure reduction Adverse Effects: Somnolence</td>
</tr>
<tr>
<td>Sperling et al. (2017)</td>
<td>Double blind, placebo controlled RCT, N=147,18-49 y/o</td>
<td>Uncontrolled partial-onset seizures</td>
<td>Ganaxolone 1500mg/day, Treatment period = 10 weeks (2 weeks of titration + 8 weeks of maintenance)</td>
<td>Reduced average numbers of weekly seizures, mean percentage change in seizure frequency is -17.6% in the treatment group Adverse effects: dizziness, fatigue, somnolence</td>
</tr>
</tbody>
</table>
Table 1.4 Progesterone as an anticonvulsant in animal studies. (modified and adapted from Zhong, 2015). PTZ: pentylenetetrazole, SQ: subcutaneous, IP: intraperitonial

<table>
<thead>
<tr>
<th>Author</th>
<th>Subjects</th>
<th>Seizure Model</th>
<th>Treatment</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Selye (1942)</td>
<td>White rats, male, immature, 45g</td>
<td>PTZ 2 doses of ~70 mg/kg each, SQ</td>
<td>Progesterone (110 mg/kg, IP)</td>
<td>Increases seizure threshold Adverse effects: sedation, anesthesia</td>
</tr>
<tr>
<td>Craig (1966)</td>
<td>Swiss-Webster mice, male and female, 20-30g</td>
<td>PTZ 85 mg/kg, SQ</td>
<td>Progesterone (0-1000 mg/kg, PO)</td>
<td>Suppresses seizures ED_{50} = 200 mg/kg Adverse effects: sedation, motor impairment</td>
</tr>
<tr>
<td>Mohammad et al (1998)</td>
<td>White rats, male, 260-300g</td>
<td>Amygdala kindling</td>
<td>Progesterone (0, 10, 30, 60, 75, 90 mg/kg, IP)</td>
<td>Suppresses generalized seizures at 75 mg/kg Adverse effects: sedation</td>
</tr>
<tr>
<td>Kokate et al (1999)</td>
<td>NIH Swiss mice, male, 25-30g</td>
<td>PTZ, 85 mg/kg, SQ</td>
<td>Progesterone (50-200 mg/kg, IP)</td>
<td>Suppresses clonic seizures ED_{50} = 94 mg/kg</td>
</tr>
<tr>
<td>Frye &amp; Saclise, (2000)</td>
<td>Ovariectomized Long-Evans rats, 55 days old upon arrival</td>
<td>Kainic acid, 32mg/kg, SQ</td>
<td>Progesterone (0, 4, 8 mg/kg, SQ)</td>
<td>Decreases the duration of partial and full seizures at 4 and 8 mg/kg</td>
</tr>
<tr>
<td>Van Luijtelaar et al. (2001)</td>
<td>WAG/Rij mice, female</td>
<td>WAG/Rij mice, genetic absence model</td>
<td>Progesterone (20mg/kg, 30mg/kg, IP)</td>
<td>Increases the number and duration of spike-wave discharge</td>
</tr>
<tr>
<td>Lonsdale &amp; Burnhan (2003)</td>
<td>Wistar rats, female, 210-260g</td>
<td>Amygdala kindling</td>
<td>Progesterone dose-response (0, 10, 20, 40, 120, 160 mg/kg, SQ)</td>
<td>Suppresses generalized seizures (ED_{50} = 103mg/kg) and focal seizures (Rmax = 20%). Suppresses generalized seizures at 100% 20min post-treatment Adverse effects: sedation</td>
</tr>
<tr>
<td>Reddy et al (2004)</td>
<td>Adult C57BL6/129SvEv mice, male and female, 25-30g</td>
<td>PTZ, 85 mg/kg, SQ</td>
<td>Progesterone (10-150 mg/kg, IP)</td>
<td>Suppresses clonic seizures male: ED_{50} = 106 mg/kg female: ED_{50} = 70 mg/kg Adverse effects: sedation, motor impairment</td>
</tr>
<tr>
<td>Lonsdale et al (2006)</td>
<td>Wistar rats, male, 300-400g</td>
<td>Amygdala kindling</td>
<td>Progesterone dose-response (0, 40, 80, 120, 160 mg/kg, SQ)</td>
<td>Suppresses generalized seizures (ED_{50} = 65.3mg/kg) and focal seizures (ED_{50} = 114mg/kg). Suppresses generalized seizures at 100% and suppresses of focal seizures at 37.5% at 40min post-treatment Adverse effects: sedation</td>
</tr>
<tr>
<td>Akula et al (2009)</td>
<td>Albino mice, Laka strain, mice, 22-30g</td>
<td>PTZ i.v. timed infusion</td>
<td>Progesterone (20-80mg/kg, SQ)</td>
<td>Increases threshold for tonic extensor seizure by 50% at 30mg/kg</td>
</tr>
<tr>
<td>Jeffrey et al. (2014)</td>
<td>C57 black mice, male, 6-10 months</td>
<td>Hippocampal kindling</td>
<td>Progesterone (10, 35, 100, 160mg/kg)</td>
<td>Suppresses generalized seizures and reduce the duration of focal seizures in a dose-dependent manner</td>
</tr>
<tr>
<td>Zhong (2015)</td>
<td>Wistar rats, female</td>
<td>Amygdala kindling</td>
<td>Progesterone (0-160mg/kg, SQ)</td>
<td>Only suppresses generalized seizures in 20% subjects at 100mg/kg and above, adverse effect: ataxia, respiratory depression</td>
</tr>
</tbody>
</table>
1.7.2.2 Allopregnanolone (ALLO)

Like progesterone, allopregnanolone has also demonstrated anticonvulsant activity in a number of different animal seizures models. Table 1.5 summarizes these studies. The reported ED50s have varied, but most studies have reported ED50s below 20mg/kg - lower than the ED50s reported for progesterone. Side effects, especially sedation, however, have also been reported even at these lower doses (Table 1.5).

As indicated by Table 1.5, allopregnanolone suppresses generalized seizures in the PTZ model (Kokate et al, 1994; Reddy et al, 2004), the kainic acid model (Frye & Scalise, 2000), and the 6Hz model (Kaminski et al, 2004). It also suppresses the secondarily generalized seizures in the rat amygdala kindling model (Lonsdale et al, 2006; Lonsdale & Burnham, 2007; Zhong, 2015) and the mouse hippocampal kindling model (Jeffrey et al, 2014).

However, allopregnanolone has not proven to be effective in all of the models of generalized seizures. For example, it has failed in the maximal electroshock model (Kokate et al, 1994). It also exacerbates the absence seizures modeled by the WAG/Rij rats (Budziszewska et al, 1999).

The results related to the suppression of limbic focal seizures (“afterdischarges”) in kindled subjects have been more mixed. Kindled limbic focal seizures model drug-resistant complex partial seizures (Albright & Burnham, 1980). Thus, suppression of the kindled focus is of great interest to drug development. Jeffrey et al. (2014) reported that allopregnanolone produced a reduction in the duration of focal hippocampal afterdischarges in mice. Lonsdale and Zhong, who use a stricter criterion and scored focal seizures as “present” or “absent”, reported no suppression in amygdala-kindled rats (Lonsdale et al, 2006; Lonsdale & Burnham, 2007), or less than 40% of suppression at the highest dose (Zhong, 2015). Jeffrey et al. would have
reported only shortening but no suppression of focal seizures if they had used the criteria of Lonsdale and Zhong.

1.7.3 5α-Dihydroprogesterone (5α-DHP)

The anti-seizure effects of 5α- DHP are the major focus of this thesis. The anti-seizure effects of 5α -DHP was first described by Landgren et al (1987). Before work began in the Burnham laboratory, however, there had been no other research in this area. There was already preliminary evidence, however, to suggest that 5α- DHP might be active.

**Preliminary Evidence from the Progesterone Studies** It had been known for some time that the anti-seizure effects of progesterone were largely mediated by its metabolites. Part of the evidence came from animal studies. In animal studies, when the 5α metabolism is blocked by finasteride, the anticonvulsant effects of progesterone are nearly abolished (Frye, 1998; Kokate et al, 1999). Lonsdale had also noted that there was a delay in the onset of anticonvulsant action after progesterone administration (Lonsdale & Burnham, 2003). Progesterone has a high oil/water partition coefficient and can easily cross the blood-brain barrier to reach its site of action. This delay suggested that some metabolism might be taking place before the compound became active (Lonsdale & Burnham, 2003).

There was also a clinical case study showing that a woman who had good seizure control on progesterone, relapsed when the doctor prescribed her finasteride that blocks 5α metabolism. Finasteride was later removed from her treatment, and she achieved good seizure control once more (Herzog & Frye, 2003).
Table 1.5 Allopregnanolone (ALLO) as an anticonvulsant in animal studies. PTZ: pentylenetetrazole, MES: maximal electroshock SQ: subcutaneous, IP: intraperitoneal.

<table>
<thead>
<tr>
<th>Author</th>
<th>Subjects</th>
<th>Seizure Model</th>
<th>Treatment</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kokate et al. (1994)</td>
<td>NIH Swiss mice, male, 25-30g</td>
<td>PTZ, 80mg/kg, SQ MES</td>
<td>ALLO (-up to 100mg/kg, IP)</td>
<td>ED\textsubscript{50}=13.7mg/kg in PTZ model, Not effective against MES-induced seizures at any dose. Adverse effect: sedation, motor toxicity (TD\textsubscript{50} = 42.0mg/kg)</td>
</tr>
<tr>
<td>Budziszewska et al. (1999)</td>
<td>WAG/Rij rats 10022363</td>
<td>WAG/Rij, genetic absence model</td>
<td>ALLO (5-20mg/kg, IP)</td>
<td>Increases number and duration of spike wave discharges</td>
</tr>
<tr>
<td>Frye &amp; Scalise, (2000)</td>
<td>Ovariectomized Long- Evans rats, 55 days old upon arrival</td>
<td>Kainic acid, 32mg/kg, SQ</td>
<td>ALLO (0, 4, 8mg/kg, SQ)</td>
<td>Increases latency to initial partial seizures, decreases number and durations of full and partial seizures</td>
</tr>
<tr>
<td>Kaminski et al. (2004)</td>
<td>NIH Swiss mice, male, 25-30g</td>
<td>6Hz corneal stimulation</td>
<td>ALLO (2-100mg/kg, IP)</td>
<td>ED\textsubscript{50}= 14.2mg/kg against 6Hz seizures</td>
</tr>
<tr>
<td>Reddy et al. (2004)</td>
<td>Adult C57BL6/129SvEv mice, male and female, 25-30g</td>
<td>PTZ, 85mg/kg, SQ</td>
<td>ALLO (0-100mg/kg, IP)</td>
<td>Suppresses of clonic seizures, male: ED\textsubscript{50}=15mg/kg, female: ED\textsubscript{50}=7mg/kg</td>
</tr>
<tr>
<td>Lonsdale et al. (2006)</td>
<td>Wistar rats, male, 300-400g</td>
<td>Amygdala kindling</td>
<td>ALLO dose-response (0, 1.25, 5, 10, 20, or 30 mg/kg, IP)</td>
<td>Suppresses generalized seizures (ED\textsubscript{50}=15.2mg/kg), Adverse effect: ataxia (TD\textsubscript{50}=26.6mg/kg)</td>
</tr>
<tr>
<td>Lonsdale &amp; Burnham, (2007)</td>
<td>Wistar rats, female, 240-265g</td>
<td>Amygdala kindling</td>
<td>ALLO dose-response (0, 0.625, 1.25, 2.5, 5.0, 7.7 10, or 20 mg/kg, SQ) THP time-response (10, 20, 30, 60, 120, 240 min, SQ)</td>
<td>Suppresses generalized seizures (ED\textsubscript{50}=1.1mg/kg), Suppresses generalized seizures at 75% from 15 to 60min post-treatment Adverse effect: ataxia (TD\textsubscript{50}=8.6mg/kg)</td>
</tr>
<tr>
<td>Jeffrey et al. (2014)</td>
<td>C57 black mice, male, 6-10 months old</td>
<td>Hippocampal kindling</td>
<td>ALLO (1, 3.5, 10, 30mg/kg, IP)</td>
<td>Suppresses generalized seizures and reduces the length of focal seizures, completely suppresses generalized seizure at 10 and 30mg/kg</td>
</tr>
<tr>
<td>Zhong (2015)</td>
<td>Wistar rats, female</td>
<td>Amygdala kindling</td>
<td>ALLO (0-20mg/kg, SQ)</td>
<td>Suppresses generalized seizures (ED\textsubscript{50}=24mg/kg), Adverse effect: ataxia (TD\textsubscript{50}=27mg/kg)</td>
</tr>
</tbody>
</table>
**Direct Evidence from the 5α-DHP Studies**  Landgren et al. (1987) reported that 5α-DHP reduced the frequency of epileptiform interictal spiking in penicillin-induced seizures in adult ovariectomized cats. Lonsdale then began to research the anti-seizure properties of 5α-DHP. Lonsdale et al. (Lonsdale & Burnham, 2003; Lonsdale et al, 2006) found that 5α-DHP suppressed both amygdala-kindled focal seizures and secondarily generalized seizures at non-sedating doses. These effects were seen in both male and female rats.

Not all subsequent studies have supported Lonsdale’s work. Jeffrey (2014) reported no effects of 5α-DHP on focal seizures in hippocampal-kindled mice. It worth noting that Jeffrey’s study and Lonsdale’s study differed in the animal species, the kindling site, and the route of administration, disallow researchers to make meaningful comparisons. Zhong (2015), however, working in the Burnham laboratory, also failed to see this strong anticonvulsant effects of 5α-DHP. While suppression of generalized seizures was seen (at higher doses), effects against the kindled amygdala focus were weak. The extrapolated ED50 against focal seizures was 10 times greater than in Lonsdale’s study (Zhong never reached 50% suppression, even at the maximum soluble doses.) (Zhong, 2015).

1.8 5α-DHP as a Promising AED for Complex Partial Seizures

5α-DHP showed strong anticonvulsant activity in Lonsdale’s study (2008), both against generalized seizures and focal seizures in the amygdala-kindled rats. These were seen at non-sedating doses. The focal seizures in amygdala-kindled rats model drug-resistant partial seizures in humans. A compound that could treat drug-resistant seizures without sedation could be an asset in the clinical setting.
The control of complex partial seizures is one of the most important problems in the field of epilepsy therapy. Patients with uncontrolled seizures often have a low quality of life (Fazel et al, 2013; Hamid et al, 2014). A drug effective against complex partial seizures would represent a very great advance.

For these reasons, the present study was designed to continue the work of Lonsdale and Zhong as it related to the anti-seizure effects of 5α-DHP. It was hoped that we could resolve the conflicting results, and replicate Lonsdale’s original findings. If we could find that 5α-DHP was active and evolve an effective analog of 5α-DHP, there is every reason to believe that it would be carried forward into full preclinical animal testing, and, eventually, clinical trials.

1.9 Objectives and Research Hypothesis

The current study continued the investigation of the anti-seizure effects of 5α-DHP, using different routes of administration, solvents and post-injection time points.

Based on the work of Lonsdale, we hoped and hypothesized that 5α-DHP would be effective against both generalized seizures and focal seizures at non-sedating doses.
Chapter 2 General Methods

The research protocol for the present study was approved by the Animal Care Committee of the Faculty of Medicine of the University of Toronto, and follows the guidelines laid down by the Canadian Committee for Animal Care.

2.1 Subjects

Adult female Wistar rats, 50 days old, were obtained from a commercial breeder (Charles River, Quebec, Canada) and housed individually in 19 x 15 x 9 inches transparent, plastic cages. The animals were provided with food (rat chow) and water ad libitum. The vivarium was kept at a constant temperature (21°C) and maintained on a 12-h light/dark cycle (lights on at 07:00).

2.2 Drugs and Vehicle

5α-DHP was obtained from Toronto Research Chemical (Toronto, ON). β-cyclodextrin was obtained from AK Scientific Inc. (Union City, CA). Benzyl alcohol was obtained from Fisher Chemicals (Toronto, ON). Benzyl benzoate and cottonseed oil were obtained from Acros Organics (Toronto ON).

5α-DHP was dissolved at 3.2mg/mL in 45% (w/v) β-cyclodextrin in sterilized saline in the experiment described in Chapter 3, and 2.5mg/mL in 45% (w/v) β-cyclodextrin) in sterile filtered water in the experiment described in Chapter 5. Those solutions were prepared one day prior to the drug testing trials and sonicated overnight to promote solubility. They were then diluted to appropriate concentrations according to dosage on the day of testing.
In the Chapter 4 experiment, a different solvent was used, the “benzyl vehicle”. Fifteen mg of 5α-DHP was dissolved first in a mixture of 150µL benzyl alcohol and 150µL benzyl benzoate. This solution was then heated in a 60°C water bath for 10 minutes to promote solubility. Once 5α-DHP was completely dissolved, 700µL of cottonseed oil were added to dilute the final concentration to 15mg/mL. The solution was prepared fresh on the day of drug testing.

A vehicle control group, matched in volume to the volume of the largest drug dose, was used given as part of each dose-response study.

2.3 Procedure for Stereotaxic Surgery

One week after arrival from the breeding farm, during which the animals were handled daily, the subjects were surgically implanted with a chronic bipolar electrode in the right basal lateral amygdala.

For surgery, the subjects were anesthetized with isoflurane (induction 5%, maintenance 2.5%). Once the animal was fully anesthetized, the fur on the scalp was shaved. The skin was sanitized with two alternating wipes of alcohol and betadine. Eye ointment was applied to prevent corneal drying. The rat’s body was placed on a heating pad set to 39°C, covered with surgical drape, and injected with ketoprofen (3mg/kg, SQ) to minimize post-surgical discomfort.

The head was rigidly fixed on a stereotaxic instrument (DKI900, David Kopf Instruments, Tujunga, USA). An incision was made then along the midline of the scalp, the skull was cleaned, and holes were drilled for the electrode and screws. The screws were first affixed to the skull, and then a bipolar electrode (MS303/1; Plastics One, Roanoke, VA, U.S.A.) was lowered into
place using the following coordinates relative to bregma and derived from the atlas of Paxinos and Watson (AP: 2.5mm, ML: 5.0mm, DV: -8.7mm from bregma). The incisor bar was adjusted to provide a flat skull (bregma and lambda were at the same height) (Paxinos & Watson, 2009). The whole assembly was cemented into place with dental acrylic (Repair Material, Dentsply, York, P.A, U.S.A). The entire surgery was carried out using sterile techniques. An additional injection of ketoprofen (3mg/kg SQ) was given 24 hours, 48 hours and 72 hours following the surgery.

2.4 Procedure for Kindling

Kindling was begun 1 week (minimum) after surgery. The kindling stimulus was a 1-s train of 400µA (peak-to-peak) 60 Hz biphasic square-wave pulses, and was produced using a Grass Model S-88 stimulator in series with two PSIU 6 stimulus isolation units (Grass Instruments, Quincy, MA, USA). EEG was recorded 30 seconds before, and after each stimulation until the last `spike` in the EEG. All subjects were kindled to a criterion of 15 stage V seizures based on the Racine scale: stage I, facial clonus; Stage II, head nodding; Stage III, forelimb clonus; Stage IV, rearing; Stage V, loss of postural control (Racine, 1972).

2.5 Procedures for Afterdischarge Threshold (ADT) Determination and Stability Testing

Two days after reaching criterion, the afterdischarge threshold (ADT) of each subject was assessed using the ascending-series technique (Pinel, 1976). Stimulation was started at 60µA, and continued at 2 minute intervals with increments of 20µA. The current that first produced an afterdischarge that was more than 2s in duration was considered the ADT of the subject. After ADT determination, all subjects were then stability tested at 120% of ADT every second day.
The rats that had five consecutive stage V seizures were considered stable and proceeded to the next stage of testing.

2.6 Procedure for Drug Testing and Toxicity Evaluation

The procedures for drug testing varied between experiments and are described in the Specific Methods section in each experimental chapter.

Immediately before each seizure test, the animals were assessed for toxicity using Loscher’s ataxia scale, defined as follows: Stage I) slight ataxia in hind legs, no decrease in abdominal muscle tones; Stage II) more pronounced ataxia, slight decrease in abdominal muscle tone, with flat body position; Stage III) further increase in ataxia and more marked decrease in abdominal muscle tone, with the flat body posture more pronounced, splayed hind legs, crawling during forward locomotion; Stage IV) marked ataxia, animal loses balance during forward locomotion, total loss of abdominal muscle tone, with flat body posture, splayed hind legs, and dragging during forward locomotion; and Stage V) very marked ataxia with frequent loss of balance during forward locomotion, total loss of abdominal muscle tone (Loscher et al, 1987).

Stage 2 and above were considered to indicate “toxicity present” (Lonsdale et al., 2006; Lonsdale & Burnham, 2007).

2.7 Electrode Placement Verification

Following the completion of drug testing, subjects were deeply anesthetized with isoflurane, and then perfused transcardially with physiological saline, followed by 3% potassium
ferricyanide and 3% potassium ferrocyanide trihydrate in formalin. The electrode tip was marked by a cathodic direct current pulse of 300uA for 20 seconds for Prussian blue staining.

Brains were extracted and post-fixed overnight in formalin, and then stored in 0.1% sodium azide in 25% sucrose solution for at least 2 days before slicing.

All brains were sliced coronally at a thickness of 40um using a cryostat (Jung CM3000, Leia Inc., Canada) at -21°C.

The sections were stained using the cresyl violet method. Slides were immersed in solutions in the following order: 100% ethanol for 15 minutes, 95% ethanol for 2 minutes, 70% ethanol for 2 minutes, distilled water for 2 minutes, 0.5% cresyl violet and 0.3% acetic acid in distilled water for 2 minutes, distilled water for 2 minutes, 70% ethanol for 2 minutes, 95% ethanol with 0.8% acetic acid for 2 minutes, 95% ethanol for 2 minutes. The slides were then immersed twice into 100% ethanol for 2 minutes, each time with a fresh solution. The slides were finally soaked in xylene twice for five minutes each. A cover slip was fixed onto each slide using CytosealTM (Thermo Fisher Scientific, USA).

Stained sections were examined under a light microscope and compared to figures in the Paxinos and Watson atlas. Only subjects with correct placements were included in the subsequent data analysis.

2.8 Data Analysis

All data analysis was done using GraphPad Prism 6.0 unless otherwise indicated. Dose-response and time-response data were graphed for each study. Both graded results and quantal results were presented.
Chapter 3. The Anticonvulsant Effects of $5\alpha$-Dihydroprogesterone

Administered via the Subcutaneous Route in Amygdala-kindled Rats

3.1 Rationale

Previous studies on $5\alpha$-DHP have reported conflicting results. Lonsdale and Burnham (2003) reported an ED50 of 2.9 mg/kg against generalized seizures and of 4.3 mg/kg against focal seizures in the female amygdala-kindled rats. $5\alpha$-DHP was injected via the subcutaneous (SQ) route in this study. Jeffery et al. (2014), however, reported no effect of $5\alpha$-DHP against focal or generalized seizures in hippocampal-kindled mice. Jeffrey tested $5\alpha$-DHP at 5 and 10 mg/kg injected intraperitoneally (IP) in hippocampal-kindled male C57 black mice. Finally, Zhong (2015) reported an ED50 of 13 mg/kg against generalized seizures and 43% suppression of focal seizures at 45 mg/kg in the female amygdala-kindled rats, using the SQ route.

Neither Zhong (2015) nor Lonsdale (2006) observed any signs of ataxia at any dose tested. Jeffrey (2014), however, reported a mild sedating effect of $5\alpha$-DHP at 10 mg/kg.

It is hard to compare Lonsdales’s and Jeffrey’s data since their experiments differ in species, kindling sites, and route of administration. Both Lonsdale and Zhong reported positive results in female rats, however, although their ED50’s varied. The present study was designed to confirm Lonsdale’s and Zhong’s results in female amygdala-kindled rats using the SQ route and to determine our own ED50’s in preparation for a future time-response study.
3.2 Specific Methods

3.2.1 Subjects

Ten adult, female Wistar rats were obtained from Charles Rivers (QC, Canada) at the age of 50 days. Their housing conditions have been described in the General Methods section. This group of animals was reused in the study described in Chapter 4.

3.2.2 Drugs

5α-dihydroprogesterone (5α-DHP) (Toronto Research Chemical, Toronto, ON, Canada) was dissolved at 3.2 mg/mL in a solution of 45% β-cyclodextrin (AK Scientific Inc., Union City, CA) using sterile physiological saline.

Drug solutions were prepared one day prior to drug testing and sonicated in a water bath overnight to promote solubility.

3.2.3 Surgery, Kindling, Stability Testing, Electrode Placement Verifications

All of the above procedures were performed using the procedures outlined in the General Methods section. One week (minimum) following arrival from the breeding farm, the subjects were surgically implanted with an amygdala electrode, and then kindled to 10 Stage Vs according to the Racine scale. Following determination of ADTs and stability testing, they were used for drug testing (below).
3.2.4 5α-Dihydroprogesterone (5α-DHP) Dose-Response Study

After the completion of stability testing, drug testing was begun. The subjects were injected with various doses of 5α-DHP SQ every second day. Six doses (0, 4, 8, 16, and 32 mg/kg) were injected in randomized order. The concentration of 5α-DHP remained constant (3.2 mg/ml), and volume of injection varied with the different doses. Dose 0 mg/kg was a vehicle control with a volume matched to the volume of the largest dose (32mg/kg).

Fifteen minutes after drug injection, the rats were stimulated at 120% of their ADT and the presence or absence of afterdischarge and behavioral seizures were recorded for each rat. The behavioral seizure was considered to be absent if no forelimb clonus, no rearing and/or no falling (Racine stages 3-5) were observed. The focal seizure was considered to be absent if the stimulus was followed by less than 4s of afterdischarge in the EEG.

All experiments were conducted between 13:00 and 16:00h.

3.3 Results

Figure 3.1A presents the quantal dose-response data related to total seizure suppression by 5α-DHP. Within the range of doses tested, 5α-DHP suppressed generalized seizures in only 30% of the subjects even at the highest dose, and it did not suppress focal seizures at any dose. The ED50 for the suppression of generalized seizures was not reached, but would have been higher than 32 mg/kg.

Figure 3.1B presents the graded data related to the strength of the generalized motor seizures at the various different doses. A weak dose-response was seen, with average Racine scores dropping from an average of 5 at the lowest doses to 2.8 ± 0.4 at the highest dose.
Figure 3.2 presents the EEG recording of subject A16-2 in this study. Complete suppression of focal seizure, defined as shortening of ADD to 4 seconds and below in our study was not observed at any dosage. Only a shortening of ADD at the highest dose was observed.

None of the subjects displayed any signs of ataxia at any dose, as rated by the Löscher ataxia scale.

3.4 Discussion

The present study showed weak anticonvulsant effects of 5α-DHP against generalized seizures and no strong anticonvulsant effects of 5α-DHP against focal seizures.

These data appear to contrast with the results of Zhong’s (2015) study, which reported a lower ED50 against generalized seizures and a partial suppression of focal seizures. This discrepancy relative to generalized seizures may result in part because a stricter criterion for generalized seizure suppression was used in the present study – a criterion more consistent with past studies in the literature (Albright and Burnham, 1980). In the present study, generalized seizures were scored as “absent” when stages 3, 4 and 5 were suppressed. In Zhong’s study, generalized seizures were scored as “absent” when stages 4 or 5 were suppressed – but suppression of stage 3 was not required. These results of these two studies’ would be more comparable if we had used Zhong’s criterion. If we had, we would have obtained an 80% suppression of generalized seizures at our dose of 32mg/kg and an ED50 of 25.7mg/kg.
Figure 3.1 Dose-response curve for 5α-Dihydroprogesterone SQ at 15 min (N=10 at each point). A) % of subjects with seizure suppression (generalized seizures with Racine scales Stage 2 or lower are considered suppressed). B) Generalized seizure strength ranked by the Racine scale. A dose-dependent effect against generalized seizures was observed, but there were no rats with complete suppression of focal seizures. No sign of ataxia was observed.
Figure 3.2 EEG recordings from subject A16-2 at different doses of 5α-DHP. *Only at the highest dosage given in this study, shortening, but not complete suppression, of afterdischarge was observed. No ataxia was observed at any dose.*
Our results would still differ from Zhong’s, however, in that we did not see any suppression of focal seizures. Our failure to see the suppression of focal seizures might have resulted from several different reasons. First, we used a lower maximum dose of 5α-DHP in our dose-response study. We didn’t have a 45mg/kg dose. Finally, the difference in effects on focal seizures may reflect a difference in absorption. In the present study, subjects had been injected repeatedly. Chronic SQ injection may cause scar tissue formation at the injection site, which might lead to inconsistent absorption and a higher ED50.

Our dose-response data are also in disagreement with Lonsdale and Burnham (2003), who reported very low ED50s of 2.9 mg/kg for generalized seizures and 4.3 mg/kg for focal seizures, without ataxia. The reason for this difference is not clear, and will be discussed in the General Discussion.

Finally, our data do not agree with Jeffery et al. (2014) who failed to find any effect in focal or generalized seizures in hippocampal-kindled mice. Like Zhong and Lonsdale, we found suppression of generalized convulsive seizures with 5α-DHP. Thus Jeffery’s data seem to be in the minority.
Chapter 4. The Anticonvulsant Effects of 5α-Dihydroprogesterone in Amygdala-kindled Rats: Trouble-Shooting Route of Drug Administration and Solvent / Time-Response Study via the Intraperitoneal Route

4.1 Rationale

Following our dose-response study (Chapter 3), we next began a time-response study. We first analyzed our technical procedures, and decided to make changes to our route of administration and to our solvent.

4.1.1 Inconsistent Absorption following SQ Injections – Change to the IP Route

We first decided to switch from the SQ route to the intraperitoneal (IP) route. Previous studies on SQ injections had shown large differences in absorption. These were related to the site of injection, depth of injection, body fat, etc. (LeWitt, 2004). In particular, based on our informal observations, repeated drug injections via the SQ route can create scar tissue that interferes with drug absorption.

Since our protocol involved repeated injections, we decided to switch to the IP route to avoid this pharmacokinetic problem. The IP route affords a larger number of possible injection sites. The IP route, of course, is subject to first pass effects. We were not sure whether these would occur with 5α-DHP; to the best of our knowledge, no one had attempted DHP via IP route in rats before. We decided to proceed, and take possible first pass effects into account when analyzing our data.
4.1.2 Solubility – Change to the Benzyl Solvent

In our hands (Chapter 3) - and in Zhong’s study (Zhong, 2015) - fairly high doses of 5α-DHP had been required to suppress seizures. These high doses caused problems with solubility, and - once we switched to the IP route - with osmolarity.

With respect to solubility, 5α-DHP is hard to dissolve since it is not very soluble in either water or in oil. Testing of higher doses was impossible in our previous experiments because higher doses could not be kept in solution at the volumes allowed for injection - even with the use of β-cyclodextrin and overnight sonication.

With respect to osmolarity, in our previous experiments (Chapter 3) - and in Lonsdale’s and Zhong’s experiments - β-cyclodextrin had been used to promote the water solubility of 5α-DHP. β-cyclodextrin forms a ring-shaped polymer with a hydrophobic interior and a hydrophilic exterior. The hydrophobic interior encapsulates hydrophobic chemicals such as 5α-DHP, and the hydrophilic exterior interacts with aqueous environment (Brewster and Loftsson, 2002).

When we began IP injections, we found that our 5α-DHP injections were causing our subjects abdominal discomfort. We believed that this was caused by the high osmolarity of the β-cyclodextrin in our drug solutions. At 3.2mg/ml, 5α-DHP in 45 % β-cyclodextrin/physiological saline has an osmolarity of 1375 ± 86 mOsm, which is almost 4 times greater than the osmolarity of body fluid. IP injection of this hypertonic solution not only caused pain in subjects, but we also feared it might alter the course of drug absorption.

Thus, we began to search for a new vehicle. From a literature search, we found the following solvent formulation: benzyl benzoate plus benzyl alcohol plus cottonseed oil (1.5 : 1.5 : 7, v: v: v) (Scholtz et al., 2014). This formulation – which we’ll call the “benzyl vehicle” – proved successful and allowed us to avoid the high osmolarity of β-cyclodextrin and also to
achieve a higher concentration of 5α-DHP (15mg/ml), which allowed smaller injection volumes and made testing of higher doses of DHP possible.

4.2 Methods

4.2.1 Subjects

Adult, female Wistar rats were obtained from Charles Rivers (QC, Canada) at age 50 days. They were housed and maintained as described in the General Methods section. These were the same subjects used in Chapter 3.

4.2.2 Drugs

Every 15mg of 5α-dihydroprogesterone (Toronto Research Chemical, Toronto, ON, Canada) was dissolved at 150uL of benzyl benzoate (Acros Organics, Toronto, ON) and 150uL of benzyl alcohol (Fisher Chemicals, Toronto, ON), heated in a water bath of 60°C for 15 minutes to promote solubility. Once DHP was completely dissolved, 700uL of cottonseed oil (Acros Organics, Toronto, ON) was added to dilute the final concentration to 15mg/mL. The drug solutions were prepared fresh on the day of testing and were cooled to room temperature before injection.

4.2.3 Surgery, Kindling, Stability Testing, Electrode Placement Verifications

As described above, the subjects had been surgically implanted with electrodes, kindled to 10 Stage Vs, and stability and drug tested. Stability testing was repeated before the start of the time-response study. At the end of all testing, they were perfused and the electrode placements were verified histologically. All above procedures were carried out according to the procedures outlined in the General Method section.
4.2.4 5α-Dihydroprogesterone Time-Response Study

Following stability testing, time-response testing was begun. Time-response testing was done at 48-hours (minimum) intervals. The subjects were injected IP with 30mg/kg of 5α-DHP dissolved in the benzyl vehicle. This dose - 30mg/kg - had been the ED75 based on pilot experiments involving the new vehicle. Following injection, the kindling stimulus was applied at 120% of the ADT at various different time points after injection. Only one time point was tested in each animal on each test day, with test days separated by 48 hours (minimum).

Initially, eight post-injection time points were tested in randomized order: 10, 30, 50, 70, 90, 110, 130 and 150 minutes. After gathering N = 4 data points at each of these times, further testing was done at 10 minute intervals near the points of inflection in the time-response curve in order to better define the times of onset and offset of action. Finally, at the end of time-response testing, volume-matched vehicle control injections were done at the times of peak effect:

The presence or absence of afterdischarge(AD) and behavior seizures was recorded at each time point. The behavior seizure was considered to be “absent” if no forelimb clonus, no rearing or no falling was observed (Racine stages 3-5). The focal seizure was considered to be absent if the stimulus was followed by less than 4s of AD in the EEG. Behavioural seizures (if any) were scored according to the Racine scale. Ataxia was rated one minute before stimulation using the Löscher ataxia scale.

All experiments were conducted between the hours of 10:00 to 13:00.

4.3 Results

Time-Response Data The quantal time-response data related to seizure suppression at different post-injection times are presented in Figure 4.1A (% total suppression of generalized
seizures, % total suppression of focal seizures and % with L"oscher ataxia Stage 2 and above). The graded data are presented in Figure 4.1B (average seizure stages, average afterdischarge duration, and average L"oscher scale rating; mean ± S.E.M). Representative EEG recordings are presented in Figure 4.2.

As indicated by the figures, two different peaks of action for DHP were found. The first peak occurred almost immediately after injection and involved suppression of roughly 80% of generalized seizures and 66% of focal seizures (Figure 4.1A). The mean seizure stage was reduced to rank 2 (Figure 4.1B). At this time, however, the seizure suppression was accompanied by strong ataxia, ranking above L"oscher scale 3 (Figure 4.1B). Seizure suppression and ataxia declined to near baseline by about 50 minutes.

A later peak of action occurred at around 120-130 minutes. At this time, only generalized seizures were suppressed. At the time of maximum late effect, around 83.3% of subjects showed generalized seizure suppression (Figure 4.1A), and the average seizure stage was less than 2 (Figure 4.1B). No subjects showed focal seizure suppression, although the AD duration dropped from a baseline average of about 100 seconds to less than 50 seconds (Figure 4.2B). This peak was short-lasting, with an onset at about 110 minutes and offset at about 140 minutes. No ataxia was seen during this period of seizure suppression.

**Benzyl Vehicle Control Data** As a control, the benzyl vehicle, matched in volume, was tested without 5α-DHP. The data related to our vehicle control trials are presented in Table 4.1. Vehicle effects were examined at 15 and 130 minutes post injection, corresponding to the major peaks of action in the time-response study.
Figure 4.1 Time–response curve for 30mg/kg 5α-dihydroprogesterone IP at 30 mg/kg (For generalized seizures, and focal seizures N=6 at 10, 20, 30, 40, 50, 120, 130 140, 150min, N=4 at 70 and 90 minutes; For ataxia, N=10 at 10, 20, 130 minutes, N=9 at 30 minutes, N=8 at 40, 50, 110, 120 minutes, N=6 at 140, 150 minutes, N=4 at 70, 90 minutes). A) % of subjects with seizure suppression and ataxia vs. time. B) Generalized seizures in Racine scales (left-axis), afterdischarge (AD) duration (right-axis), and ataxia in Löscher scale vs. Time. Two peaks of action were observed, one during the first 30 minutes and the other around two hours.
Figure 4.2 Representative EEG recordings around major peaks of anticonvulsant action, DHP in benzyl vehicle. The EEG trace from a well-kindled animals are usually around 100 seconds. A) At 10 minutes post-injection (subject A16-2), it was shortened to 3 seconds. B) At 30 minutes post-injection (subject A16-2), it was shortened to 13 seconds. C) At 130 minutes post-injection (subject A3-2), it was 50 seconds.
Surprisingly we found that at 15 minutes the benzyl vehicle alone suppressed generalized seizures in 71% of animals and focal seizures in 42% of animals. Ataxia was seen in one out of seven animals. At this time, average AD duration dropped from over 100 seconds to less than 50 seconds, the average motor seizure stage dropped from 4-5 to less then 2, and the average Loscher ataxia score was less than 1 (Table 4.1). Except for the ataxia, these effects were similar to the effects seen with the 5α-DHP.

At 130 minutes, no effects on seizure occurrence or strength were seen and there was no ataxia (Table 4.1).

4.4 Discussion

The present study was designed to provide time-response data related to the effects of 5α-DHP on kindled seizures. A new route of administration and a new solvent were used.

Strong anticonvulsant effects were seen at two times: 1) immediately after injection and 2) at about 130 minutes after injection. Both generalized and focal seizures were suppressed at 0-30 minutes, but only generalized seizures were suppressed at 130 minutes. Ataxia was seen at the earlier time point, but not at the later time point.

Surprisingly, the benzyl vehicle alone was as effective as vehicle plus DHP at suppressing generalized seizures at 0-30 minutes, and about half as good at suppressing focal seizures. It also caused much less ataxia. These data raise questions both about the effects of DHP and about the effects of the benzyl vehicle.
Table 4.1 The effect of equal volumes of benzyl vehicle (IP) on seizures and ataxia at 15 and 130 minutes. (N=7 at 15 minutes, and N=6 at 130 minutes, data presented as mean ± S.E.M).

<table>
<thead>
<tr>
<th></th>
<th>15 minutes (N=7)</th>
<th>130 minutes (N=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Quantal Response</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Generalized seizure suppression</td>
<td>71.42</td>
<td>0.00</td>
</tr>
<tr>
<td>% Focal seizure suppression</td>
<td>42.86</td>
<td>0.00</td>
</tr>
<tr>
<td>% Ataxia</td>
<td>14.28</td>
<td>0.00</td>
</tr>
<tr>
<td><strong>Graded Response</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Generalized seizure stage</td>
<td>1.71 ± 0.78</td>
<td>4.33 ± 0.42</td>
</tr>
<tr>
<td>Afterdischarge duration (s)</td>
<td>47.00 ± 22.06</td>
<td>127.0 ± 38.63</td>
</tr>
<tr>
<td>Ataxia (Löscher scale)</td>
<td>0.57 ± 0.43</td>
<td>0.00 ± 0.00</td>
</tr>
</tbody>
</table>
**Early Effects of 5α-DHP** The first question is whether 5α-DHP has anti-seizure effects independent of the benzyl vehicle. At the early time point of 0-30 minutes, several past studies have shown that 5α-DHP causes seizure suppression in the absence of the benzyl vehicle (Lonsdale and Burnham, 2003; Lonsdale et al. 2006; Zhong 2015). Early effects were also seen in our own past work, although they were weak (Chapter 3). It should also be noted that in the present study, 5α-DHP plus the benzyl vehicle was more effective against focal seizures than the benzyl vehicle alone. Thus, it appears that 5α-DHP does have anti-seizures effects at 0-30 minutes in the absence of the benzyl vehicle.

A later chapter in this thesis will explore these early effects of 5α-DHP using the intravenous (IV) route. The IV route allows the use of lower doses 5α-DHP that will stay in solution without the benzyl vehicle.

One clear difference between the present study and past reports relates to the occurrence of ataxia. In contrast to the previous studies of Lonsdale (2006) and Zhong (2015), ataxia was associated with the early effects of 5α-DHP in the present study. Ataxia had not been seen in the earlier studies of Lonsdale and Zhong at this time period. It was also not seen in our own earlier work (Chapter 3).

One might argue that Lonsdale worked at lower 5α-DHP doses, but the Zhong doses were as high as or higher than the doses used in the present study. Alternatively, one might blame the ataxia on the benzyl vehicle, but the benzyl vehicle alone caused ataxia in only one subject. Future studies might investigate the possibility that ataxia results from an interaction of 5α-DHP with the benzyl vehicle, or that the first-pass effect resulting from changing the route of administration from SQ to IP is responsible for the ataxia, perhaps by creating sedative metabolites.
**Late Effects of DHP** The anti-seizure effects seen at 130 minutes have not been previously reported. This is a novel finding. This later onset of anticonvulsant activity is clearly due to 5α-DHP injection – not the benzyl vehicle - since the benzyl vehicle control without 5α-DHP at this point displayed no pharmacological activity. It is interesting that these late effects involved suppression of generalized – not focal - seizures and that they were not associated with ataxia.

These late effects of 5α-DHP had not previously been reported by Lonsdale or by Zhong. The duration of these late effects of 5α-DHP is short and easy to miss, and neither Lonsdale nor Zhong tested 5α-DHP’s effect on seizure at around 130 minutes.

It is very possible that these late effects of 5α-DHP relate not to 5α-DHP itself but to a metabolite. We hypothesize that 5α-DHP would have been metabolized two hours post injection and that the active compound at this time may be a break-down product. This is hard to state with certainty, however, because the time course of 5α-DHP’s metabolism, at this high dosage, has not been measured in the rat. A future study could attempt to establish a time-course for 5α-DHP metabolism in the rat and to determine what compound relates to these late-developing effects.

**Effects of the Benzyl Vehicle** Surprisingly, the benzyl vehicle proved to be almost as strong as 5α-DHP plus the vehicle. This is a novel finding, since anti-seizure effects of the benzyl vehicle had not been reported previously.

The discovery of the anti-seizure effect of the benzyl vehicle opens up a new venue for research. Since this vehicle suppressed almost 50% of focal kindled seizures, a model of drug-resistant complex partial seizures in human, the vehicle might be explored as a possible therapy for complex partial seizures, as well as for secondarily generalized convulsive seizures.
A later chapter in this thesis will explore several components of the benzyl vehicle to search for the active compound in the mixture, and will test the anticonvulsant effects of that compound.
Chapter 5. The Anticonvulsant Effects of Intravenous 5α-Dihydroprogesterone in Amygdala-kindled Rats

5.1 Rationale

In our previous experiment (Chapter 4), the benzyl alcohol solvent showed positive anticonvulsant effects at the early time points. Thus, we could not get a clear indication of the anticonvulsant effects of 5α-DHP at these times. Unfortunately, benzyl alcohol is the only solvent we have found that can keep 5α-DHP in solution at high doses without causing pain in the subjects.

To circumvent this problem, we decided to go back to β-cyclodextrin as a solvent and to administer 5α-DHP intravenously, which would allow us to use lower (soluble) doses. Drugs given intravenously are often effective at lower dosages than drugs given via the other routes. Previous studies had also demonstrated that β-cyclodextrin was a safe and tolerable solvent for intravenous injections (Pitha et al, 1994).

We began with an intravenous dose-response study and then proceeded to an intravenous time-response study. As in previous chapters, the tests were done in amygdala-kindled female rats.

Since pilot studies had suggested that peak responses would occur very soon after intravenous injections, the dose-response study was done with an injection-test interval of 5 minutes.

Our pilot experiments had also demonstrated that, the rats did not experience significant changes in afterdischarge threshold or stability after cannulation surgery (Appendix 1). Thus, in the present experiment, we proceed to drug testing immediately after cannulation surgery, and
without further stability testing. This was necessary because the cannulas stay patent for only a limited amount of time.

5.2 Specific Methods

5.2.1 Drugs and Drug Solutions

5α-dihydroprogesterone was obtained from Toronto Research Chemicals (Toronto, ON, Canada) and was dissolved at a concentration of 2.5 mg/mL in 45% β-cyclodextrin (AK Scientific Inc., Union City, CA) (w/v) in distilled water.

The drug solutions were prepared one day prior to the day of drug testing and sonicated in a warm water bath overnight to promote solubility.

5.2.2 Subjects

A new group of adult, female Wistar rats served as subjects. They were obtained from Charles Rivers (QC, Canada) at an age 50 days. They were housed, fed and maintained as described in the General Methods section. These animals were different from the animals used in Chapter 3 and Chapter 4.

5.2.3 Surgery, Kindling, Stability Testing, Electrode Placement Verifications

One week (minimum) after arrival from the breeding farm using procedure described in the General Methods section, the subjects were surgically implanted with an electrode in the right basolateral amygdala. Starting two weeks later, they were kindled to 15 Stage Vs (Racine scale), ADT tested, and then stability tested as described previously. The subjects’ ADT were re-
determined one day after the surgery using procedures described in the General Methods section. The stimulus for drug testing was 120% ADT.

After the rats had been fully kindled and stability tested, cannuli were inserted in their jugular veins, as described below. They then proceeded to drug testing without further stability tests, although afterdischarge thresholds were measured post-cannulati

5.2.4 Procedure for Cannulation Surgery

The subjects were first cannulated at the left jugular vein. If the left cannulation failed, the rats were allowed to heal for one week after which the left cannula was removed, and a new cannula was implanted on the right side.

For cannulation, the subjects were anesthetized with isoflurane (induction 5%, maintenance 2.5%), the fur on the front of the chest was shaved and the skin was sanitized with two alternating wipes of alcohol and betadine. SYSTANE® nighttime eye ointment was applied to prevent corneal drying and the rat was placed in the supine position on a heating pad at 39°C, and covered with a surgical drape. Each rat was then given an injection of ketoprofen (3mg/kg, SQ) to minimize post-surgical discomfort.

Subsequently, a 2cm incision was made approximately 1cm lateral to the trachea. The left jugular vein was blunt dissected out using tweezers and a tight ligature was made at the rostral end using a 4.0 silk non-absorbable suture to obstruct the blood flow from the head. A loose tie was made on the caudal ends, the branches between the rostral and caudal ends was tied shut. Between the rostral end and the caudal end, an incision large enough to pass the catheter were made using a microsurgical scissor. The catheter was inserted into the vessel, and fixed, using 4.0 non-absorbable ligatures at the rostral and caudal ends, and to surrounding tissues.
The cannula was then guided subcutaneously to the dorsal nape of the neck using a 16G needle to create the incision and guide the cannula. The cannula was passed through a 5mm segment of 16G needle tube, with blunt ends. The 16G tube, with the cannula inside, was cemented onto the headcap (Repair Material, Dentsply, York, P.A, U.S.A).

Once the surgery was completed, 0.1ml of locking solution was injected into the cannula to prevent blood clotting. The cannula was sealed then using a blunt-end dresser pin. The locking solution consisted of 500U/mL Heparin (Sigma, ON) and 120ug/mL gentamicin sulfate (Sigma, ON) in 1:1 sterilized physiological saline: glycerol (Luo et al., 2000). The patency of the cannula was tested each step by attempting to draw blood from it.

The cannulas were made of a 11-12cm long piece of BTPE-50 polyethylene tubing (0.023in x 0.038in) (INSTECH, Plymouth Meeting, PA) and a 3.5 - 4.0cm BTSIL-047 Dow Corning Silastic® silicone tube (0.025in x 0.047in) (INSTECH, Plymouth Meeting, PA). The two types of tubes were linked together via toluene; the overlap region was 1.0cm (Thrivikraman et al, 2002).

5.2.5 Procedures 5α-Dihydroprogesterone Drug Testing

Procedure for the Dose-Response Study

Starting four days after cannulation surgery, the subjects were injected with various doses of 5α-DHP intravenously (IV) every second day in randomized order. Five doses were injected: 0, 1.25, 2.5, 5.0 and 7.5 mg/kg:

Injections were administered at a rate of 1mL/min using a SAGE infusion pump Model 351 (Orion, Boston, MA). The infusion volume was kept constant, at 3mL/kg for all doses.
Immediately after the infusion, 0.1mL of locking solution was injected into the cannula. The cannula was then sealed with a dresser pin.

The rats were then stimulated at 120% of the ADT at 5 minutes after the start of the drug infusion.

All experiments were conducted during 13:00 to 16:00.

*Procedure for the Time-Response Study*

A different batch of kindled animals were used in the time-response study. Four days (minimum) after cannulation surgery, time-response testing was begun. Every second day, the subjects were injected with 5α-DHP at the ED75 dose for focal seizure suppression. They were then seizure tested (stimulation at ADT + 20%) at one of the following post-injection intervals, administered in randomized order: 5, 15, 30, 45, 60 and 80 minutes. The ED75 dose, (5mg/kg), was derived from the previous IV dose-response study.

Injections were administered at a rate of 1mL/min using a SAGE infusion pump Model 351 (Orion, Boston, MA). The infusion volume was kept constant, at 2mL/kg for all doses. Immediately after the infusion, 0.1mL of locking solution was injected into the cannula. The cannula was then sealed with a dresser pin.

All experiments were conducted during 13:00 to 16:00.

*Procedure for Seizure Scoring*

The presence or absence of afterdischarge and behavioral seizures was recorded for each rat, and the duration of the afterdischarge and the rank of the behavioral seizures were also recorded. Ataxia was scored using the Loscher scale immediately before administration of the kindling stimulus.
Figure 5.1 5α-Dihydroprogesterone (5α-DHP) dose-response and time-response study via IV route experiment flow.
The behavioral seizure was considered to be suppressed if no forelimb clonus, no rearing and/or falling (Racine stages 3-5) was observed. The focal seizure was considered to be significantly reduced if the stimulus was followed by less than 8s of afterdischarge in the EEG. (Percentage focal seizure suppression using Lonsdale’s previous criteria – afterdischarge duration shorter than 4 seconds were reported in Appendix II). Ataxia was scored as “present” if the Loscher score was 2 and higher.

5.3 Results

5.3.1 5α-DHP via IV route: Dose Response Study

Figure 5.2A illustrates the effects of an IV 5α-DHP in a single, representative subject. As indicated, we observed a dose-dependent suppression of the generalized seizure and a shortening of the afterdischarge duration, accompanied by a dose-dependent increase in ataxia. Figure 5.2B presents a record from the same subject after a vehicle injection. After the vehicle injection, there was no change in convulsive behaviour, afterdischarge duration or ataxia.

Figure 5.3A presents a dose-response curve based on group data for the quantal suppression of generalized and focal seizures when 5α-DHP was administered via the IV route. The presence or absence of ataxia is also indicated. An ED50 of 1.69mg/kg for generalized seizure suppression was found, and an ED50 of 3.48mg/kg for focal seizure suppression. Ataxia was seen at doses above 2 mg/kg and had a TD50 of 3.57mg/kg. The therapeutic indices for generalized and focal seizures were 2.1 and 1.0 respectively.

Figure 5.3B presents group data for graded dose-response curves related to motor seizure strength (Racine scale rating), afterdischarge duration (seconds) and severity of ataxia (Löschler scale rating). When the data were analyzed this way, we found an ED50 of 0.48 mg/kg for
Figure 5.2 EEG recordings from subject A18-4. A) EEG recordings at various dose of 5α-DHP, a dose-dependent shortening of afterdischarge duration (ADD and motor seizure stage) was observed. There was also a dose-dependent increase in ataxia B) EEG recordings at vehicle control, the vehicle has no effect on afterdischarge duration or motor seizure stage.
Figure 5.3 Dose – response curve for 5α- Dihydroprogesterone IV on seizures and ataxia at 5 min (N=7 at 7.5mg/kg, N=6 at 1.25, 2.5, 5.0 mg/kg, N=5 at 0mg/kg). A) % subjects with seizure suppression, and % subjects with ataxia stage 2 or higher vs. time. B) Seizure stages, afterdischarge duration, ataxia vs. doses (mean ± S.E.M.). Seizure stages and ataxia are graphed along the left Y-axis, whereas the afterdischarge duration is graphed along the right Y-axis.
shortening of the afterdischarge duration (seconds), an ED50 of 2.50 mg/kg for decreasing the strength of the motor seizure (Racine scale) and a TD50 of 4.63mg/kg for ataxia (Lösch scale). This analysis shows that the first effect of 5α-DHP, seen at the lowest doses, is a shortening (but not suppression) of the focal discharge. That is followed, at higher doses, by the weakening (and then disappearance) of motor seizures. At slightly higher doses, ataxia appears. The therapeutic indices, based on graded data, for generalized and focal seizures were 1.9 and 9.7 respectively.

5.3.2 5α-DHP via IV route: Time Response Study

Figure 5.4 presents EEG recordings from a single, representative subject at different times after IV injections of 5 mg/kg of 5α-DHP. As indicated, ADs were shortened 5 and 15 minutes after injection. Racine scores were lower from 5 to 45 minutes post-injection but had regained pre-injection levels by 60 minutes. A Lösch score of 1 was seen at 5 minutes and a Lösch score of 3 was seen at 15 minutes, after which no more ataxia was seen.

Figure 5.5A presents quantal group data (N = 4 at 5 and 80 minutes, N=5 at other points) for the presence of: 1) focal seizure suppression, 2) generalized seizures suppression, and 3) ataxia (Stage 2) at different times after IV injections of 5 mg/kg of 5α-DHP. As indicated, the onset of anti-seizure effects and ataxia was rapid following IV 5α-DHP. Seizure suppression and ataxia all peaked around 5 to 15 minutes post-infusion. Generalized seizures were better suppressed than focal seizures. Ataxia and focal seizure suppression disappeared at about 40 minutes, whereas generalized seizure suppression lasted longer, disappearing at about 60 minutes.

Figure 5.5B presents graded group data (N = 4 at 5 and 80 minutes, N=5 at other points) for the strength of: 1) focal seizure suppression, 2) generalized seizures suppression, and 3)
Figure 5.4 EEG recordings from subject A3-5. Lower Racine seizure stages, shorter afterdischarge durations and higher ataxia in Loscher scale were observed at earlier time points after 5α-DHP infusion.
Figure 5.5 Time-response curves for 5α-Dihydroprogesterone IV on seizures and ataxia at 5mg/kg (N=4 at 5 and 80 minutes, N=5 at 15, 30, 45, 60 minutes). A) % subjects with complete seizure suppression and % subjects with ataxia stage 2 or higher vs. time. B) Seizure stages, afterdischarge duration, ataxia vs. doses (mean ± S.E.M.). Seizure stages and ataxia are graphed along the left Y-axis, whereas the afterdischarge duration is graphed along the right Y-axis.
ataxia (Stage 2) at different times after IV injections of 5 mg/kg of 5α-DHP. As indicated, the graded effect data are fairly similar to the effects seen in the quantal data, with maximal effects for all parameters being seen at 5-15 minutes after injection. There was a rapid decline in the anti-seizure effects and ataxia between 15 to 30 minutes, as indicated by the steep slope between these two points. Subsequently, both ataxia and the anticonvulsant effects decline gradually over time, and return to baseline at around 80 minutes.

5.4 Discussion

The present study investigated the dose-response and time-response effects of 5α-DHP administered via the IV route with β-cyclodextrin as a solvent.

Dose-Response Study Dose-dependent suppression of both generalized and focal seizures was seen. ED50s were low, being 1.69mg/kg for generalized seizures and 3.48mg/kg for focal seizures. Ataxia was also seen, with a TD50 for stage 2 ataxia of 3.57mg/kg.

These results related to the suppression of generalized seizures confirmed and extended the past studies of Lonsdale et al. (2006) and Zhong (2015), both of whom reported that generalized seizures were suppressed by 5α-DHP. They are not in agreement with Jeffrey (2014), who reported no effects of 5α-DHP on generalized or focal seizure suppression. Jeffrey’s study used mice instead of rats and kindled the hippocampus instead of the amygdala.

In combination, these three positive studies make it clear that 5α-DHP has definite anticonvulsant effects against generalized motor seizures. It is interesting that, in the present study, the ED50 for generalized seizure suppression was below 5 mg/kg. A similar low ED50 had been seen in Lonsdale’s study using the SQ route.

We also found the suppression of amygdala focal seizures at relatively low doses. These
data support Lonsdale’s previous finding of low-dose suppression of amygdala focal seizures. As in Lonsdale’s study, the ED50 for focal suppression was not much higher than the ED50 for the suppression of generalized seizures.

In the present study, we also saw marked ataxia. As noted earlier, the TD50 for stage 2 ataxia (3.57mg/kg) was similar to the ED50 for suppression of the focal seizure. These data do not agree with the earlier data of either Lonsdale or Zhong, both of whom found seizure suppression with no ataxia - at least during the early post-injection intervals. (Lonsdale reported the development of ataxia which occurred later, presumably due to the metabolism of 5α-DHP to ALLO).

It is not clear why ataxia was seen in the present study but not in the previous studies. The most obvious difference between the present experiment and the previous experiments is the route of administration (IV) and the very short injection-test interval. Ataxia is known to occur with ALLO, but at 5 minutes post-infusion there should not have been much conversion to ALLO.

**Time-Response Study** The present study was designed to measure 5α-DHP’s anti-seizure and ataxic effects 5α-DHP’s at different times after IV injections of 5 mg/kg of 5α-DHP.

Both anti-seizure effects and ataxia were seen shortly after IV injection. Both generalized and focal seizures were suppressed at 5-15 minutes and this suppression was accompanied by severe ataxia. Both seizure suppression and ataxia gradually disappeared over time, and returned to baseline level by around 60-80 minutes.

With regard to generalized seizures, 5α-DHP’s effects on generalized seizures reached their peak at early time points, then declined overtime, and largely disappeared around 40-60 minutes post-infusion. Lonsdale (2008) and Zhong (2015) reported relatively longer effects of
5α-DHP on generalized seizures (SQ route). Lonsdale reported nearly 100% of generalized seizure suppression at 30 to 80 minutes post-injection; Zhong reported nearly 100% generalized seizure suppression from 40 to 160 minutes. These differences in duration probably reflect pharmacokinetic differences related to different routes of administration (SQ vs. IV).

With regard to focal seizures, 5α-DHP’s effects on focal seizures were short. This finding is in agreement with the study of Lonsdale and Burnham (2003), who reported strong focal seizure suppression from 0 to 20 minutes after SQ injection. Future studies might assay the concentrations of 5α-DHP in blood after IV injection. It seems that blood concentrations may drop rapidly.

These early effects of 5α-DHP are not likely to be due to the vehicle or to the injections per se, since β-cyclodextrin vehicle injections did not suppress seizures (or cause ataxia) in our IV dose-response study (see Chapter 5.3.1).

A continued difference between our study and past studies is that our anti-seizure effects were accompanied by strong ataxia, whereas Lonsdale’s were not (Lonsdale and Burnham, 2003; Lonsdale et al, 2006). As noted above, future studies will be necessary to address this difference in findings. It is interesting to note, however, that in our single, representative subject, considerable suppression of the focal discharge occurred at 5 minutes post injection, at which time ataxia was not fully established.
Chapter 6. The Anticonvulsant Effect of Benzyl Vehicle

6.1 Rationale

In Chapter 4, we unexpectedly found that the benzyl vehicle suppressed both generalized and focal seizures with limited ataxia. To the best of our knowledge, this is a novel finding. Any compound that suppresses focal seizures – and particularly amygdala focal seizures, a model of drug-resistant human complex seizures - is of interest.

The present study, therefore, was designed to determine the component of the benzyl vehicle that suppresses seizures in the kindling model, and then to test its effects in the pentylenetetrazole (PTZ) model, a model commonly used to screen for potential anticonvulsants (Krall et al, 1978). The PTZ model is less labor intensive and time consuming than the kindling model.

Two Experiments were performed. Experiment 1 was designed to determine the component of the benzyl compound that had anti-seizure activity, and Experiment 2 was designed to test the active component in the PTZ model.

6.2 Experiment 1. Determining the Active Components in the Benzyl Vehicle

6.2.1 Specific Methods

6.2.1.1 Drugs

The following compounds were purchased from commercial sources: 1) sodium chloride (Bioshop, Burlington, ON); 2) benzyl alcohol (Fisher Chemicals, Toronto, ON); 3) benzyl benzoate (Acros Organics, Toronto, ON); and 4) cottonseed oil (Acros Organics, Toronto, ON). These were the components of the benzyl vehicle used in Chapter 4.
These components were tested in combination and alone, following IP injections. The following solutions were tested, each at a volume of 2 ml/kg:

1) saline alone (0.9% sodium chloride in sterile water),

2) the benzyl vehicle (15% benzyl alcohol: 15% benzyl benzoate: 70% cottonseed oil, v:v:v),

3) benzyl alcohol alone (15% benzyl alcohol: 85% cottonseed oil, v:v)

4) benzyl benzoate alone (15% benzyl benzoate: 85% cottonseed oil, v:v), and

5) cottonseed oil.

All solutions were prepared fresh on the day of testing.

6.2.1.2 Subjects

The adult, female, amygdala-kindled Wistar rats from Chapter 5 were reused in this experiment.

All procedures were approved by the Animal Care Committee of the Faculty of Medicine of the University of Toronto and followed the guidelines of the Canadian Council on Animal Care.

6.2.1.3 Drug Testing

Drug testing was begun a week (minimum) after the end of the tests described in the last chapter. Subjects were injected IP on a 48-hour schedule with 2mL/kg of either: 1) saline, 2) benzyl vehicle, 3) benzyl alcohol, 4) benzyl benzoate, 5) cottonseed oil. This volume (2 ml/kg) was same as the volume used in Chapter 3. Following injections, the kindling stimulus was applied at 120% of the ADT at 15 minutes after injection. Only one drug was tested in each animal on each test day, with test days separated by 48 hours (minimum).
Seizures were scored in both quantal and graded fashion. Quantally, the presence or absence of afterdischarge and behavioral seizures was recorded at each time point. The behavioral seizure was scored “absent” if no forelimb clonus, rearing or falling was observed (Racine stages 3-5). The focal seizure was considered to be absent if the stimulus was followed by less than 4s of afterdischarge in the EEG. For graded scoring, behavioural seizures (if any) were scored according to the Racine scale. Ataxia was rated one minute before stimulation using the Löscher ataxia scale.

All experiments were conducted between the hours of 13:00 to 16:00h.

6.2.1.4 Data Analysis

Fisher exact test was used to analyze the quantal data in R3.40. GraphPad Prism 6 was used to analyze the graded data. Graded data for generalized seizure stages, afterdischarge durations, and ataxia were compared using one-way ANOVAs, followed by post hoc Tukey multiple comparison test against the saline control. Data are presented at mean ± SEM.

6.2.2 Experiment 1. Determining the Active Components in the Benzyl Vehicle Results

Figure 6.1 presents the effects of the different components of benzyl vehicle against secondarily generalized seizures (F_{4,26} =13.04, p<0.0001), amygdala-kindled focal seizures (F_{4,26} =6.091, p=0.0013), and Löscher ataxia scores (F_{4,26} =3.476, p=0.021).

As shown in Figure 6.1A, Fisher’s exact test revealed that the benzyl vehicle and benzyl alcohol suppressed generalized seizures in most animals (p<0.001). In terms of graded data, the benzyl vehicle (p<0.01 vs. saline, benzyl benzoate and cottonseed oil group) and benzyl alcohol (p<0.001 vs. saline, benzyl benzoate, and cottonseed oil group) decreased motor seizure stage in treated animals, while saline benzyl benzoate and cotton seed oil injections had no effect.
Figure 6.1 Anticonvulsant effects of various component in benzyl vehicle at 15 min (N=6 for each group). Anticonvulsant effects on A) generalized seizure, quantal, B) generalized seizure, graded, C) focal seizure, quantal, D) focal seizure, graded, D) ataxia, quantal, E) ataxia, graded. All graded responses were graphed in mean ± S.E.M format, p-values of Fisher exact test (A, C, E) and ANOVA test (B, D, F) are indicated on the graph (* p < 0.05, ** p < 0.01, *** p <0.001 against saline group in Tukey’s multiple comparison.)
Figure 6.1B showed that the benzyl vehicle and benzyl alcohol also suppressed focal seizures in some animals (p<0.01). As shown in Figure 6.1D, the benzyl alcohol treated group shortened afterdischarge duration (p<0.05 against saline).

However, both benzyl vehicle and benzyl alcohol produced ataxia in some subjects (p<0.05, Figure 6.1E). As indicated in Figure 6.1F, the benzyl alcohol treated group also experienced statistically significant ataxia score (p<0.01 vs saline, benzyl benzoate, and cottonseed oil group). More ataxia was seen with the benzyl alcohol injections than with the benzyl vehicle injections (p<0.05).

6.3 Experiment 2. Anticonvulsant Effects of Benzyl Alcohol

6.3.1 Specific Methods

6.3.2.1 Drugs

Pentylenetetrazole (PTZ) was purchased from Tocris Bioscience (Bristol, UK), and was kept at 20°C until use. It was diluted to 10mg/mL in sterile physiological saline. Benzyl alcohol (Fisher Chemical, ON, Canada) was diluted in cottonseed oil (Acros Organics, ON, Canada) at the following concentrations: 0%, 2.5%, 5.0%, 7.5%, 10% and 20% (v/v).

All solutions were prepared fresh on the day of the experiment.

6.3.2.2 Subjects

Male, CF-1 mice were purchased from Charles River (QC, Canada). They were housed in groups of 3-4 per cage, at a constant temperature of 21°C, on a 12/12h light/dark cycle (lights on at 7 am), with food and water available ad libitum. The mice weighed 34.74 ± 5.24 at the time of drug testing.
All procedures were approved by the Animal Care Committee of the Faculty of Medicine of the University of Toronto and followed the guidelines of the Canadian Council on Animal Care.

6.3.2.3 Drug Testing

**Procedure for Dose-Response Testing** Dose-response testing was initiated one week (minimum) after the mice had arrived from the breeding farm. Each mouse was injected with a single dose of benzyl alcohol (0, 100, 200, 400, and 800mg/kg IP). The injection volume was kept constant and benzyl alcohol was therefore diluted in cottonseed oil to the following concentrations: 0%, 2.5%, 5.0%, 10%, and 20%. Ten minutes after the benzyl alcohol injection, each subject was injected with 90mg/kg PTZ IP. Mice were then placed in a test chamber and monitored for convulsive seizure activity (below) for 15 minutes. Mice were euthanized via CO2 followed by cervical dislocation immediately after the start of tonic hindlimb extension or at the end of the observation period.

All tests were carried out between 13:00 and 16:00h.

**Procedure for Time-Response Testing** Time-response testing was initiated in a different group of mice one week (minimum) after arrival from the breeding farm. Each mouse was injected IP with 300mg/kg of benzyl alcohol in cottonseed oil (7.5% v/v). At various times following the benzyl alcohol injection (5, 10, 15, 30, 60 minutes), each subject was injected with 90mg/kg PTZ IP. Mice were then placed in a test chamber and monitored for convulsive seizure activity (below) for 30 minutes. Mice were euthanized via CO2 followed by cervical dislocation immediately after the start of tonic hindlimb extension or at the end of the observation period.

All tests were carried out between 13:00 and 16:00h.
**Procedure for Seizure Scoring** Latencies to following convulsive seizure behaviors were recorded: 1) the first myoclonic jerk, 2) facial and forelimb clonus, and 3) tonic hindlimb extension. If a type of seizure behavior did not occur during the period of behavioral observation, it was scored as “absent” in the quantal response and its latency was recorded as the maximal observation time in graded response.

6.3.2 Experiment 2. Anticonvulsant Effects of Benzyl Alcohol Results

**Dose-Response Data** Figures 6.2A and B presents dose-response data, with Figure 6.2A presenting data for total seizure suppression (quantal) while Figure 6.2B presents seizure latency data (graded). As indicated, benzyl alcohol was effective in delaying the onsets of all of the types of motor seizures (Figure 6.2B), and completely suppressed them in many animals (Figure 6.2A). Effects were against tonic extension at the lowest doses. Higher doses were required to suppress face and forelimb clonus, and still higher doses to suppress myoclonic jerks.

In terms of quantal response, the ED50 was around 300mg/kg for hindlimb extension and 600mg/kg against facial and forelimb clonus. While no formal attempt was made to score ataxia, it was clearly evident at the higher doses, and, at the highest dose of 800mg/kg, all subjects experienced severe respiratory depression with complete loss of righting reflex.

However, even at zero dose of benzyl alcohol, 20% of subjects did not display hindlimb extension. This may reflect the variability in the PTZ-induced seizures.

**Time-Response Data** Figures 6.3 A & B present the time-response data for seizure suppression after an IP injection of 300mg/kg of benzyl alcohol, the ED50 for tonic hindlimb extension in our previous dose-response study. Figure 6.3A presents data related to total
Figure 6.2 Dose-response for benzyl alcohol in IP pentylenetetrazole(PTZ) model. (N=10 at each A) Quantal and B) graded dose-response curve, a dose-dependent suppression of face and forelimb clonus and hindlimb extension was observed. Its ED50 against hindlimb extension was around 300mg/kg.
Figure 6.3 Time-response for benzyl alcohol in IP. pentylenetetrazole(PTZ) model. (N=10 at each points) A) Quantal and B) graded time-response curve. Benzyl alcohol showed strong anticonvulsant activity at early time points, its half-minimum effect against hindlimb extension was around 20 minutes.
suppression (quantal) while Figure 6.3B presents data for latencies to onset of the different seizure types (graded).

As indicated by Figure 6.3A, the anticonvulsant action of benzyl alcohol had a rapid onset, with maximal suppression of tonic hindlimb extension (80%) within 5 minutes. The effect gradually declined over time, and was gone by about 60 minutes. The effect was half maximal at around 20 minutes.

As indicated by Figure 6.3A, the effects on tonic hindlimb extension lasted longest, the effects on face and forelimb clonus were of shorter duration, and the effects on myoclonic jerk lasted the shortest.

6.4 Discussion

The present study was designed to find the active anticonvulsant ingredient in benzyl vehicle, and to provide preliminary dose- and time-response for its effects in the PTZ model.

Benzyl alcohol was found to be the only active ingredient in the benzyl vehicle. It is a commonly used solvent and drug excipient, used both to enhance solubility and as a bacteriostatic agent. Its structure is presented in Figure 6.3. It is found in many commercially available formulations, such as progesterone intramuscular injectables. The World Health Organization (WHO) has set an acceptable oral daily intake for humans at 5mg/kg. (Xie et al, 2015). In kindled rats, a single dose suppressed both the generalized and focal seizures. In the PTZ model, benzyl alcohol suppressed motor seizures in a dose-dependent fashion, showing almost immediate effects (after IP injection) that lasted about an hour.

To our knowledge, the present study is the first report of the anti-seizure effects of benzyl alcohol. Numerous past studies, however, have shown anticonvulsant effects following the acute administration of other alcohols. Acute ethanol injection at 1.5g/kg IP, for instance, has been
shown to suppress generalized seizures and to shorten focal afterdischarge in amygdala-kindled rats (Pinel et al, 1985). At 1250mg/kg, isopropyl alcohol increases seizure threshold up to 65% in the electroconvulsive shock model, part of its anticonvulsant activity being attributed to its metabolite acetone (Chu et al, 1948).

Other chemicals with structures similar to benzyl alcohol (Fig. 6.4) also have anticonvulsant properties. Vanillyl alcohol, a benzyl alcohol with a methoxy group substituted at meta position and an hydroxyl group at para position, have been found to be protective against ferric chloride-induced seizures, perhaps due to its radical scavenging property (Hsieh et al, 2000). Vanillin, an oxidized version of vanillyl alcohol, suppresses generalized seizures in amygdala-kindled animals with an ED50 of 292mg/kg and shortens the afterdischarge durations (Wu et al, 1989).

While benzyl alcohol’s anti-seizure effects are impressive, it is unlikely that it could be developed as an anticonvulsant therapy due to its toxicity. The doses that suppressed seizures in kindled animals also produced ataxia, and the higher doses used in the PTZ study actually caused respiratory depression. These effects would limit the clinical usage of benzyl alcohol.

Curiously, one past toxicity study of benzyl alcohol listed seizures as one of its toxic effects (McCloskey et al, 1986). This report seems to be in conflict with the anti-seizure effects seen in the present study. The McCloskey study, however, was done at extremely high doses of benzyl alcohol that must have cause respiratory suppression. It is possible that seizures seen were hypoxia-induced seizures caused by the respiratory depression.

The mechanism of benzyl alcohol’s anti-seizure actions is not clear. Past studies have looked into its actions in in vitro preparations, but most of them have used anesthetic concentrations. At anesthetic concentrations (15-30mM in brain homogenates), benzyl alcohol
inhibits diazepam receptor binding, but not GABA and muscimol binding (Speeg et al, 1980). It also protects cells against NMDA toxicity at 2mM (Takadera & Ohyashiki, 2008), decreases conductance and increases mean opening time of nicotinic acetylcholine channels at 40mM (Bouzat & Barrantes, 1991) and interacts with potassium (Kv1.1) ion channels at 20mM (Elliott & Elliot, 1997). Studies done at these anesthetic concentrations, however, may or may not be relevant to the anti-seizure effects seen in the present study. We did not know the corresponding in vitro doses of our present experiment; we believe that the ED50 for tonic hindlimb extension has not yet reached the anesthetic doses.

Even though the toxicity of benzyl alcohol would limit its clinical use, future experiments might test structurally similar compounds that were less toxic. Future studies might also investigate whether low doses of benzyl alcohol could potentiate the anticonvulsant effect of other drugs, thus developing better formulations for existing anticonvulsant agents.
Figure 6.4 Chemical structures of benzyl alcohol and other compounds with similar structure
Chapter 7 General Discussion

7.1 Objectives

The primary objective of the present studies was to investigate the anticonvulsant effects of 5α-DHP in dose-response and time-response studies in amygdala–kindled rats. Different routes of administration, solvents and post-injection time points were used.

A secondary objective was to document the newly discovered anticonvulsant effects of benzyl alcohol in in dose-response and time-response studies in mice.

7.2 Major findings

Our major findings were that:

1) Our initial SQ dose-response studies showed weak anticonvulsant effects of 5α-DHP against generalized seizures, and no anti-seizure effects of 5α-DHP against focal seizures (Chapter 3). It seemed possible, however, that absorption was poor via the SQ route. Our vehicle was β-cyclodextrin.

2) Subsequent time-response studies using the IP route and a new vehicle – the benzyl vehicle – showed good anticonvulsant effects at two time points: 1) immediately after injection, and 2) at about 130 minutes after injection (Chapter 4). Both generalized and focal seizures were suppressed at the early time point, but only generalized seizures were suppressed at 130 minutes. Ataxia was seen at the earlier time point, but not at the later time point.

Surprisingly, when the benzyl vehicle was tested alone (without 5α-DHP), it was just as good at suppressing generalized seizures at 0-30 minutes, and about half as good at suppressing focal seizures. Some ataxia was seen. Thus, our data involving 5α-DHP dissolved in the benzyl vehicle were hard to interpret, since the vehicle itself was active.
We decided to change to the IV route, which required lower (soluble) doses of 5α-DHP, and went back to β-cyclodextrin as a vehicle.

3) When our route of administration was changed to the IV route – which allowed 5α-DHP administration without the benzyl vehicle - dose-dependent suppression of both generalized and focal seizures was seen with 5α-DHP (Chapter 5). ED50s were low, being 1.69mg/kg for generalized seizures and 3.48mg/kg for focal seizures. Ataxia was also seen, with an TD50 for stage 2 ataxia of 3.57mg/kg.

In our IV time-response study using the ED75 for focal seizure suppression, we observed both generalized and focal seizure suppression almost immediately after 5α-DHP infusion. The effects disappeared within an hour.

4) In follow-up studies, benzyl alcohol was found to be the only active ingredient in the benzyl vehicle. In kindled rats, a single IP dose suppressed both the generalized and focal seizures. In the PTZ model, IP benzyl alcohol suppressed motor seizures in a dose-dependent fashion - showing almost immediate effects that lasted about an hour.

7.3 Comparison of the Present Results to Past Studies

The anticonvulsant effects of 5α-DHP were first described by Landgren et al. (1987). These researchers reported that 5α-DHP suppressed penicillin-induced seizures in cats, but that 5α-DHP was less potent than progesterone and much less potent than ALLO.

In subsequent studies by Lonsdale and collaborators (2003; 2006), it was found that 5α-DHP was actually more potent than other progesterone family compounds when injected SQ in kindled rats. It effectively suppressed both amygdala focal seizures and secondarily generalized seizures with no ataxia.
Zhong (2015), however, failed to replicate the results of Lonsdale et al. (2003, 2006). In Zhong’s hands, 5α-DHP showed only limited effects against generalized seizures and negligible effects against focal seizures in the amygdala-kindling model.

In the present study, after moving to the IV route, it was found that 5α-DHP was clearly effective against both generalized and focal amygdala-kindled seizures. Thus, we have more or less replicated the findings of Lonsdale and collaborators (2003; 2006), although we had to switch the route of administration to do so.

We should note that Lonsdale et al. reported a complete suppression of the focal ADs in some subjects, whereas we have found only a shortening of the focal AD. The difference in these results probably relates to technical factors. Due to a prolonged “switch artifact”, present in our system at the time Lonsdale worked, Lonsdale would not have been able to see ADs shorter than 4 seconds. If very short ADs occurred in her studies, she would have scored them as “absent”. Due to an improved recording technique, we can now see very short ADs and we score them as “present”. Thus, we report “shortening” of focal ADs, whereas she reported “suppression”. Still, we can report that 5α-DHP greatly shortened focal ADs, even if it did not completely suppress them.

We should also note that Lonsdale et al. saw seizure suppression without ataxia in her SC studies. Ataxia was clearly present in our IV studies, however. The ataxia seen in our studies may relate to injecting high levels of 5α-DHP rapidly via the IV route. Future studies might explore whether it is possible to obtain seizure suppression without ataxia, perhaps by using very slow IV infusions.

Why did Zhong et al. – and why did we at first - fail to see these anti-seizure effects of 5α-DHP? The most likely answer relates to solubility and route. 5α-DHP is extremely hard to
keep in solution. It was not until we switched to the IV route – which allowed us to work at lower concentrations and 100% bioavailability – that we began to see clear effects.

The IV route should probably be used in future animal studies of 5α-DHP. The direct infusion of 5α-DHP via implanted cannulas has high bioavailability and complete absorption and, thus, lower (soluble) doses can achieve good blood levels of high partition-coefficient molecules - as suggested by a previous study from our laboratory (Trépanier et al, 2015). In our hands, chronic SQ injections probably led to scar tissue formation around the injection sites, causing limited and slow absorption. IP injections with β-cyclodextrin caused pain to the animals, probably due to the high osmolarity required to dissolve DHP and high volume of injection. Benzyl alcohol, which made IP injections tolerable, proved to be anticonvulsant in itself. Thus, the IV route seems best for future studies.

### 7.4 Clinical Relevance of the Present Results

Patients with uncontrolled seizures often suffer from psychiatric and somatic comorbidities (Fazel et al, 2013; Hamid et al, 2014), as well as socioeconomic problems and stigmatization (Burnham, 2006). Achieving good seizure control can greatly improve the patient's quality of life. Given the drug-resistant nature of human complex-partial seizures, novel drugs with new mechanisms are needed for this seizure type. 5α-DHP may have the potential to be developed as an anticonvulsant drug for complex partial seizures.

An ideal complex partial drug would suppress both secondarily generalized seizures and focal seizures without ataxia. In our hands, 5α-DHP satisfied the first two requirements, but unfortunately caused ataxia. Future studies might explore the question of whether 5α-DHP can suppress seizures without causing ataxia, as in Lonsdale’s studies. Alternately, non-sedating 5α-
DHP analogs might be developed as novel compounds for the treatment of complex partial seizures.

Relative to analogs, it should be noted that 5α-DHP cannot be patented, and, therefore, 5α-DHP itself would never be developed as a novel anti-seizure drug. Novel analogs of 5α-DHP, however, could be patented and developed.

We hope in future studies to develop 5α-DHP analogs that are more effective and more tolerable than DHP itself. In the next section, I will describe some limitations of the present study and propose future studies that will aid in the rational design of 5α-DHP analogs.

7.4.1 Proposed Future Studies Related to Therapeutic Effects and Ataxia

7.4.1.1 Metabolic Profile of DHP

The metabolic profile of 5α-DHP would be worth studying. 5α-DHP has at least one active metabolite – allopregnanolone (ALLO) (see Chapter 1.7). ALLO is a strong GABA-A receptor agonist (Lambert et al, 2003) and binds to membrane progesterone receptors (mPRs) as well (Singh et al, 2013).

One question that could be approached in future studies would be whether ALLO is responsible for the ataxia seen in some of our studies. ALLO, a GABA-A receptor agonist is known to produce sedative side effects (Lonsdale, 2005; Zhong, 2015). While ALLO could not be responsible for the short-term ataxia seen in our IV studies, it might be responsible for the ataxia seen with other, slower routes. A second question would be whether ALLO might be responsible for the second cycle of anti-seizure effects seen at 130 minutes in our IP time-course study.
A future pharmacokinetic study might assay the concentrations of 5α-DHP and ALLO at various time points after IV and IP injection. This would help us to differentiate the relative anticonvulsant contributions of each progesterone metabolite and aid in the rational design of 5α-DHP analogs.

In addition, future drug development studies might attempt to design 5α-DHP analogs that do not produce this sedative metabolite. Analogs with longer half-lives would also be desirable.

7.4.1.2 Mechanism Studies

Knowing the mechanism of a drug’s therapeutic actions can greatly facilitate drug development processes. The goal of producing analogs of a compound is to improve the compound pharmacokinetically without losing its therapeutic effects.

5α-DHP has several possible molecular targets, including both intracellular and cell-surface receptors (Singh et al, 2013). The classical nuclear progesterone receptor (nPR) is the best-known target for 5α-DHP. Once activated, nPRs bind to progesterone response elements in the DNA and promotes gene transcription.

5α-DHP does bind to the nPRs, with an affinity weaker than progesterone but stronger than allopregnanolone (Iswari et al, 1986; Rupprecht et al, 1996). Yet the anticonvulsant effects of 5α-DHP are, in all probability, unrelated to its genomic actions. In our hands, the onset of 5α-DHP’s anticonvulsant actions began as early as 5-minutes post-injection – an interval too short for genomic transcription. The nPR pathway thus probably does not contribute to 5α-DHP’s anti-seizure effects, but might produce unwanted hormonal effects. Future researchers will perhaps want to develop 5α-DHP analogs that do not bind to the nPRs.
In addition to the nuclear receptor, 5α-DHP has other molecular targets. Two types of cell-surface progesterone receptors have been identified: the membrane progesterone receptors (mPRs) and progesterone membrane receptor components (PGMRCs) (Tischkau & Ramirez, 1993; Singh et al, 2013). Binding to these cell-surface receptors is more likely to explain 5α-DHP’s rapid anti-seizure effects. 5α-DHP has a stronger affinity for the mPRs than for the classical nuclear PRs.

mPRs are a G-protein coupled receptors, and are associated with many downstream pathways, such as extracellular-signal regulated kinases (ERK), classical Gi and Gs protein (Singh et al, 2013) and L-type calcium channels (Luoma et al, 2011). Future studies might concentrate on these cell-surface pathways in attempts to determine the mechanism of 5α-DHP’s anti-seizure actions.

7.4.2 Proposed Studies Related to Hormonal/Toxic Effects

Patients take anticonvulsant anti-seizure drugs to achieve seizure control. Hormonal effects would be unwanted and possibly dangerous. In this section, DHP’s effects on the reproductive system and its possible tumorigentic properties will be discussed. As noted above, eliminating these would be a direction for 5α-DHP analog development.

7.4.2.1 Effects on the Reproductive System

5α-DHP has hormonal properties, and may have effects on female reproductive cycling and mating behaviors. Progesterone itself has been used as a contraceptive for many years (Zimmerman et al, 1973), and 5α-DHP, like progesterone, might suppress the female reproductive cycle. Indirect evidence suggests that 5α-DHP affects lutenizing hormone (LH) (Mahesh et al, 1987) and follicle stimulating hormone (FSH) (Sanyal & Todd, 1972) secretion,
two hormones that regulate female reproductive cycling. Direct evidence of 5α-DHP’s effects on the reproductive cycle, however, is lacking. A future study could investigate whether 5α-DHP alters the reproductive cycle in rats.

In addition, 5α-DHP affects female reproductive behavior. Although it is less potent than progesterone, 5α-DHP is more potent than other progesterone metabolites in inducing lordosis in Sprague-Dawley rats (Wahlen & Gorzalka, 1972). Krebs et al (2000) have suggested that this effect may be regulated by the progesterone receptor membrane component 1 (PGRMC1).

Similarly, chronic administration of 5α-DHP at 500μg to estrogen-primed, ovariectomized female mice increases receptivity a strain-dependent manner (Gorzalka & Wahlen, 1974). It has been found that this 5α-DHP-induced receptivity is a dominant trait in mice (Gorzalka & Wahlen, 1976).

It would be desirable to eliminate all of these hormonal effects in 5α-DHP analogs evolved for anti-seizure therapy.

7.4.2.2 Effects on Mammary Tumors

Tumorigenesis in mammary tissues is another possible undesired side effect of 5α-DHP. 5α-DHP promotes cellular proliferation and decreases cell adhesion in the nM to μM range in vitro (Wiebe & Muzia, 2001; Wiebe et al, 2016). It is also responsible for the neoplastic transformation of non-tumor C4HD murine mammary cells in vivo (Wiebe et al, 2015). The serum content of 5α-DHP in significantly higher in animals with tumors, and is even higher in tumor tissue (Wiebe et al, 2015).

Wiebe’s group hypothesizes that 5α-DHP stimulates the cell-surface progesterone receptors, which then activate the mitogen-activated protein kinase (MAPK) pathway and
stimulate cell proliferation and metastasis. This membrane binding is specific to progesterone and 5α-DHP - none of other progesterone metabolites - or estrogen or testosterone metabolites - binds to the same extent (Wiebe et al, 2015).

Since it is hypothesized that the membrane progesterone receptors are also responsible for 5α-DHP’s anticonvulsant effects, it will be challenging to develop 5α-DHP analogs that are anticonvulsant but not tumorigenic. Three possible strategies could be considered: 1) selective delivery of 5α-DHP analogs to the brain but not to the periphery, 2) creating biased-ligands for mPRs that selectively activate some pathways and inhibit other pathways, and/or 3) generating tissue-specific 5α-DHP analogs that activate receptors in the brain but not in other tissues.

7.5 Summary of Proposed Future Studies

Below is a summary of proposed future studies:

1. Pharmacokinetics studies. We would wish to identify the contributions of 5α-DHP and its metabolites to the onset and duration of anticonvulsant action. This would be a second way to approach mechanism.

2. Mechanism studies. We would wish to investigate the binding sites of 5α-DHP and its downstream pathways at anticonvulsant doses. Identifying the anticonvulsant receptors for 5α-DHP could aid in rational drug development.

3. Safety studies. We would wish to investigate 5α-DHP's effects on the reproductive system and mammary tissues at anticonvulsant doses.

4. Analog studies. All of the above would contribute to the design of novel analogs of 5α-DHP. These could then be tested in the kindling model and in other animal seizure
models to investigate their possible potency against complex partial seizures, and also to
determine whether they would have broad-spectrum anticonvulsant effects.
References


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Figure A1. Afterdischarge duration (ADD) before and after cannulation, pilot testing (N=10). *The difference in afterdischarge threshold (ADT) before and after cannulation are not statistically significant. All subjects that were stable before cannulation are stable after cannulation. Cannula remains patent for around 1 week.*
Figure A2 5α- Dihydroprogesterone IV route on seizure suppression, using Lonsdale’s criterion. A). Dose- response curve, % focal seizure suppression vs doses (mg/kg) (N=7 at 7.5mg/kg, N=6 at 1.25, 2.5, 5.0 mg/kg, N=5 at 0mg/kg). B) Time- response curve, % focal seizure suppression vs time (min) (N=4 at 5 and 80 minutes, N=5 at 15, 30, 45, 60 minutes).