Progesterone as an Anti-convulsant

By

Afshin Shahzamani

A thesis submitted to the Department of Pharmacology
in conformity with the requirements for
the degree of Masters of Science

University of Toronto
Toronto, Ontario, Canada
July, 2001

© Copyright Afshin Shahzamani, 2001
The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author’s permission.

L’auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

L’auteur conserve la propriété du droit d’auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

0-612-63177-X
Acknowledgements

There are many people that I’d like to thank for helping me in this long journey.

First, I would like to thank Dr. Burnham. His love for life is contagious. He opens doors when others close them. All along, despite competing responsibilities, he has been helpful.

I would like to thank the following “lab-related” people who have, in many ways, helped me complete this project. Some of these individuals have helped more than professional duty requires; some have become friends.

Ted Brown  Antonio Mendonca
Jerome Cheng  Brian Scott
Sergei Likhodi  Nancy Mingo
Janet  Eric Law
Elizabeth Ng  David Lai
John Misek  Heather Edwards
Emmanuel Ho  Sue Ambrosini

I would like to thank Alla Vilner. She cheers me up, always.

I would like to thank Paula Thiessen who never “doodles all-day-long” when friends need her.

I would like to thank David Petroff who studies Gravity – we can sleep a sound sleep, some things are still as they should be.

I would like to thank my aunt’s entire family for having open hearts.

I would like to thank my sister and her family, who gave me a second chance.

I would like to thank my parents, Ali and Azar, and my brother, Ramin, who have always – without equivocation – believed in my innate abilities and my integrity.
Most of all, I would like to thank my wife, Andrea Griggs, who has already been there for me and with me, in the tough times, and in the good times. She gives meaning to what is otherwise absurd, and I could not have done anything meaningful without her.
Progesterone as an Anti-convulsant

Master of Science, 2001

Afshin Shahzamani

Department of Pharmacology

University of Toronto

Abstract

Progesterone (P₄) has been successfully used as an anticonvulsant. Its use, however, has been limited to patients whose seizures are exacerbated by varying hormonal states in the menstrual cycle. Its anticonvulsant mechanism of action is unclear. Therefore, the following studies were designed to increase the understanding of the mechanism of action of P₄ and promote its broader use in a clinical setting: 1) Effect of P₄ on Amygdala-kindled seizure 2) Effect of chronic low doses of P₄ in various seizure models 3) Effect of gender and age on the anticonvulsant activity of P₄ 4) The role of GABA and the classic intracellular receptor in mediating P₄'s anticonvulsant action.

These studies indicate that P₄ is an effective anticonvulsant in males and infants. Clinical trials in non-catamenial adults and children would be warranted. Its mechanism of action may involve the newly discovered cell-surface P₄ receptor and may provide the possibility of new targets for future drug development.
Table of Contents

1 INTRODUCTION 6
1.1 Epilepsy 6
1.1.1 Definitions 6
1.2 Types of Seizures 7
1.2.1 Common Seizure Types 7
1.3 Therapy for Epilepsy 8
1.3.1 Antiepileptic Drugs (AED's) 8
1.3.2 Prognosis for Seizure Control 9
1.4 Steroid Sex Hormones and Epilepsy 9
1.4.1 Catamenial Seizures 10
1.4.2 Steroid Sex Hormones: General Background 11
1.4.3 Progesterone and Estrogen as Neurosteroids 14
1.4.4 Synthetic and Metabolic Pathways 14
1.4.5 Commercially Available Progesterone, MPA and Ganaxolone 17
1.4.6 Sex Steroids and the Brain: Receptors 18
1.4.7 Some Other Notable Neuro-active Steroids 28
1.5 Animal Seizure Models 29
1.5.1 Electroconvulsive Shock Seizures: Maximal (MES) and Threshold (ECS) 31
1.5.2 Pentylenetetrazol: Maximal (MMT) and Threshold (MET) 32
1.5.3 Kindling 34
1.6 Past studies of Progestin Effects on Seizures 35
1.6.1 Clinical Studies 36
1.6.2 Animal Studies 36
1.7 Objectives 42

2 GENERAL METHODS 45
2.1 Animals 45
2.2 Drugs 45
2.3 Procedures for the Kindling Experiments 45
2.3.1 Implantation of Electrodes 46
2.3.2 Kindling Procedure 46
2.3.3 Determination of After-discharge Threshold 47
2.3.4 Determination of Stability 48
2.3.5 Procedure for Drug Testing 49
2.3.6 Verification of Electrode Placement 49
2.3.7 Procedure for Ovariectomy and Gonadectomy 50
2.4 Procedure for Vaginal Smears 51
2.5 Procedure for the MET Test 52
2.6 Procedure for the MMT Test 53
2.7 Procedure for the ECS Test

2.8 Procedure for the MES Test

2.9 Procedure for Pentylenetetrazol Infusion

2.10 Procedure for Tests of Sedation and Ataxia

2.11 Statistical Analysis

3.1 Rationale

3.2 Methods

3.3 Results

3.4 Summary

4 ANTICONVULSANT EFFECTS OF CHRONIC LOW-DOSE PROGESTERONE AND MPA IN SIX SEIZURE MODELS

4.1 Rationale

4.2 Methods

4.3 Results

4.4 Summary

5 THE ANTICONVULSANT EFFECTS OF PROGESTERONE IN ADULT AND 15-DAY-OLD MALE AND FEMALE RATS IN THE MMT AND THE MES SEIZURE MODELS

5.1 Rationale
5.2 Methods
Drug Preparation and Administration
Testing and Seizure Scoring
Data Analysis

5.3 Results
5.3.1 Experiment 3a: Adult Female Subjects
5.3.2 Experiment 3b: Adult Male Subjects
5.3.3 Experiment 3c: Immature Female Subjects
5.3.4 Experiment 3d: Immature Male Subjects

5.4 Summary

6 MECHANISTIC STUDIES OF THE ANTICONVULSANT EFFECTS OF PROGESTERONE

6.1 Rationale

6.2 Methods
Subjects
Drugs and Drug Administration
Testing and Seizure Scoring
Data Analysis

6.3 Results
6.3.1 Experiment 4a: Indomethacin
6.3.2 Experiment 4b: RU486

6.3 Summary

1. DISCUSSION

7.1 The Kindling Data: Experiment 1

7.2 The “Chronic, Low-Dose” Data: Experiment 2

7.3 The Influence of gender and Age: Studies at 15-Minute Injection/Test Interval

7.4 Studies of Mechanism: Experiment 4

7.5 How Does Progesterone Stop Seizures? : The Role of Allopregnanolone

7.6 How Does Progesterone Stop Seizures? : Non-GABA-Mediated Actions
1) Non-Specific Effects
2) Binding to the Intracellular Receptor
3) Other Metabolites
4) Binding to the Membrane Receptor

7.7 How Does Progesterone Stop Seizures? Threshold Rises

7.8 How Does Progesterone Stop Seizures: Clinical Actions
1) Contraceptive effects
2) Anti-estrogen Effects
List of Figures

Figure 1  Metabolic Pathways of Sex Hormones  13
Figure 2a Systemic Plasma Hormone Concentrations During the Menstrual Cycle in the Human  16
Figure 2b Systemic Plasma Hormone Concentrations During the 4-Day Estrous Cycle in the Rat  16
Figure 2c Feedback Control of the Hypothalamus and the Pituitary by Progesterone and Estrogen a Different Times of the Fertility Cycle  17
Figure 3  The Anti-convulsant Effects of Progesterone in the Kindling Model Progesterone Was Administered IP or SC  68
Figure 4  The Anti-convulsant Effects of Progesterone in the Kindling Model Progesterone Was Administered in Cyclodextrin and Corn Oil  70
Figure 5a The Effect of Estrogen on After-discharge Threshold  71
Figure 5b The Anti-convulsant Effects of Progesterone in the Kindling Model Subects Were Estrogen-Primed  71
Figure 6  The Anti-convulsant Effects of Progesterone in the Kindling Model Intact Subjects Were Used  73
Figure 7  The Anti-convulsant Effects of Progesterone in the Kindling Model Subects Were Gonadectomized and Hormone-Replaced Males  75
Figure 8 The Effect of Chronic Progesterone and MPA on the Generalized Convulsive Threshold in Amygdala-Kindled Rats  84
Figure 9  The Effect of Chronic Progesterone and MPA on the Threshold for ECS Seizures  86
Figure 10 The Effect of Chronic Progesterone and MPA on the Threshold for MES Seizures  87
Figure 11  The Effect of Chronic Progesterone and MPA on the Latencies to the Onset of MET Clonic Seizures  89
Figure 12 The Effect of Chronic Progesterone and MPA on the Latencies to the Onset of MMT Tonic (Forelimb Extension) Seizures  90
Figure 13  The Effect of Chronic Progesterone and MPA on the Threshold Dose of Pentylenetetrazol Required to Induce Clonic Seizures (FLC)  

Figure 14  The Anti-convulsant Effects of Progesterone in Adult, Female Wistar Rats  

Figure 15  The Anti-convulsant Effects of Progesterone in Adult, Male Wistar Rats  

Figure 16  The Anti-convulsant Effects of Progesterone in 15-day-old, Female Wistar Rats  

Figure 17  The Anti-convulsant Effects of Progesterone in 15-day-old, Male Wistar Rats  

Figure 18  The Influence of Indomethacin (15mg/kg) on the Anticonvulsant Effects of Progesterone (100mg/kg)  

Figure 19  The Influence of RU486 (20mg/kg) on the Anticonvulsant Effects of Progesterone (100mg/kg)
List of Tables

Table 1  Brain Distribution of Sex Hormones and Related Enzymes  23
Table 2  Past Studies on the Anticonvulsant Effects of Progesterone  40
Table 3  The Anticonvulsant ED50s of Progesterone for the Suppression of Kindled Seizures  77
Table 4  The Anticonvulsant ED50s of Progesterone for the Suppression of MMT and MES Seizures  107
**List of Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>µA</td>
<td>micro-Amps</td>
<td></td>
</tr>
<tr>
<td>µg</td>
<td>microgram</td>
<td></td>
</tr>
<tr>
<td>3-alpha HSD</td>
<td>3-alpha hydroxy-steroid dehydrogenase</td>
<td></td>
</tr>
<tr>
<td>ADT</td>
<td>afterdischarge threshold</td>
<td></td>
</tr>
<tr>
<td>AED</td>
<td>antiepileptic drug</td>
<td></td>
</tr>
<tr>
<td>aMPN</td>
<td>anterior medial preoptic nucleus</td>
<td></td>
</tr>
<tr>
<td>CA</td>
<td>ammon's horn</td>
<td></td>
</tr>
<tr>
<td>CAmg</td>
<td>central amygdaloid nucleus</td>
<td></td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine mono-phosphate</td>
<td></td>
</tr>
<tr>
<td>cIMPN</td>
<td>caudal parts of the lateral periphery of the preoptic nucleus</td>
<td></td>
</tr>
<tr>
<td>Cyclodextrin</td>
<td>2 hydroxypropyl-β-cyclodextrin</td>
<td></td>
</tr>
<tr>
<td>Cyclodextrin</td>
<td>hydroxypropyl-beta-cyclodextrin</td>
<td></td>
</tr>
<tr>
<td>DHEA</td>
<td>Dihydroepiandrosterone</td>
<td></td>
</tr>
<tr>
<td>E EB</td>
<td>estradiol benzoate</td>
<td></td>
</tr>
<tr>
<td>ECS</td>
<td>threshold electroconvulsive shock seizure test</td>
<td></td>
</tr>
<tr>
<td>ED₅₀</td>
<td>effective dose (50%)</td>
<td></td>
</tr>
<tr>
<td>EEG</td>
<td>electroencephalogram</td>
<td></td>
</tr>
<tr>
<td>ER</td>
<td>estrogen receptor</td>
<td></td>
</tr>
<tr>
<td>ERE</td>
<td>estrogen receptor elements</td>
<td></td>
</tr>
<tr>
<td>ERα</td>
<td>estrogen receptor alpha</td>
<td></td>
</tr>
<tr>
<td>ERβ</td>
<td>estrogen receptor beta</td>
<td></td>
</tr>
<tr>
<td>FEBP</td>
<td>feto/neonatal estrogen binding protein</td>
<td></td>
</tr>
<tr>
<td>FLC</td>
<td>forelimb clonus</td>
<td></td>
</tr>
<tr>
<td>FLE</td>
<td>forelimb extension</td>
<td></td>
</tr>
<tr>
<td>FSH</td>
<td>Follicular stimulating hormone</td>
<td></td>
</tr>
<tr>
<td>GABA-A</td>
<td>gamma-aminobutyric acid</td>
<td></td>
</tr>
</tbody>
</table>
GCT  generalized convulsive threshold  
GD gestational day 
GDX gonadectomized 
GnRH generalized releasing hormone 
GRIP glucocorticoid receptor interacting protein 1 
HLE hindlimb extension 
HPOA Hypothalamus 
ICV intracerebral ventricular 
ILS lateral septal nucleus 
IP intraperitoneal 
kg kilogram 
LH Luteinizing hormone 
mA milli-Amps 
MES maximal electroconvulsive shock seizure test 
MET threshold pentylenetetrazol seizure test 
mg milligram 
MMT maximal pentylenetetrazol seizure test 
MPA medroxy progesterone acetate 
mRNA messenger RNA 
ND<sub>v</sub> neurotoxic dose (50%) 
ovBNST oval nucleus of the bed nucleus of the stria terminalis 
OVX ovariectomized 
PdMA<sub>mg</sub> posterodorsal part of the medial amygdaloid nucleus 
P<sub>1</sub> Progesterone 
PND Post-natal day 
PO oral 
PR progesterone receptors 
PRA progesterone receptor A 
PRB progesterone receptor B 
prBNST principle nucleus of the bed nucleus of the stria terminalis 
R<sub>M</sub> maximal response 
rMPN rostral portion of the medial preoptic nucleus 
SC subcutaneous 
SMRT1 silencing mediator for retinoid and thyroid hormone receptors
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>SRC1</td>
<td>steroid receptor coactivator 1</td>
</tr>
<tr>
<td>StPA</td>
<td>strial part of the preoptic area</td>
</tr>
<tr>
<td>TERP-1</td>
<td>truncated estrogen receptor product-1</td>
</tr>
</tbody>
</table>
1 INTRODUCTION

1.1 Epilepsy

1.1.1 Definitions

The "epilepsies" are a group of neurological disorders characterized by spontaneous, recurrent seizures (Engel and Starkman, 1994). "Seizures" are episodes of excessive neuronal activity that can be identified by characteristic 'spike' or 'spike and wave' abnormalities in electroencephalographs (EEGs) (Burnham, 1997).

Epilepsy is a very common disorder of the central nervous system (CNS), affecting up to 1% of the population (Berg et al., 1996; Hauser et al., 1991).

Seizures occur in epileptic patients due to the abnormally low seizure thresholds seen in these patients (Scher, 1997). All brains have the circuitry necessary to produce seizures, and drugs that block inhibition or enhance excitation will induce a seizure in anyone (Engel and Starkman, 1994). In non-epileptic people, seizure thresholds are high, however, and spontaneous seizures do not occur. In epileptic patients enhanced excitation - or insufficient inhibition - leads to lowered thresholds and spontaneous seizures (Engel, 1996).
1.2 Types of Seizures

1.2.1 Common Seizure Types

Seizures can be classified as “generalized”, involving the entire brain, or “partial” involving only a part of the brain (Proposal for revised clinical and electroencephalographic classification of epileptic seizures. From the Commission on Classification and Terminology of the International League Against Epilepsy, 1981). There are a number of types of generalized seizures. The two most commonly seen are “tonic-clonic” and “absence seizures”. “Tonic-clonic” seizures involve a loss of consciousness with tonic-clonic convulsions. Constant “spiking” is seen in the EEG (Swoboda and Drislane, 1994). “Absence” seizures involve a loss of consciousness without convulsions. A pattern of three per second “spike and wave” is seen in the EEG (Mirsky et al., 1986).

There are only two categories of partial seizures: “simple” partial and “complex” partial (So, 1995). “Simple” partial seizures involve abnormal sensations, emotions or movements. They do not involve an impairment of consciousness. EEG “spiking” is seen in neo-cortical or limbic regions (Devinsky et al., 1988). “Complex” partial seizures are episodes of confused behavior, during which the patient is out of contact with the environment. Consciousness is said to be “impaired”. “Spiking” is unilateral or more commonly bilateral (Gastaut, 1970).
Partial seizures of any sort may "generalize" to the whole brain, producing a loss of consciousness and tonic-clonic convulsions. The generalized seizure is then termed a "secondarily" generalized seizure (So, 1995).

1.3 Therapy for Epilepsy

1.3.1 Antiepileptic Drugs (AED's)

Currently, pharmacological treatment is the first line of defense against seizures. In most cases, it is the best option available (Aiken and Brown, 2000).

Traditional drugs (such as phenytoin, carbamazepine and phenobarbital) stop seizures by either acting as GABA-A receptor agonists or by blocking calcium or sodium channels (Macdonald and McLean, 1986). These drugs are called "first generation" AED’s in contrast to the newer drugs which have been introduced recently. The newer drugs are called "second generation" AED’s (Loscher, 1998).

A number of "Second generation" AED’s have been introduced in the past decade. In general, they have the same mechanisms of action as the old AED’s (Loscher, 1998) (see Appendix 1). Perhaps for this reason, the new AED’s have failed to provide significantly greater control of drug-resistant seizures than the first generation AED’s.
(Loscher, 1998) - although they do have fewer side effects (Rogvi-Hansen and Gram, 1995). Clinical success with non-drug therapies (such as the ketogenic diet) has proved that drug-resistant seizures can be suppressed, and has provided hope that drugs working through novel mechanisms could be effective against drug-resistant seizures.

A discussion of both the older and newer drugs that are on the market in Canada is provided in Appendix 1.

1.3.2 Prognosis for Seizure Control

The majority of patients (about 60%) suffering from epilepsy can control their seizures adequately with current medications (Shorvon, 1996). Approximately 20% of patients attain partial control, and about 20% completely resist drug control (Shorvon, 1996). Patients with drug-resistant seizure are said to have “intractable” epilepsy.

Anticonvulsants that act through novel mechanisms may be more effective against intractable seizures, and may lead to fewer and less severe side effects. It is possible that drugs related to steroid sex hormones might provide anticonvulsant compounds that work by a novel mechanism.
1.4 Steroid Sex Hormones and Epilepsy

1.4.1 Catamenial Seizures

The possibility that sex hormones may affect seizure thresholds is suggested by the phenomenon of catamenial epilepsy.

Seizure frequency in many women is related to the phases of their fertility cycle—
and, thus, to the levels of progesterone and estrogen (Herzog, 1999). Seizures in these patients are called "catamenial". The prevalence of catamenial seizures is unclear, but, estimates suggest that they occur in ten to seventy percent of the female epileptic population (Backstrom, 1976; Bauer et al., 1995). The majority of women suffering from catamenial epilepsy have seizures of the complex partial variety (Cummings et al., 1995).

Until recently, it was thought that catamenial exacerbation was due simply to a high ratio of estrogen to progesterone, estrogen being thought to be proconvulsant and progesterone being though to be anticonvulsant (Backstrom, 1976). Herzog (Herzog et al., 1997a), however, has recently proposed that there are three distinct patterns of catamenial seizures with different hormonal profiles: 1) Pattern One is seen in women whose seizure frequency increases around the time of ovulation. This correlates with a surge in estrogen levels (Herzog et al., 1997b). 2) Pattern Two is seen in women who have increased seizures during the entire luteal (post ovulation) phase. During the luteal phase, progesterone (and estrogen) levels are normally high. Women displaying this
pattern of seizure frequency, however, may have chronically elevated levels of estrogen or may have deficient levels of progesterone (Bonuccelli et al., 1989; Murri and Galli, 1997; Narbone et al., 1990). 3) Pattern Three is seen in women whose seizures get worse during the perimenstrual period. This occurs at the end of the luteal phase when the levels of both estrogen and progesterone are are dropping. This pattern may correspond to a period of “progesterone withdrawal” (Herzog et al., 1997b).

Relative to progesterone withdrawal, Smith’s group has recently shown that progesterone withdrawal enhances excitation by rendering GABA-A receptors less insensitive to the effects of endogenous GABA (Smith et al., 1998c).

Further evidence that seizure incidence is affected by sex hormones comes from studies that show seizure onset, or an increase in seizure frequency, at puberty (Herzog, 1991). Other studies have shown that seizure frequency is reduced, or that seizures lose their catamenial pattern, after menopause (Harden et al., 1999).

The phenomenon of catamenial epilepsy suggests that estrogen is proconvulsant, and that progesterone is anticonvulsant. If progesterone is anticonvulsant, conceivably it could be used as a treatment for epilepsy. The focus of this thesis is an in-depth pharmacological study of progesterone as an anticonvulsant.
1.4.2 Steroid Sex Hormones: General Background

The steroidal metabolic pathway is illustrated in Figure 1. The best-known steroid sex hormones are progesterone and estrogen, which are secreted primarily from the ovaries in females, and testosterone, which is secreted primarily from the testicles in males. Estrogen and progesterone are not exclusively found in females, however, and testosterone is not exclusively found in males. Low levels of testosterone are found in females (McNatty, 1981). Likewise, males produce low levels of progesterone and estrogen (Berruti, 1998).

Peptide hormones released from the hypothalamus and the pituitary control the release of steroid sex hormones in the periphery. The hypothalamus releases the peptide gonadotropin-releasing hormone (GnRH) (Reindollar et al., 1985). GnRH controls the release of the peptides follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary. FSH and LH control spermatogenesis in males, which involves the production of testosterone (Franz, 1988). They also control the development of follicular growth and the corpus luteum in females, which results in the production of progesterone and estrogen (Baird and McNeilly, 1981).

Steroid sex hormones released in the periphery enter the brain and provide positive and negative feedback to both the hypothalamus and the anterior pituitary (McNeilly, 1988). Since they have a high partition coefficient, they easily cross the blood-brain barrier.
Figure 1
Metabolic Pathways of Sex Hormones
Hormonal release is relatively constant in males, but it is cyclical in females. Figure 2 demonstrates the variation in levels of progesterone and estrogen during the female fertility cycle. The fertility cycle in humans is divided into the follicular phase, ovulation, and the luteal phase. A primary follicle contains an ovum surrounded by several layers of granulosa and theca cells, which produce estrogen (and some progesterone) (Baird and McNeilly, 1981). In the follicular phase, a primordial follicle grows to maturity – mainly under the control of FSH (Baird and McNeilly, 1981). Just prior to mid cycle, increasing LH and FSH secretion cause rapid follicular growth and begin to alter the function of the granulosa and theca cells from the production of estrogen to the production of progesterone (Franz, 1988). This culminates in a surge in LH and FSH release, which causes ovulation (Franz, 1988). Ovulation is the expulsion of the ovum from the follicle and the conversion of the granulosa cells to lutein cells. Together, these cells make up the corpus luteum. The conversion is mainly under the control of LH (Cooke, 1988). After ovulation, the luteal phase begins. The corpus luteum produces large quantities of progesterone and estrogen (Cooke, 1988). Negative feedback from progesterone onto the hypothalamas leads to low levels of LH and FSH. The end of the luteal phase is marked by the degeneration of the corpus luteum, which results in a sharp drop in the levels of progesterone and estrogen. The degradation of the corpus luteum also causes menstration, and the start of a new ovarian cycle (Franz, 1988).
1.4.3 Progesterone and Estrogen as Neurosteroids

Progesterone and estrogen are gonadal hormones that act peripherally, and also enter the brain to provide feedback. In addition, they are "neurosteroids", which means that they are both produced and catabolized within the brain (McEwen, 1992). The enzymes that synthesize and metabolize sex hormones are widespread in the brain, and are found in many structures outside of those involved in sexual function.

1.4.4 Synthetic and Metabolic Pathways

The synthetic pathways involved in steroidogenesis are shown in figure 1. They are similar in the body and the brain. Progesterone is derived from pregnenolone, which is derived from cholesterol (figure 1). Progesterone is metabolized to allopregnanolone (figure 1). Through another pathway, progesterone is also the precursor for testosterone and estrogen. Aromatase converts testosterone to estrogen.

The enzymes involved in the catabolism of progesterone to allopregnanolone are two Cyp450 enzymes: 5-alpha reductase and 3-alpha dehydrogenase. Allopreganaolone is a potent agonist and an allosteric modulator of the alpha subunit of the GABA-A receptor (Maitra and Reynolds, 1998). Brain distribution and other pertinent details of these enzymes are provided in Appendix 2.
Figure 2b.

Figure 2a.

From Freeman, 1994.

From Vander et al., 1994.

4-day estrous cycle in the rat. The diagram is taken

Systemic plasma hormone concentrations during the

Figure 2b.

Figure 2a.

From Freeman, 1994.

From Vander et al., 1994.

4-day estrous cycle in the rat. The diagram is taken

Systemic plasma hormone concentrations during the
The diagram is taken from Sherwood, 2009.

Progression and estrogen at different times of the fertility cycle. Feedback control of the hypothalamic-pituitary system by pituitary hormones and the pituitary...

Figure 2c
In the periphery, it is primarily the gonads, which synthesize sex steroids. Catabolism occurs primarily in the liver (Feuer, 1983). In the brain, both glia and neurons contribute to steroidogenesis (Zwain and Yen, 1999). Astrocytes are the most active steroidogenic cells. They produce both progesterone and dehydroepiandrosterone (DHEA). Oligodendrocytes predominantly produce pregnenolones. Neurons predominantly produce estrogens (Zwain and Yen, 1999).

1.4.5 Commercially Available Progesterone, MPA and Ganaxolone

Progestins are synthetic compounds that mimic progesterone. Progesterone and a number of progestins are commercially available and are used clinically to treat a variety of conditions. Indications include contraception, hormone replacement therapy, amenorrhea, dysfunctional uterine bleeding and endometrial hyperplasia (Apgar and Greenberg, 2000).

Progestins are categorized according to the time of market introduction or according to structural derivation. Ethynoldiol diacetate and norethindrone are examples of “estranes”. Norgestrel, levonorgestrel, desogestrel, gestodene, and norgestimate are examples of “gonanes”. Medroxyprogesterone acetate (MPA) is an example of a “pregnane” (Apgar and Greenberg, 2000).
MPA and natural progesterone have been used successfully in clinical trials to treat catamenial seizures (Herzog, 1995; Mattson et al., 1984). MPA is available in oral tablets (Provera®) and in injectable form (Depo-Provera®). Natural progesterone is orally administered in micronized progesterone tablets (Prometrium®) and as a vaginal gel (Crinone®) (Apgar and Greenberg, 2000).

In the present studies, natural progesterone and MPA were used. An important difference between these is that natural progesterone is metabolized to allopregnanolone, which enhances GABAergic activity, whereas MPA is not.

Ganaxolone is a synthetic steroid whose structure mimics allopregnanolone, the progesterone metabolite. Like allopregnanolone, ganaxolone binds to the steroid site of the GABA-A receptor, and increases chloride flux (Carter et al., 1997). Like most GABA-A-acting drugs, ganaxolone exacerbates absence seizures (Snead, 1998). In clinical trials for epilepsy, ganaxolone has had only mild beneficial effects on intractable seizures (Kerrigan et al., 2000; Laxer et al., 2000). Allopregnanolone itself may be useful in preventing the withdrawal effects of progesterone in catamenial seizures (Reddy and Rogawski, 2000).

1.4.6 Sex Steroids and the Brain: Receptors
Many lines of evidence suggest that sex hormones affect brain excitability (McEwen, 1999) and seizure thresholds (Woolley and Schwartzkroin, 1998). Most studies have found that estrogen is excitatory (and proconvulsant) and that progesterone is inhibitory (and anticonvulsant) (Woolley and Schwartzkroin, 1998).

While the main focus of this thesis is progesterone and its receptors, estrogen and its receptors will be discussed as well, since the two hormones are intimately related. Progesterone and estrogen both cause physiological changes in the brain at the molecular and cellular levels (Smith et al., 1998a; Woolley and Schwartzkroin, 1998) and at the behavioral level (Steiner, 1992), and there is some evidence of functional antagonism between the two hormones (Brann et al., 1988). There are also instances of synergism between the two (McNicol, Jr. and Crews, 1979; Williams et al., 1981). It is difficult to discuss the effects of one hormone without delving into the effects of the other. It is possible that some of the anticonvulsant effects of progesterone relate to its functional antagonism of estrogen.

1.4.6.1 Estrogen Receptors

The best-studied estrogen receptors are the intracellular receptors, which mediate gene transcription. There are two types of the “classic” (intercellular) estrogen receptors (ER’s): alpha estrogen receptors (ERα), and beta estrogen receptors (ERβ). The two
isoforms are highly homologous in all domains except in the amino terminal (Delaunay et al., 2000). Both isoforms are capable of transcriptional activation, although ERβ has weaker activity (Delaunay et al., 2000). After binding estrogen, the ER’s homo- or heterodimerize and bind to the estrogen receptor response elements (ERE), enhancing transcription (Delaunay et al., 2000).

Estrogen is involved in the regulation of a multitude of genes, including the induction of progesterone receptors (Blankenstein et al., 1995), c-fos (Giannakopoulou et al., 2001), glanin (Howard et al., 1997), gonadotropin receptors (Xiong et al., 1994) and oxytocin (Richard and Zingg, 1990). Interestingly, estrogen quickly down-regulates its own receptors (Brown et al., 1996a).

In the rat brain, both ERα and ERβ are widely distributed (table 1) (Shughrue et al., 1998). In the limbic structures, ERα and ERβ mRNA are most abundant in the nuclei of the amygdala. The lateral nuclei, however, lack both isoforms, and the central nuclei lack ERβ. Piriform, entorhinal, and isocortex are weakly labeled for ERα, but are more intensely labeled for ERβ. The same pattern is seen in the hippocampus, with greater concentration of receptors in the ventral region. It should be noted that Shughrue’s data contrast with Orikasa’s findings that ERα, but not ERβ, are present in the hippocampus (Orikasa et al., 2000). Orikasa, however, used intact rats, whereas Shughrue used ovariectomized rats. Within the hippocampus, McEwen’s group has found that ER are
highly concentrated in the hilus and that they are found only in interneurons (Weiland et al., 1997a).

Gender and age do not seem to affect ER distribution (Orikasa et al., 2000). Males and females have similar levels of ER’s (in males testosterone is metabolized into estrogen, which then binds the ER’s) (Brown et al., 1996b). Sex differences in ER distribution, however, are found in the hypothalamus (Brown et al., 1996b), with females having higher levels than males. Raab et al. found that in the midbrain ERα is present in both the pre and postnatal periods, whereas ERβ is only expressed postnatally (Raab et al., 1999). ERα and ERβ are both found in the brains of infants and children, although levels of stimulation are low until puberty.

Significant interspecies differences do exist in ER distribution (Osterlund et al., 2000b). As compared to the rat, primates lack ERα mRNA in the medial nuclei of the amygdala. Primates also lack ERα mRNA in the amygdoloidal areas related to fear (central and basolateral nuclei). The highest expression of ER in the amygdala of primates (including humans) is in the amygdala-hippocampal area, the posterior cortex nucleus, the accessory basal, and the periamygdala cortex. There are significant species differences in the hypothalamus and cerebral cortex also. In the hypothalamus, ERα mRNA levels are very high in the paraventricular and supraoptic regions of primates as compared to rats. In rats, ERβ is highly expressed in these regions. In the cerebral cortex, there is no laminar pattern of ERα distribution in rats. In the human, there are
<table>
<thead>
<tr>
<th>Hemisphere</th>
<th>Posterior Nucleus</th>
<th>Medial Nucleus</th>
<th>Ventral Nucleus</th>
<th>Amygdala</th>
<th>Bed Nucleus of the Stria Terminalis</th>
<th>Hippocampal Formation</th>
<th>Layer 6</th>
<th>Layer 4-5</th>
<th>Dentate Gyrus</th>
<th>CA1-CA3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lateral</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + +</td>
<td>+ + + +</td>
<td>+ + +</td>
<td>+ +</td>
<td>+</td>
<td>+ +</td>
<td>+ + + +</td>
</tr>
<tr>
<td>Medial</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + +</td>
<td>+ + + +</td>
<td>+ + +</td>
<td>+ +</td>
<td>+</td>
<td>+ +</td>
<td>+ + + +</td>
</tr>
<tr>
<td>Sephun</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ +</td>
<td>+</td>
<td>+ +</td>
<td>+ + + +</td>
</tr>
<tr>
<td>Insular</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ +</td>
<td>+</td>
<td>+ +</td>
<td>+ + + +</td>
</tr>
<tr>
<td>Subicular</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ +</td>
<td>+</td>
<td>+ +</td>
<td>+ + + +</td>
</tr>
<tr>
<td>Cingulate</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ +</td>
<td>+</td>
<td>+ +</td>
<td>+ + + +</td>
</tr>
<tr>
<td>Prefrontal</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ +</td>
<td>+</td>
<td>+ +</td>
<td>+ + + +</td>
</tr>
</tbody>
</table>

Table 1: Brain Distribution of Sex Hormones and Related Enzymes
<table>
<thead>
<tr>
<th>Brain Region</th>
<th>ER</th>
<th>ERα</th>
<th>ERβ</th>
<th>Mem. ER</th>
<th>PR</th>
<th>PRA</th>
<th>PRB</th>
<th>Mem. PR</th>
<th>Aromatase</th>
<th>Reductase</th>
<th>HSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subthalamic nucleus</td>
<td>+</td>
<td>+³</td>
<td>+³</td>
<td>+³</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Habenula</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medial (Habenula)</td>
<td>+³</td>
<td>+³</td>
<td>+³</td>
<td>+³</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lateral (Habenula)</td>
<td>+³</td>
<td>+³</td>
<td>+³</td>
<td>+³</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+³</td>
<td>+³</td>
<td>+³</td>
<td>+³</td>
<td>+³, +³, +³, +³, +³</td>
<td>+³, +³, +³, +³</td>
<td>+³</td>
</tr>
<tr>
<td>Preoptic area</td>
<td>+³</td>
<td>+³</td>
<td>+³</td>
<td>+³</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lateral</td>
<td>+³</td>
<td>+³</td>
<td>+³</td>
<td>+³</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medial</td>
<td>+³</td>
<td>+³</td>
<td>+³</td>
<td>+³</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Periventricular</td>
<td>+³</td>
<td>+³</td>
<td>+³</td>
<td>+³</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Periventricular nucleus</td>
<td>+³</td>
<td>+³</td>
<td>+³</td>
<td>+³</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paraventricular nucleus</td>
<td>+³</td>
<td>+³</td>
<td>+³</td>
<td>+³</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Suprachiasmatic nucleus</td>
<td>+³</td>
<td>+³</td>
<td>+³</td>
<td>+³</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Supraoptic nucleus</td>
<td>+³</td>
<td>+³</td>
<td>+³</td>
<td>+³</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arcuate nucleus</td>
<td>+³</td>
<td>+³</td>
<td>+³</td>
<td>+³</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dorsalmedial nucleus</td>
<td>+³</td>
<td>+³</td>
<td>+³</td>
<td>+³</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lateral hypothalamus</td>
<td>+³</td>
<td>+³</td>
<td>+³</td>
<td>+³</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ventralmedial nucleus</td>
<td>+³</td>
<td>+³</td>
<td>+³</td>
<td>+³</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preoptic nuclei</td>
<td>+³</td>
<td>+³</td>
<td>+³</td>
<td>+³</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Supramammillary nuclei</td>
<td>+³</td>
<td>+³</td>
<td>+³</td>
<td>+³</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tuberomammillary nuclei</td>
<td>+³</td>
<td>+³</td>
<td>+³</td>
<td>+³</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mamillary nuclei</td>
<td>+³</td>
<td>+³</td>
<td>+³</td>
<td>+³</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Subcomissural organ</td>
<td>+³</td>
<td>+³</td>
<td>+³</td>
<td>+³</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pituitary</td>
<td>+³</td>
<td>+³</td>
<td>+³</td>
<td>+³</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cerebellum</td>
<td>+³</td>
<td>+³</td>
<td>+³</td>
<td>+³</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medulla</td>
<td>+³</td>
<td>+³</td>
<td>+³</td>
<td>+³</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 George, Ojeda, 1982
2 Lauber, 1994
3 Lephart, 1992
4 MacLusky, 1985
5 Sanghera, 1991
6 Pelletier, 1994
7 Guerra-Araiza, et al. 2000
8 Shughre et al. 1997
9 Shughre et al. 1998
10 Orikasa et al. 2000
11 Hagihara, et al. 1992
12 Shughre, Lane, 1997
13 Weiland, Orikasa, 1997
14 Compagnone, Mellon, 2000
15 Camacho-Arroyo et al. 1998
16 Lauber et al. 1991
17 Szabo et al. 2000
18 Cammancho-Arroyo, 1998
19 Shinoda, 1994
20 Brown et al., 1987
21 Lauber, Lichensteiger, 1994
22 MacLusky et al. 1994
23 Lauber, Lichensteiger, 1996
24 Peletti, Martini, 1999
25 Cheng, 1994

24
low levels of ERα in all cortical areas, and layer five of the temporal cortex expresses higher levels of ERα mRNA. In the human hippocampus, both ERα and ERβ are present (with ERβ being more prominent in the hippocampus than in any other region of the brain) (Osterlund et al., 2000a). In the rat, however, only ERα is present in the hippocampus (Orikasa et al., 2000). Osterlund notes that ER distribution in human brains is in areas involved in emotion, memory and cognition (and also endocrine function of course) and may reflect estrogen’s role in these functions. Furthermore, estrogen levels are significantly different between primates (including the human) and rodents - primates having much higher levels than rodents. Therefore, estrogens or anti-estrogens may have differential effects on behavior in rats and humans.

Recently, cell-surface (membrane) receptors for estrogen have also been described (Ramirez et al., 1996). These receptors do not have transcriptional activity and presumably mediate changes in membrane excitability (Ramirez et al., 1996). The “fast” or “non-genomic” effects of estrogen have been attributed to these membrane receptors (Wong et al., 1996).

Functionally, at a molecular level, cell-surface estrogen receptors have been found to: 1) activate the adenylate cyclase system (Morozova et al., 1990), 2) induce the phosphorylation of the cAMP response element (Zhou et al., 1996), and 3) stimulate nitric oxide synthase activity by increasing intracellular calcium concentrations (Baldi et al., 2000; Stefano et al., 2000). Their net effect on ion channels is to: 1) decrease L-type
calcium currents (Mermelstein et al., 1996), and 2) increase kainate-induced currents (Gu et al., 1999). These effects are excitatory in nature (Joels, 1997). The relevance of these receptors to the effects of estrogen on behavior, or brain excitability, is not well understood yet.

1.4.6.2 Progesterone Receptors

Like the ER's, the "classic" progesterone receptors (PR) are intracellular. They also come in two forms: progesterone receptor A (PR-A) and progesterone receptor B (PR-B). PR-A is identical to PR-B except that PR-A is amino-terminal truncated (Giangrande et al., 1997). PR-A is missing the first 164 amino acids that are present in PR-B. The two isoforms are obtained from the same copy of the PR gene, but they have distinct estrogen-inducible promoters (Giangrande and McDonnell, 1999).

PR-B is a transcriptional activator. PR-A, on the other hand, functions as a transcriptional repressor (Giangrande and McDonnell, 1999). PR-A inhibits the transcriptional activity of all steroid hormone receptor genes. The opposing transcriptional activities of the two isoforms are mediated through distinct signaling pathways (Wen et al., 1994). This is due to differential cofactor binding. PR-A has a higher affinity for the corepressor Silencing Mediator for Retinoid and Thyroid hormone receptors (SMRT). PR-B has a higher affinity for the coactivators Glucocorticoid
Receptor Interacting Protein 1 (GRIP1) and Steroid Receptor Coactivator 1 (SRC1) (Giangrande et al., 2000).

PR-A and PR-B are estrogen-inducible, but they also engage in 'cross-talk' by affecting ER transcriptional activity (Katzenellenbogen, 2000). PR-A is a trans-dominant repressor of the transcriptional activity of other sex and stress hormones. This effect involves a noncompetitive interaction through distinct cellular targets or distinct contact sites within the same target (Wen et al., 1994). The repressor effects of PR-A, including its anti-estrogenic effects, can be induced by progesterone receptor antagonists such as RU486 (mifepristone) (McDonnell and Goldman, 1994) or ZK98299 (onapristone) (Vegeto et al., 1993). Hence it appears that these compounds have agonist activity with PR-A.

PR’s are involved in the regulation of many genes. These include the GnRH (Kepa et al., 1996) and FSH genes (O’Conner et al., 1997), as well as the genes related to ER’s.

Similar to the ER, PR-A and PR-B have a pattern of brain distribution that extends far beyond the hypothalamic nuclei involved in sexual function. Within the isocortex, layers two and four express high levels of PR. The cortical nucleus of the amygdala also expresses high levels of PR (Hagihara et al., 1992) (note: Hagihara did not distinguish between receptor sub-types). Both PR-A and PR-B are equally expressed in the hippocampus (Guerra-Araiza et al., 2000). Within the hippocampus, the pyramidal
layers of CA1 and CA3 fields express high levels of PR (Hagihara et al., 1992). The normal function of these widespread receptors is not clear. They may, however, play a role in progesterone’s anticonvulsant effects.

PR levels change during the estrous cycle. This is probably due to the estrogen-inducible nature of the PR. Therefore, the question arises as to whether estrogen synergizes progesterone’s anticonvulsant effects or whether progesterone antagonizes estrogen’s proconvulsant effect? The interaction between estrogen and progesterone may be important in progesterone is actions as an anticonvulsant. It is important to note that both PR-A and PR-B expression can be estrogen sensitive or insensitive (Camacho-Arroyo et al., 1998b). This sensitivity is determined by the region of the brain and not necessarily (but possibly) by the receptor isoform. The PR levels vary during the estrous cycle in the hypothalamus and the frontal cortex, but the hippocampal receptors are not affected (Guerra-Araiza et al., 2000). In the hypothalamus, both isoforms are induced by estrogen and down-regulated by progesterone(Camacho-Arroyo et al., 1998a). Estrogen treatment does not affect PR mRNA in the amygdala of either male or female rats (Lauber et al., 1991).

PR’s are found in the brains of both females and males. There are some male/female differences in the distribution and levels, however. These differences are most evident in regions of the brain related to sexual function such as the hypothalamus (Khisti et al., 1998). There is no significant gender-related difference in estrogen-induced PR in cortical regions.
PR’s are also found in pre-pubertal brains. In the cortex, the intracellular PR is detectable at post-natal day one. PR levels rapidly increase, starting on day seven, and reach maximum levels (higher than adult) at day ten. Thereafter, PR expression drops gradually to adult levels. A similar early pattern of expression is seen in the hypothalamic preoptic area, except that levels remain constant after day fourteen (Gee et al., 1988). The ontogenic development of PR’s is similar in both sexes (with the exception of particular nuclei mentioned above).

Recently, cell-surface PR’s have been discovered. These are distinct from the “classic” intracellular PR’s. Little is known as yet about the function of these cell surface receptors, although the “fast” effects of progesterone have been attributed to them (Joels, 1997). These include inhibition of purkinje neurons (Smith, 1991) and stimulation of sperm functions (Calogero et al., 2000). Recently, Morrison’s group has shown that membrane PR’s stimulate tyrosine kinase activity, which results in phospholipase C activation (Morrison et al., 2000). In sperm, progesterone enhances calcium currents through its membrane receptor (Foresta et al., 1993). While these effects should enhance excitability, membrane PR’s are curiously associated with decrease excitability in neurons (Joels, 1997).

1.4.7 Some Other Notable Neuro-active Steroids
Progesterone, allopregnanolone and estrogen are not the only neuro-active steroids involved in the normal and abnormal function of the brain. Stress hormones, androgens and pregnenalone can also alter neuronal excitability (Rupprecht and Holsboer, 1999).

Administration of stress hormones (i.e., corticosterone) generally increases seizure thresholds in both animals (Stitt and Kinnard, 1968) and in humans (Aird 1951). Paradoxically, stress can trigger seizures in some epileptic patients (Frucht et al., 2000; Spector et al., 2000).

Testosterone appears to be proconvulsant (Rosse et al., 1990). This may be due to the conversion of testosterone to estrogen (Edwards et al., 1999a).

Pregnenolone sulfate is proconvulsant and decreases seizure thresholds (Kokate et al., 1999; Maione et al., 1992; Reddy and Kulkarni, 1998). Pregnenolone does this in two ways. First, pregnenolone antagonizes GABA-A receptors (Majewska et al., 1988; Majewska and Schwartz, 1987; Mienville and Vicini, 1989). Second, it allosterically potentiates NMDA receptors (Wu et al., 1991).

1.5 Animal Seizure Models
The fact that sex hormones affect excitability in many regions of the brain - and that some of the effects are inhibitory - suggests that sex hormones might be used pharmacologically to control seizures. The focus of the present thesis was the pharmacological antagonism of seizures with progesterone. The work was done using animal seizure models. It was done to supplement past clinical studies, and to provide a basis for future clinical work.

Animal seizure models are used in drug development because unproven potential AED's cannot be tested legally or ethically on epileptic patients. These models should reliably produce seizure activity. They should also be easy to use and have clinical relevance (Fisher, 1989).

A number of animal models are used to study seizures and the epilepsies (Fisher, 1989). Most commonly, acute "seizure" models are used. These involve electrical brain stimulation or the systemic administration of various chemical convulsants. In these models, seizures do not occur spontaneously. They occur only when the experimenter applies the epileptogenic stimulus (Fisher, 1989).

Some investigators have argued that these acute preparations do not fully model clinical epilepsy since the seizures are not spontaneous (Avanzini, 1995). A number of chronic "epilepsy" models - where the seizures occur spontaneously - do exist (Fisher, 1989). Some of these are genetic models, whereas others involve post-status "spontaneous recurring seizures" (SRS) (Nissinen et al., 2000).
While these spontaneous models have some attractive features, they have never been validated pharmacologically. To test the anticonvulsant properties of new compounds, models with known and valid pharmacological profiles are necessary. Therefore, acute, validated models have been used in the present studies. These models are discussed below.

1.5.1 Electroconvulsive Shock Seizures: Maximal (MES) and Threshold (ECS)

The maximal electroconvulsive shock seizure (MES) model is the standard animal model for human tonic-clonic seizures (Woodbury, 1972). High-intensity electrical stimulation is passed through the subject's brain using corneal or pinnaeal electrodes. Usually, mice or rats are used. Standard current parameters are 60 Hz sine-wave current for 0.2 seconds at 50 mA in mice or 150 mA in rats (Swinyard, 1972). The seizures begin with a brief period of tonic flexion, followed by forelimb and hind limb tonic extension. Anticonvulsants that suppress the tonic hindlimb extension in rodents – such as phenytoin and phenobarbital – also suppress tonic-clonic seizures in man (Woodbury, 1972).

Lower-intensity electrical currents – applied in the same manner – produce seizures that are exclusively clonic (Woodbury, 1972). These submaximal seizures are called “threshold” electroconvulsive shock seizures (ECS). Anticonvulsants that suppress
forelimb clonus in the "threshold" ECS model – such as ethosuximide and valproic acid – also suppress absence seizures in man (Woodbury, 1972).

In administering ECS or MES, investigators should be aware of species, gender and age differences. With regard to species, the threshold for ECS and MES is higher in rats than in mice (Swinyard, 1972).

With regard to gender, females tend to have lower seizure thresholds than males (Kokka et al., 1992; Sackeim et al., 1987). The ECS threshold for female rats is 16 mA, for instance, and for males it is 19 mA (Kokka et al., 1992).

With regard to age, developmental variations are particularly dramatic (London and Buterbaugh, 1978). Immediately after birth, rats have remarkably high ECS and MES seizure thresholds (over 40 mA for the clonic threshold, over 160 mA for the tonic threshold). The threshold drops rapidly to base-line levels over the first three weeks of life. At day 21, the threshold for clonic seizures is approximately 15 mA and for tonic seizures, approximately 30 mA. Thereafter, thresholds rise slightly into adulthood (London and Buterbaugh, 1978).

1.5.2 Pentylenetetrazol: Maximal (MMT) and Threshold (MET)

Pentylenetetrazol (also called "Metrazol" and "PTZ") is a chemical convulsant, which antagonizes GABA-A receptors non-selectively (Pellmar and Wilson, 1977).
A subcutaneous injection of a high dose of pentylenetetrazol (85mg/kg in rat) produces forelimb and hindlimb tonic flexion and extension in rats. This is the "maximal metrazol seizure" model (MMT) (Fisher, 1989). Like the tonic seizures induced by electrical currents, MMT seizures mimic tonic-clonic seizures in man (Woodbury, 1972).

A lower subcutaneous injection of 70mg/kg in rats (85mg/kg in mice) of pentylenetetrazol produces clonic seizures. This is the "threshold metrazol seizure" model (MET) (Stone, 1972). Like the clonic seizures produced by electrical stimulation, MET seizures mimic absence seizures in man (Ferrendelli et al., 1989).

There are also species, gender and age differences in the MMT and MET models. With regard to species, rats require slightly lower doses of the convulsant than mice to produce clonic or tonic seizures (Fisher, 1989).

With regard to gender, females have higher seizure thresholds than males (Kokka et al., 1992). Twenty-two mg/kg of tail-vein infused pentylenetetrazol produces clonic and tonic seizures in female rats. The same response is seen with 17mg/kg in male rats (Kokka et al., 1992).

With regard to age, two conflicting alterations occur. Infant rats have very high seizure thresholds that drop over the first three weeks of life. After that they rise again.
Curiously, although the thresholds are high in infants, the latency to the onset of seizures is much shorter in infants than in adults (Velisek et al., 1992).

1.5.3 Kindling

Kindling is a technique for producing focal seizures with secondary generalization (Goddard et al., 1969; Racine, 1972). In the kindling model, an electrode is permanently implanted in the brain of an animal subject (often a rat). The subjects are then stimulated at intervals (often daily) with a low level of electrical stimulation. At first, only a localized (“focal”) seizure occurs. With repeated stimulations, however, the seizure activity spreads to other sites (or “generalizes”), as measured by the electroencephalograph (EEG). Eventually, after motor structures have become involved convulsions occur (Racine, 1972).

When the kindling electrode is implanted in limbic structures, such as the amygdala or the hippocampus, the kindling preparation models complex partial seizures with secondary generalization (Albright and Burnham, 1980). Albright and Burnham showed that focal limbic discharge in kindled subjects has a pharmacological profile similar to human complex partial seizure (Albright and Burnham, 1980). They found that anticonvulsants successfully suppressed the generalized component of seizures, but were unable to raise seizure thresholds in the focus at clinically relevant doses (Albright and Burnham, 1980; Albright, 1983). In humans, anticonvulsants also suppress secondarily
generalized seizures, but fail to suppress limbic focal (complex partial) activity. Hence, the kindling model is a pharmacologically valid model of human complex partial seizures.

Species, gender, and age also affect kindling. All species that have been tested (including primates) kindle (Wada et al., 1978), (Wada, 1981). Seizure thresholds and the rate of kindling, however, vary with the species. Higher animals, including primates, tend to kindle more slowly (Wada, 1981).

With regard to gender, differences in kindling rate or seizure threshold between males and females have not been studied. There are, however, reports on the effect of the sex hormones on kindling thresholds and kindling rates. With regard to estrogen, Edwards et al. found that it lowered seizure threshold and increased the kindling rate (Edwards et al., 1999b). Buterbaugh also saw a similar increase in kindling rate with estrogen (Buterbaugh, 1987). Wahnschaffe, however, did not observe variations in seizure threshold across the estrous cycle (Wahnschaffe and Loscher, 1992). With regard to progesterone, Edwards et al. (1999) have found that it raises the seizure threshold and decreases the kindling rate.

With regard to age, Moshe (1998) has shown that rat pups kindle at a faster rate and to higher seizure stages than adult subjects. Kindling threshold in rat pups has not been reported. Recently, however, Edwards et al. (unpublished data) have shown that rat pups (fourteen days old) have remarkably high seizure thresholds.
1.6 Past studies of Progestin Effects on Seizures

1.6.1 Clinical Studies

Clinicians have used progestin therapy to control intractable catamenial seizures for more than half a century. Table 2a presents a summary of the case studies and open trails that have used progesterone or medroxyprogesterone acetate, a synthetic progestin. Positive results have been reported in all studies. Although the earlier studies were only case reports, Herzog (1988, 1995) and Matson's (1984) studies were actual clinical trials. Matson used medroxyprogesterone acetate (MPA), whereas Herzog used natural progesterone. Significant and similar anticonvulsant effects were shown in both studies.

Despite this success, progestins have not found widespread use even in the treatment of catamenial epilepsy. Moreover, no clinician has yet ventured to try them on the non-catamenial epileptic population. Part of the reason for this failure may be that no "blind" (single or double) clinical studies have as yet been performed to evaluate the anticonvulsant effects of progesterone. Equally lacking are convincing animal studies to support the clinical observations.

1.6.2 Animal Studies
A number of past animal studies have investigated the anticonvulsant effects of progesterone. Unfortunately, many of these have used unreasonably high (sedating) doses. When lower (therapeutic) doses have been used, the data have been inconsistent. Table 2b and table 2c provide a summary of these studies. Table 2b summarizes studies involving high doses of progesterone (over 30 mg/kg) and table 2c summarizes studies involving low doses (under 5 mg/kg).

The high dose data (Table 2b) are not contradictory. High doses of progesterone are clearly anticonvulsant (Table 2b). These anticonvulsant effects can be seen in the MET, the MES and the kindling models. The anticonvulsant doses used in these studies, however, are highly sedative (Table 2b), and they produce blood levels that are well above physiological levels (Finn and Gee, 1994). These studies do not resemble the clinical trials in which women have received relatively low doses of progesterone and progestins (Table 2a), which produce plasma levels within the physiological range. In clinical trials, patients have not suffered from excessive sedation (Table 2a).

The low dose data (Table 2c) are less complete and consistent. In the kindling model, for instance, Holmes and Weber (1984) found that progesterone retarded quick kindling in infants, but not in adult male rats. Edwards et al. (1999) reported that progesterone retards kindling in adult female rats. Also in this model, Mohammad et al. (1998) found that progesterone doses below 75 mg/kg are not anticonvulsant in male rats. Edwards et al. (1999), however, found significant anticonvulsant activity at 5 mg/kg in
female rats. These data seem to suggest that progesterone, at low doses, works in female rats, but not in male rats.

Wooley and Timaras (1962), testing electroshock threshold, found that low dose progesterone partially countered the small threshold drops caused by estrogen in ovariectomized adult female rats. Stitt and Kinnard (1968), however, found that low dose progesterone in female rats was not protective in the MES model. These data might suggest that progesterone works against threshold, but not maximal seizures. Once again, no direct comparisons have been made.

In the threshold pentylenetetrazol model, dose-response studies have shown anticonvulsant effects of progesterone at high doses only. Kokate (1998) did these studies in adult male mice, and Craig (1966) did them in both male and female mice.

In kainic acid seizures, Nicoletti et al. (1985) found anticonvulsant effects using low doses of medroxyprogesterone acetate. In this model, Frye and Bayon (1999) have also found anticonvulsant effects using low doses of progesterone. These investigators, however, have used latency measures. Changes in latency generally imply small changes in seizure thresholds.

To summarize, at low doses of progesterone – which resemble the doses used in clinical studies – the findings are incomplete and inconsistent.
Table 2a: Clinical studies of progesterone (P₄) as anticonvulsant

<table>
<thead>
<tr>
<th>Author</th>
<th>Patients</th>
<th>Epilepsy</th>
<th>Treatment</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zimmerman, A.W., et al., (1973)</td>
<td>Case report adult woman</td>
<td>Generalized Seizures (catamenial)</td>
<td>Primidone 250mg, Phenobarbital 30mg &amp; 1. Povera*(10, 20, 50 mg/day) 2. Depo-Provera (250, 250, 150 mg/2 week intervals)</td>
<td>1. No effect; 50 mg/kg showed slight efficacy 2. Complete cessation of seizures for 4 months Adverse effects: amenorrhea</td>
</tr>
<tr>
<td>Hall, S., (1977)</td>
<td>Case report adult woman</td>
<td>Generalized tonic-clonic seizures (catamenial)</td>
<td>90mg Phenobarbital &amp; Progesterone (0.35mg/day)</td>
<td>Complete seizure control over 7 months Adverse effects: amenorrhea</td>
</tr>
<tr>
<td>Mattson, R.H., et al., (1984)</td>
<td>Open clinical trial 14 adult women</td>
<td>13 complex partial, 1 absence all intractable</td>
<td>Prior medication &amp; Provera (in 8 patients, 10mg, q2-4d, PO) Depo-Provera (in 6 patients, 120-150mg, IM)</td>
<td>39% reduction in seizure frequency, (3 patients withdrew from study) Adverse effects: amenorrhea, spotting</td>
</tr>
<tr>
<td>Backström, T., et al., (1984)</td>
<td>Open clinical study 7 adult women 22-43 years old</td>
<td>Complex partial with one distinct focus, selected based on greater than 1 epileptic discharge per 5 minutes of EEG recording</td>
<td>Prior medication &amp; P₄ (IV in alcohol &amp; Ringers Glucose solution, 0.5-3 mg bolus + 4-12 mg/hr infusion, achieved luteal phase levels, 72nmol/L)</td>
<td>Significant reduction in frequency of epileptic discharges (4/7 patients)</td>
</tr>
<tr>
<td>Herzog, A., (1986)</td>
<td>Open clinical trial 8 adult women 16-41 years old</td>
<td>Complex partial, (focal paroxysmal of temporal origin, catamenial)</td>
<td>Prior medication &amp; 50-400 mg natural P₄ q2d during times of high seizure frequency (achieved luteal phase levels, 5-25ng/mL)</td>
<td>68% reduction in seizure frequency Adverse effects: transient tiredness, depression (4/8 patients)</td>
</tr>
<tr>
<td>Herzog, A., (1995)</td>
<td>Open clinical trial 25 adult women 18-40 years old</td>
<td>Complex partial (with secondary generalization, 13/25) (catamenial)</td>
<td>200 mg natural P₄ lozenges q2d during times of high seizure frequency (achieved luteal phase levels, 5-25ng/mL)</td>
<td>54% reduction in seizure frequency Adverse effects: asthenia, depression (2/25 patients)</td>
</tr>
<tr>
<td>Herzog, A., (1999)</td>
<td>Open clinical trial 15 adult women (3 year follow-up)</td>
<td>Complex partial (catamenial)</td>
<td>200 mg natural P₄ lozenges q2d during times of high seizure frequency (achieved luteal phase levels, 5-25ng/mL)</td>
<td>62% reduction in seizure frequency</td>
</tr>
</tbody>
</table>
Table 2b: Animal studies involving high-dose progesterone (P₄)

<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, H., (1942)</td>
<td>White rats young ♂</td>
<td>Pentylenetetrazol</td>
<td>P₄ (≈33 mg/kg)</td>
<td>↑ Seizure threshold</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Adverse effects: anesthesia</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2. P₄ (65 mg/kg)</td>
<td>2. ↑ Adverse effects:</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>hypnosis, anesthesia</td>
</tr>
<tr>
<td>Craig, C.R., (1966)</td>
<td>Swiss-Webster mice 20-30 g ♂ &amp; ♀</td>
<td>1. Pentylenetetrazol 85 mg/kg SC</td>
<td>P₄ dose-response: (0-1000 mg/kg)</td>
<td>↑ Seizure threshold</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1. ED₉₀=200 mg/kg</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2. Adverse effects: acute neuro-toxicity,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ND₉₀=720 mg/kg</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Craig, C.R., &amp; Deason, J.R., (1968)</td>
<td>Swiss-Webster mice 20-30 g ♂ &amp; ♀</td>
<td>Pentylenetetrazol 85 mg/kg SC</td>
<td>P₄ dose-response: (0-1000 mg/kg)</td>
<td>↑ Seizure threshold</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1. ED₉₀=200 mg/kg</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2. Adverse effects: acute neuro-toxicity,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ND₉₀=720 mg/kg</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Selye, H., (1970)</td>
<td>Holtzman rats 100 g ♀</td>
<td>Picrotoxin 3.5 mg/kg SC</td>
<td>P₄ (chronic, 100 mg/kg, q2d)</td>
<td>↑ Seizure threshold</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Adverse effects: catatonic</td>
</tr>
<tr>
<td>Mohammad, S., et al, (1998)</td>
<td>White rats 260-700g ♂</td>
<td>Kindling (amygdala) (amygdala) 5 consecutive stage 5s</td>
<td>P₄ dose-response: (0,10,30,60,75,90 mg/kg)</td>
<td>↑ Seizure threshold only @ 75 mg/kg</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Adverse effects: mild sedation @30 mg/kg,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>severe sedation @90 mg/kg</td>
</tr>
<tr>
<td>Kokate, T.G., et al., (1998)</td>
<td>NIH Swiss mice 25-50 g ♂</td>
<td>1. Pentylenetetrazol 85 mg/kg SC</td>
<td>P₄ dose-response: (50-200 mg/kg)</td>
<td>↑ Seizure threshold</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1. ED₉₀=94 mg/kg</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2. ↑ ED₉₀=250 mg/kg</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Adverse effects: sedation</td>
</tr>
</tbody>
</table>
Table 2 C: Animal studies involving low-dose progesterone (P₄)

<table>
<thead>
<tr>
<th>Author</th>
<th>Subjects</th>
<th>Seizure Model</th>
<th>Treatment</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Woolley, D., &amp; Timaras, P.</td>
<td>Long Evans Rats Immature &amp; mature</td>
<td>Electroshock seizure threshold (Woodbury paradigm, 1952)</td>
<td>1. Chronic E₂B (40,100μg/kg/day) 2. Chronic P₄ (5mg/kg/day) 3. Chronic E₂B+P₄</td>
<td>1. ↓↓ Seizure threshold 2. ↑ 3. ↓ Adverse effects: amenorrhea in intact adult♀</td>
</tr>
<tr>
<td></td>
<td>♀ (OVX) &amp; ♂</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stitt, S.L., &amp; Kinnard, W.J.</td>
<td>Wistar Rats 125-150g ♀</td>
<td>Electroshock seizure threshold (Woodbury paradigm, 1952)</td>
<td>1. P₄ (acute, 70mg/kg) 2. P₄ (chronic, 5mg/kg) 3. MPA (chronic, 10mg/kg) 4. MPA+E₂ (chronic, 10mg/kg)</td>
<td>1. ↔ Seizure threshold 2. ↔ 3. ↔ 4. ↓ Adverse effects: amenorrhea</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Holmes, G.L., &amp; Weber, D.A.</td>
<td>Sprague-Dawley rats Immature Γ (15 &amp; 30 days) &amp; Adult ☢ (56-70 days)</td>
<td>Kindling (amygdala) Kindled hourly and daily</td>
<td>P₄ (5.25 mg/kg, hourly &amp; daily)</td>
<td>↓ Kindling rate in immature rats ↔ in mature rats Adverse effects: sedation @25mg/kg in immature Γ</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nicoletti, F., et al.</td>
<td>Wistar rats 200g ♂ &amp; ♀</td>
<td>Kainic acid 15 mg/kg SC</td>
<td>MPA (chronic, 2.5 mg/kg)</td>
<td>♀, ↓ Severity ↔ latency ♀, ↑ &amp; ↔</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Landgren, S., et al.</td>
<td>Cat ♀ (OVX)</td>
<td>Penicillin focus (cerebral cortex)</td>
<td>P₄ (=100ng/mL, IV infusion)</td>
<td>↓ Spontaneous interictal spikes</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frye, C., &amp; Bayon, L.</td>
<td>Long Evans rats Young Adult (aprox. 60 days) ♀ (OVX)</td>
<td>Kainic acid, 32 mg/kg, SC 1. Kainic acid (Woodbury paradigm, 1952)</td>
<td>1. E₂B+P₄ &amp; withdrawal; (E₂B: 10μg/kg@0hr, P₄: 0.5mg/kg @44hrs, kianic acid @48hrs) 2. Silastic implants (cholesterol, E₂B+P₄, intermittent E₂B+P₄)</td>
<td>1. ↓ Seizure duration 2. ↓</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Edwards, H., et al.</td>
<td>Wistar rats 200-225g ♂</td>
<td>Kindling (amygdala) 15 consecutive stage 5s</td>
<td>P₄ (5 mg/kg)</td>
<td>↑ Seizure threshold</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
1.7 Objectives

1. Past experiments from this laboratory have suggested that progesterone is an effective anticonvulsant at a low dose (5mg/kg) in the kindling model of complex partial epilepsy (Edwards et al., 1999b). Adult female rats were used and only a single dose of progesterone was tested. The first objective of these studies was to perform a full pharmacological study of progesterone in the kindling model. Dose-response studies were done. These data are described in Experiment 1.

2. The second objective of these studies was to determine whether low dose progesterone was an effective anticonvulsant in other seizure models, especially when applied chronically.

In addition, MPA was tested. MPA has pharmacokinetic advantages over progesterone, and it is not converted to allopregnanolone (Sturm et al., 1991). Therefore, it was hoped that MPA would provide valuable information about the mechanism of the anticonvulsant action of progesterone. These data are reported in Experiment 2.

3. The third objective of these studies was to perform dose and time-response studies in both immature rats and in adult males. This was to determine whether
progesterone could be used in childhood epilepsies, or in adult men. These data are reported in Experiment 3.

4. The forth objective of these studies was to determine whether progesterone’s anticonvulsant effects were mediated via binding to progesterone receptors (intracellular or cell surface), or whether they related to progesterone's neuroactive metabolite, allopregnanolone. Progesterone’s anticonvulsant effects were assessed in the presence of RU486 – which blocks the “classic” progesterone receptors – and in the presence of indomethacin – which blocks progesterone’s conversion to allopregnanolone. These data are reported in Experiment 4.
Chapter 2:

General Methods
2 General Methods

2.1 Animals

Wistar rats (Charles River, Canada) served as subjects. The age and sex of the subjects varied and is specified for each experiment. Subjects were housed in transparent, plastic cages (24x24x45 cm) in a vivarium maintained at 18°C on a 12-hour light/dark cycle (lights on at 7:00 a.m.). Adult subjects were housed individually, while immature subjects were housed with their dams. All subjects were given free access to food (Purina Rat Chow®) and water. On test days, subjects were transported to the test room in their home cages and were allowed to acclimatize for 30 minutes prior to experimentation.

2.2 Drugs

Medroxyprogesterone acetate and indomethacin were obtained from a local pharmacy. All other drugs including progesterone, estrogen, testosterone, and beta-hydroxy cyclodextrin were obtained from Sigma-Aldrich.

2.3 Procedures for the Kindling Experiments
2.3.1 Implantation of Electrodes

Two weeks (minimum) after arrival in the animal facility, subjects were anesthetized with sodium pentobarbital (Somnitol®, 40mg/kg for female subjects, 60mg/kg for male subjects, IP) and their heads were fixed in a stereotaxic instrument. Heads were sterilized with 70% alcohol, and an iodine solution. A sagittal incision (1.5 cm) was then made in the scalp, and the superficial muscles were separated from the skull with Q-tips®. The skull was washed with normal saline solution, and bregma was located. Three holes were drilled (each 5 mm from bregma) in three quadrants of the skull and jeweler’s screws were inserted to act as anchors for the implant. A hole was then drilled at the implant site and a bipolar electrode was lowered into the right amygdala using the following coordinates: 1 mm caudal from bregma, 4.8 mm lateral from the sagittal suture, 8.5 mm ventral from the dura, The incisor bar was set at +5 mm. The electrode was cemented in place with a mixture of cranioplastic powder and cranioplastic liquid (Cranioplastics One Inc., Roanoke, VA).

Electrodes consisted of two Polyamide®-insulated, stainless wires (0.25mm diameter) (MS303/1, Plastic One, Roanoke, Virginia). Subjects were allowed 9 days for post-surgical recovery, and then daily handling was begun.

2.3.2 Kindling Procedure
Fourteen days after surgery, kindling was initiated. Kindling was performed in a plastic stimulation cage (width: 40cm, length: 50cm, height: 30cm). Daily stimulations were administered between 12:00 and 6:00 PM. The stimulus consisted of a 1s train of 1ms, 60Hz biphasic (positive – and negative – going), square-wave pulses, 500 uA (peak to peak), generated by Grass Model S88 stimulator coupled to two Grass PSIU6 constant-current stimulus-isolation units (Grass Instruments Co., Quincy, MA). Convulsive responses were scored according to Racine’s (1972) 5-stage scale of motor seizures: stage 1) mouth clonus; stage 2) head nodding; stage 3) forelimb clonus; stage 4) rearing; stage 5) loss of postural control. Electroencephalographic (EEG) activity was recorded for the duration of the after-discharge, using a Grass Model 7D polygraph. Subjects were kindled until 30 stage 5 seizures had been evoked.

2.3.3 Determination of After-discharge Threshold

After-discharge threshold (ADT) was determined using slight variations of the ascending series technique (Pinel et al., 1976) or half-split technique (Racine, 1972). The ascending series technique was used in Experiment 1. The half-split technique was used in Experiment 2. ADT was determined before kindling began and also after the subjects had had 5 stage 5 seizures.

The ascending series technique of threshold measurement involves the initial administration of a sub-threshold electrical stimulus followed by subsequent, step-wise
increases in the electrical stimulus separated by a short “inter-stimulus interval”. In the present study, ADT determination began with a stimulus of 20 uA (peak to peak). The stimulus intensity was then increased in 20 uA steps up to 320 uA; then the intensity was increased in 40 uA steps. A 1-minute interval was allowed between stimuli. ADT was defined as the minimum stimulus intensity needed to provoke an after-discharge of at least 4 seconds in non-kindled subjects or a generalized seizure in well-kindled subjects.

The half-split technique is also called the “up and down” method (Kokaia et al., 1994). The electrical current-interval between a stimulus that produces seizures and one that doesn’t is halved to obtain a new current-interval. This procedure is continued until the threshold is determined within an established margin of error. Long inter-stimulus intervals are used. In the present study, the ADT was determined over several days. On the first day, a current of 100 uA was used. Depending on whether this current induced a seizure (after-discharge of at least 4 seconds in non-kindled subjects or a generalized seizure in well-kindled subjects), the current intensity was halved or doubled on the second day. This process was continued until the interval between the lowest effective stimulus and the highest ineffective stimulus was 20 uA or less. ADT was defined as the halfway point within this interval.

2.3.4 Determination of Stability
After the ADT was determined, seizure stability at ADT plus 40% was assessed. Subjects were stimulated daily at a current intensity equal to ADT plus 40%. ‘Stability’ was defined as 5 consecutive stage 4 or 5 seizures. If subjects were not stable, the stimulus intensity for kindling was increased by an additional 40%.

2.3.5 Procedure for Drug Testing

The procedure for drug testing is specified for each experiment in the specific methods section.

2.3.6 Verification of Electrode Placement

Once kindling experiments were complete, subjects were anesthetized with sodium pentobarbital (Somnitol®, 80mg/kg) and perfused. After loss of consciousness, the site of the implantation was lesioned by a current of 8mA, lasting 30 seconds. The thoracic cavity was then opened via a medial incision, exposing the heart. A cut was made in the right auricle and a cannula (gauge 16) was inserted into the left ventricle. Perfusion began with 100mL of normal saline. The perfusion rate was 15mL/minute. Perfusion was completed with 100mL of 4% formaldehyde solution (9g NaCl, 108g 37% formaldehyde, in 1L of distilled water). Brains were then extracted and stored in 20% sucrose solution containing 4% formaldehyde. Forty-eight hours later, the brain was
withdrawn from the fixative and stored until sectioning at -80°C in a sealed plastic container.

At the time of sectioning, the brains were attached to a specimen disc using embedding medium (Tissue Tek®, Sakura Finetechnical Co., Ltd., Tokyo). They were then transferred to a cryostat (Jung CM3000, Leica Inc., Canada), maintained at -25°C, and allowed to equilibrate for 20 minutes. Sections (30um) were cut coronally. Every fifth section in the area of the electrode tip was thaw-mounted on poly-L-lysine coated glass slides and allowed to air dry.

Dried sections were stained with cresyl violet. Slides were immersed for 3 minutes in each of each of the following media: 100% ethanol, 95% ethanol, 70% ethanol, distilled water, 0.5% cresyl violet, distilled water, and 70% ethanol. Slides were then incubated for 2 minutes in 95% ethanol containing 0.1% acetic acid, and then twice in 100% alcohol. Finally, slides were incubated twice for 5 minutes each in xylene. The slides were then covered using a cover slip and Permount® (Fisher Chemicals).

The electrode tract was examined with a magnification-projection system (Research Analysis System model 421251, Amersham, MI). Only subjects with verified electrode placements in the targeted region were used in subsequent data analyses.

2.3.7 Procedure for Ovariectomy and Gonadectomy
Ovariectomy and gonadectomy were performed on some of the subjects in the kindling experiments.

Twenty-four hours after the 10th stage 5 seizures, female subjects were ovariectomized. Subjects were anesthetized with 4% halothane and then anesthesia was maintained using 1.5% halothane. Bilateral flank incisions (1 cm) were made. The uterine horn attached to the ovary and the accompanying fat were pulled through the incision. The ovary and the accompanying fat and vessels were tied (5-0 silk suture) and the oviduct severed and removed with a single cut. The uterine horn was returned to the peritoneal cavity. The muscle and skin incision were closed with one and two sutures, respectively. Subjects were allowed a 14-day post-operative recovery period before further procedures.

Male subjects were gonadectomized at the time of electrode implantation. Under general anesthesia, a single, sagittal incision (1 cm) was made in the scrotal sac. The testes and epididymes was pulled through the incision. Both tissues were severed and removed by a single cut, following suturing of the tissue above the cut. The muscle and skin incision were closed with one and two sutures respectively. Subjects were allowed 14-day post-operative recovery period before further procedures.

2.4 Procedure for Vaginal Smears
In some experiments, daily vaginal smears were performed (between 10:00 h and 10:30 h) to characterize each day of the 4-day estrous cycle in the intact female rat (Freeman, 1996). Smears were obtained by placing a blunted, plastic 20 µl Eppendorf tip on the vaginal opening and flushing the orifice twice with 10 µl of 0.9% saline. Tips were rinsed thoroughly with 70% ethanol after each use. The saline flush was displayed on a clean microscope slide and observed at 20X magnification. Phases of the estrous cycle were classified according to the following criteria: 1) diestrus – smears were characterized by a heterogeneous mixture of polymorphic leukocytes and epithelial cells; 2) proestrus – smears had predominantly large round nucleated epithelial cells; 3) estrus – smears consisted primarily of cornified epithelium with few or no nuclei visible, and 4) metestrus – smears with exclusively leukocytes in high density.

2.5  Procedure for the MET Test

The threshold pentylenetetrazol test (MET) was administered according to a modification of the protocol of Krall et al. (Krall et al. 1978). This test was used in Experiment 2 on adult female Wistar rats. Subjects were injected with 70mg/kg of pentylenetetrazol (SC) in the rough of the neck. Pentylenetetrazol was dissolved in normal saline to a concentration of 19 mg/kg. The volume of injection was 4mL/kg. Following injection, the subjects were placed in a test chamber and observed for 30 minutes. Seizures were scored as “present” or “absent”. The latency to the onset of
2.6 Procedure for the MMT Test

The maximal pentylenetetrazol test (MMT) was administered according to a modification to the protocol of Desmedt et al. (Desmedt et al., 1976). This test was used in Experiments 2, 3, and 4. Adult subjects were injected with 85mg/kg of pentylenetetrazol (SC) in the rough of the neck. Pentylenetetrazol (21mg/mL) was dissolved in normal saline. The volume of injection was 4mL/kg. Rat pups were injected with 150mg/kg of pentylenetetrazol (SC) in the rough of the neck. Pentylenetetrazol (15mg/mL) was dissolved in normal saline. The volume of injection was 10mL/kg. Following injection, the subjects were placed in a test chamber and observed for 30 minutes. The seizures were scored as "present" or "absent". The latency to the onset of seizures was also recorded. Seizure absence was defined as the absence of forelimb extension (FLE). Forelimb extension was measured as opposed to hind-limb extension (HLE), because subjects did not produce hind-limb extensions reliably in this model.

2.7 Procedure for the ECS Test
The threshold electroconvulsive shock test (ECS) was administered according to a modification to the protocol of Woodbury (1972). This test was used in Experiment 2 on adult female Wistar rats. The ECS stimulus was administered via corneal electrodes. Electrodes were wetted with normal saline prior to application to the cornea. The following parameters were used for stimulation: pulse configuration - sine wave, pulse frequency-60 Hz, train duration-0.2 seconds, pulse intensity - variable (10-50 mA). Variable pulse intensity was used because the threshold for ECS seizures was being measured. To determine ECS threshold, the half split method (as described previously) was used. The seizures were scored as "present" or "absent". Seizure absence was defined as the absence of forelimb clonus.

2.8 Procedure for the MES Test

The maximal electroconvulsive shock test (MES) was administered according to a modification to the protocol of Krall et al. (Krall et al. 1978). This test was used in Experiments 3 and 4. The ECS stimulus was administered via corneal electrodes. Electrodes were wetted with normal saline prior to application to the cornea. The following stimulus parameters were used: pulse configuration-sine wave, pulse frequency - 60 Hz, train duration-0.2 seconds, pulse intensity - 150 mA. Seizures were scored as "present" or "absent". Seizure absence was defined as the absence of hindlimb extension (HLE).
In Experiment 2 the threshold for MES was determined in adult female Wistar rats. The same procedure was followed as in the standard MES test except that variable pulse intensity (10-100mA) was used. To determine this threshold, the half split method, as described previously, was used.

2.9 Procedure for Pentylitenetetrazol Infusion

The pentylitenetetrazol infusion test allows accurate threshold measurements of the clonic or tonic seizures resulting from the administration of pentylitenetetrazol. This test was used in Experiment 2 in adult female Wistar rats. It was administered according to the protocol of Thavendiranathan et al. (2000). Pentylitenetetrazol was dissolved in normal saline to a concentration of 10mg/mL. The mixture was administered through the tail vein at an injection rate of 1 mL/minute. A Sage Instrument syringe pump (model 351) was used for administration. The infusion was stopped when tonic seizures were produced. The time to the onset of tonic seizures was recorded.

2.10 Procedure for Tests of Sedation and Ataxia

In all experiments, subjects were challenged with the righting-reflex test and Loscher's ataxia rating scale immediately prior to testing. The righting reflex test involves rolling the subject onto its back and observing whether it is able to rotate 180 degrees onto its limbs.
Loscher's test of ataxia involves simple observation, and uses the following 6-stage categories of behavioral impairments:

1. Slight ataxia  
   tottering of hind limbs

2. More pronounced ataxia  
   dragging of hind limbs

3. Further increase of ataxia  
   more pronounced dragging of hind limbs

4. Marked ataxia  
   animal occasionally loses balance during forward locomotion

5. Very marked ataxia  
   animal frequently loses balance during forward locomotion

6. Extreme ataxia  
   animal, despite attempts, is unable to move forward

2.11 Statistical Analysis

Time-response and dose-response curves were plotted for both the clonic and tonic seizures. Quantal curves were constructed by plotting the percentage of animals at each time or dose that were protected against the clonic or tonic seizures. Whenever possible, dose-response curves were fitted with sigmoidal curves and using linear regression, and ED50's were calculated from the fitted curves.
Interval-ratio data (e.g., latencies) were expressed as mean ± standard error of the mean (SEM). When two groups were being compared and the data were normally distributed, a student t-test or a paired t-test was used. When the data were not normally distributed, the Mann-Whitney Rank Sum test was used. When more than two groups were being compared and the data were normally distributed, analysis of variance (one-way ANOVA) was used. Provided that the overall analysis of variance was significant, post-hoc comparison used Duncan’s Multiple Range test. When the data were not normally distributed, the Kruskal-Wallis one-way analysis on ranks was done. A critical significance level of P < 0.05 was used for all tests.
Chapter 3

Anticonvulsant Effects of Progesterone

in Amygdala-kindled Rats
3 Anticonvulsant Effects of Progesterone in Amygdala-kindled Rats

3.1 Rationale

In small, open clinical trials, progesterone has proven to be an anticonvulsant. Women with catamenial seizures, who are refractory to other treatments, consistently respond to progesterone (see table 2a). These patients generally suffer from intractable complex partial seizures. Complex partial epilepsy is the most common adult form of epilepsy, and it is often intractable (see Introduction).

The kindling preparation provides an animal model in which to study the effects of progesterone on complex partial seizures (Introduction). Past studies on the anticonvulsant effects of progesterone in the kindling model, however, have produced conflicting results. Edwards et al. (1999) found that 5 mg/kg of progesterone abolished amygdala-kindled seizures in 60% of adult female Wistar rats. Mohammad et al. (1998), however, found that in male Wistar amygdala-kindled rats, doses below 75mg/kg were ineffective at blocking generalized kindled seizures. Edwards did not perform dose-response studies, whereas Mohammad did. These data seem to suggest that progesterone may have low-dose anticonvulsant effects in females but not males.

Experiment 1 was designed to clarify the effects of progesterone on kindled seizures. Dose-response studies with progesterone were performed in amygdala-kindled
female and male Wistar rats. $ED_{50}$'s were determined, and the influences of endogenous and exogenous hormones on the response were explored. It was originally hypothesized that progesterone would be anticonvulsant at low doses (5 mg/kg), at least in females.

In an attempt to replicate the procedure of Edwards et al., adult Wistar rats were used, and progesterone was injected 2 hours before seizures were triggered. It was assumed that the responses would be genomic and mediated by the classic progesterone receptor. Genomic responses take some time to develop.

When initial trials failed to show low-dose progesterone effects (data not shown), a number of subsequent trials were performed, varying different experimental parameters (The same ovariectomized subjects were used in Experiments 1a and 1b).

**Experiment 1a**

In Experiment 1a, parameters related to administration were manipulated. To assess the possible importance of first-pass effects, the route of delivery was varied. The drug was administered IP (first pass effects) or SC (no first pass effect). To assess the possible effect of the vehicle, the drug vehicle was varied. The drug was administered either in beta-hydroxy cyclodextrin or corn oil (Edwards et al. had used oil as their drug vehicle).
Experiment 1b

In Experiment 1b, the possible role of estrogen was investigated. The effect of "estrogen priming" on after-discharge threshold (ADT) was determined. Subjects were then "estrogen-primed" before tests of the anticonvulsant effects of progesterone.

Experiment 1c

In Experiment 1c, to determine effect of endogenous hormones (including estrogen) a new group of intact (not ovariectomized) subjects were used. This allowed the evaluation of anticonvulsant effects of progesterone within a normal hormonal milieu (Edwards et al. used intact subjects). (All other kindling experiments used castrated subjects to prevent the confounding effect of endogenous hormones when testing progesterone.)

Experiment 1d

In Experiment 1d, castrated male subjects, with and without hormone replacement, were used to provide a comparison with the female subjects in Experiments 1a, 1b and 1c.

3.2 Methods
Subjects

The same subjects were used in Experiment 1a and 1b. They were 33 right amygdala-implanted, ovariectomized female Wistar rats. In Experiment 1c, the new subjects were 24 right amygdala-implanted, intact (not ovariectomized) female Wistar rats. In Experiment 1d, the subjects were 36 right amygdala-implanted, gonadectomized and hormone-replaced, male Wistar rats. Hormone replacement consisted of SC implantation of Silastic® capsules containing cholesterol, estrogen or testosterone. Subjects were housed as described in the General Methods.

Kindling and Threshold Testing

The subjects (in all experiments) were kindled daily to a criterion of 30 stage 5 seizures (procedure as in General Methods). Their thresholds were measured using the ascending series method as described in the General Methods. Thresholds were checked for stability as described in the General Methods.

Dose-Response Testing

Four days following the last kindling stimulus (stability testing), progesterone dose-response studies were initiated. In all studies, the following doses of progesterone were used: 4, 8, 16, 32, and 64 mg/kg. For all experiments (except 1a, when corn oil was used as vehicle), progesterone was dissolved in beta-hydroxy cyclodextrin to a concentration of 32mg/mL for the highest dose, followed by serial dilution for lower doses. It was administered in a volume of 2mL/kg. In part of Experiment 1b, the drug
progesterone) was prepared in a similar manner as above, except that corn oil was used as the vehicle.

In the first part of Experiment 1a, the subjects were randomized and injected SC or IP with different doses (4, 8, 16, 32, 64 mg/kg) of progesterone. Two hours after drug injection, the anticonvulsant action of progesterone was tested by administering a kindling stimulus equivalent to ADT+40%. The same subjects were tested repeatedly with different doses. A four-day interval was allowed between tests. It permits complete clearance of progesterone.

In the second part of Experiment 1a, the same subjects (ovariectomized females) were randomized and injected SC with different doses of progesterone dissolved in corn oil or beta-hydroxy cyclodextrin. Seizure testing was the same as described above.

In the first part of Experiment 1b, the same subjects (ovariectomized females) were “estrogen primed” with a SC injection of 10ug/kg estradiol benzoate 48 hours prior to ADT testing. Estradiol was dissolved in corn oil, and injected in a volume of 2mL/kg. ADTs were determined (ascending series method as described in General Methods) before and after “estrogen priming”.

In the second part of Experiment 1b, a progesterone dose-response study was then performed in “estrogen primed” subjects (ovariectomized females). Forty-six hours after estrogen priming, they were randomized and injected SC with different doses of
progesterone. Seizure testing was identical to Experiment 1a, except that a newly found ADT + 40% was used. It was based on the ADT measured in the presence of estradiol.

In Experiment 1c, new subjects (intact females) were randomized and injected SC with different doses of progesterone. Seizure testing was identical to Experiment 1a. It should be noted that the intact subjects were not cycling and exhibited persistent estrous. The kindling stimulus cause female rats to cycling arrest and the subjects perpetually appear to be in the proestrous/estrous phase. (Edwards et al., 1999).

In Experiment 1d, adult male subjects were (gonadectomized and hormone-replaced males) with cholesterol (control), estrogen or testosterone. They were then randomized and injected SC with different doses of progesterone. Seizure testing was identical to Experiment 1a.

Seizure Scoring

Two components of the kindled seizure were scored: the generalized convulsion and the focal component. Each component was marked as “present” or “absent”. Generalized seizures were defined as stage 4 and 5 seizures, as catagorized by Racine’s classification (General Methods). The generalized seizure was scored as absent if seizure stages 4 and 5 were suppressed. The focal seizure was defined as 3 or more seconds of “spiking” in the EEG record. This component was scored as absent, if a normal EEG
followed the stimulation. These definitions are consistent with Albright’s scoring technique (Albright and Burnham, 1980).

Data Analysis

Dose-response curves were plotted for progesterone’s effects on both the generalized and focal components of the seizures. Quantal curves were constructed by plotting the percentage of animals that were protected at each dose. ED₅₀’s and Rmax’s were calculated. A paired Student t-test was used to compare ADT’s in Experiment 1b.

3.3 Results

Sedation

Sedation was generally not apparent for doses of 32mg/kg or less. A dose of 64mg/kg in the SC, cyclodextrin groups from experiments 1a and 1b (data are combined), however, caused significant sedation, including loss of the righting reflex in 36 % of the subjects (N=14). Ataxia was measured on the Loscher scale (General Methods) and found to be 4.9 +/- 0.4 in these groups. This was significantly different from control subjects (P<0.001) using the paired t-test.

Experiment 1a: Progesterone Dose-Response: Route of Administration and Vehicle
Figure 3 shows the progesterone (injected IP or SC) dose-response curve for the suppression of generalized and focal seizures in well-kindled, ovariectomized, female Wistar rats. For generalized seizures in both the IP and the SC treated groups, the ED${}_{50}$ was 33 mg/kg and the maximal response (100%) occurred at 64 mg/kg. The route of administration did not matter. SC administration was therefore used in all subsequent studies.
The anticonvulsant effects of progesterone against generalized kindled convulsions (closed circles) or focal seizures (open circles) Each point represents data from at least 7 subjects. Subjects were adult, ovariectomized female Wistar rats. Figure 3a: progesterone was administered IP. Figure 3b: progesterone was administered SC.
No substantial suppression of focal seizure activity was seen at any of the doses tested in either group. No low-dose anticonvulsant effects were seen.

Figure 4 shows the progesterone (dissolved in cyclodextrin or corn oil) dose-response curve for the suppression of generalized and focal seizures in well-kindled, ovariectomized, female Wistar rats. For generalized seizures in the beta-hydroxy cyclodextrin treated group, the ED₅₀ was 33 mg/kg and the maximal response (100%) occurred at 64 mg/kg. There was no substantial suppression of generalized seizures in the corn oil treated group. Cyclodextrin was, therefore, used in all subsequent studies.

No substantial suppression of focal seizure activity was seen at any of the doses tested in either group. No low dose progesterone effects were seen.

**Experiment 1b: Progesterone Dose-Response: Estrogen Priming**

Figure 5a shows the effect of “estrogen-priming” on the ADT as measured by the ascending series method (General Methods). The same subjects were used as in Experiment 1a. The ADT was significantly reduced by 23% (P < 0.001, using t-test). Thus, estrogen priming lowers seizure thresholds at the seizure focus. In the subsequent
Figure 4: The Anticonvulsant Effects of Progesterone in the Kindling Model
Progesterone Was Administered in Cyclodextrin or Corn Oil

The anticonvulsant effects of progesterone against generalized kindled convulsions (closed circles) or focal seizures (open circles) each point represents data from at least 7 subjects. Subjects were adult, ovariectomized female Wistar rats.

Figure 4a: progesterone was administered in cyclodextrin.
Figure 3b: progesterone was administered in corn oil.
The Effect of Estrogen on After-discharge Threshold (ADT)

Data are represented as mean +/- standard error. Each group contains 33 subjects; (*) indicates a significant difference from the control group using a paired t-test (P<0.001).

Figure 5a: The Effect of Estrogen on After-discharge Threshold

The anticonvulsant effects of progesterone against generalized kindled convulsions (closed circles) or focal seizures (open circles). Each point represents data from at least 5 subjects. Subjects were adult, ovariectomized (estrogen-primed) female Wistar rats.

Figure 5b: The Anticonvulsant Effects of Progesterone in the Kindling Model Subjects Were Estrogen-Primed
dose-response study (Figure 5b), estrogen primed subjects were tested at their newly measured ADT's +40%.

Figure 5b shows the progesterone dose-response curve for the suppression of generalized and focal seizures in well-kindled, ovariectomized, "estrogen-primed" female Wistar rats. For generalized seizures in the "estrogen primed" subjects, the ED$_{50}$ was 40mg/kg and the maximal response (80%) occurred at 64mg/kg. No low-dose anticonvulsant effects were seen. Therefore, estrogen does not synergize progesterone's anticonvulsant effect in the kindling model.

No substantial suppression of focal seizure activity was seen at any of the doses tested.

As a positive control for the testing method, a dose-response of phenobarbital was preformed (data not shown). The ED$_{50}$ against generalized seizures was 12.5 mg/kg.

**Experiment 1c: Progesterone Dose-Response: Intact subjects**

Figure 6 shows the progesterone dose-response curve for the suppression of generalized and focal seizures in well-kindled, intact, female Wistar rats (These subjects were not the same as those used in previous studies). For generalized seizures in the intact subjects, the ED$_{50}$ was 30mg/kg and the maximal response (100%) occurred at
Progesterone, "intact"

![Graph showing the anticonvulsant effects of progesterone in the kindling model.](image)

Figure 6: Anticonvulsant Effects of Progesterone in the Kindling Model

Intact Subjects Were Used

The anticonvulsant effects of progesterone against generalized kindled convulsions (closed circles) or focal seizures (open circles). Each point represents data from at least 5 subjects. Subjects were adult, intact (not ovariectomized) female Wistar rats.
64mg/kg. No low-dose anticonvulsant effects were seen. Therefore, normal levels of endogenous hormones do not alter progesterone’s anticonvulsant effect in the kindling model.

No suppression of focal seizure activity was seen at any of the doses tested.

**Experiment 1d: Progesterone dose-response, male rats, hormone replacement**

Figure 7 shows the progesterone dose-response curves for the suppression of generalized and focal seizures in gonadectomized, cholesterol-replaced, estrogen-replaced and testosterone-replaced, well-kindled, male Wistar rats. For generalized seizures, the ED₁₀ was 35mg/kg in the cholesterol-replace subjects, 37mg/kg in the estrogen-replaced subjects, and 101mg/kg in the testosterone-replaced subjects. The maximal response (90%) occurred at 64mg/kg in the cholesterol-replace subjects, and also in the estrogen-replaced subjects. The maximal response (60%) occurred at 98mg/kg in the testosterone-replaced subjects. No low-dose anticonvulsant effects were seen. Hence, the anticonvulsant effects of progesterone in gonadectomized and cholesterol-replaced or estrogen-replaced rats are similar to the response in female rats. Testosterone in male rats, however, makes them less responsive to the anticonvulsant effects of progesterone.
Figure 7: The Anticonvulsant Effect of Progesterone in the Kindling Model
Subjects Were Gonadectomized and Hormone-Replaced Males

The anticonvulsant effects of progesterone against generalized kindled convulsions (closed circles) or focal seizures (open circles) Each point represents data from at least 7 subjects. Subjects were adult, ovariectomized female Wistar rats.
Figure 7a: progesterone was administered in cholesterol-replace subjects.
Figure 7b: progesterone was administered in estrogen-replaced subjects.
Figure 7c: progesterone was administered in testosterone-replaced subjects.
No substantial suppression of focal seizure activity was seen at any of the doses tested in any of the groups.

3.4 Summary

Progesterone was an effective anticonvulsant in female rats, but at doses that were higher than expected. ED₅₀s were usually in the range of 30-40mg/kg (summarized in Table 3). Manipulations of the route of drug delivery, the drug vehicle, and of exogenous (estrogen priming) and endogenous (intact subjects) hormonal states did not improve progesterone’s anticonvulsant response. Estrogen priming, however, lowered seizure thresholds, thus supporting the theory that estrogen is an important cause of catamenial seizures of the complex partial variety.

Castrated males (cholesterol or estrogen replaced) had ED₅₀s similar to the females. Testosterone-replaced castrated males, however, had high ED₅₀s, suggesting that testosterone blocks the anticonvulsant effects of progesterone.

The original hypothesis that progesterone would have anticonvulsant effects at low doses was not supported.
The anticonvulsant ED50's of progesterone for the suppression of Amygdala-generalized convulsions

<table>
<thead>
<tr>
<th>Route of Administration</th>
<th>Vehicle</th>
<th>Sex</th>
<th>Hormonal Parameters</th>
<th>Amygdala ED50 (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IP</td>
<td>Cyclodextrin</td>
<td>Female</td>
<td>Ovariectomized</td>
<td>33</td>
</tr>
<tr>
<td>SC</td>
<td>Cyclodextrin</td>
<td>Female</td>
<td>Ovariectomized</td>
<td>33</td>
</tr>
<tr>
<td>SC</td>
<td>Corn oil</td>
<td>Female</td>
<td>Ovariectomized</td>
<td>&gt;64</td>
</tr>
<tr>
<td>SC</td>
<td>Cyclodextrin</td>
<td>Female</td>
<td>Ovariectomized / Estrogen-replaced</td>
<td>40</td>
</tr>
<tr>
<td>SC</td>
<td>Cyclodextrin</td>
<td>Female</td>
<td>Intact</td>
<td>30</td>
</tr>
<tr>
<td>SC</td>
<td>Cyclodextrin</td>
<td>Male</td>
<td>Gonadectomized / Cholesterol-replaced</td>
<td>35</td>
</tr>
<tr>
<td>SC</td>
<td>Cyclodextrin</td>
<td>Male</td>
<td>Gonadectomized / Estrogen-replaced</td>
<td>37</td>
</tr>
<tr>
<td>SC</td>
<td>Cyclodextrin</td>
<td>Male</td>
<td>Gonadectomized / Testosterone-replaced</td>
<td>101</td>
</tr>
</tbody>
</table>
Chapter 4:

Anticonvulsant Effects of Chronic Low-Dose Progesterone and MPA in Six Seizure Models
4 Anticonvulsant Effects of Chronic Low-Dose Progesterone and MPA in Six Seizure Models

4.1 Rationale

The results of Experiment 1 indicated that progesterone is not anticonvulsant at low doses in amygdala-kindled rats. These findings are consistent with similar studies by Mohammad’s group who also found suppression of kindled convulsion at high doses (Mohammad et al., 1998b).

The fact that only high doses – which caused sedation – were effective suggests a GABAergic mechanism of action for progesterone’s anticonvulsant effects. These findings, however, are not consistent with the clinical data, which indicate that low doses of both progesterone and MPA (which is not metabolized into allopregnanolone) are effective anticonvulsants.

One important difference between Experiment 1 study and the clinical studies is that we administered progesterone acutely, whereas epileptic patients use progesterone or MPA chronically. It seemed possible that low doses of progesterone or MPA might be anticonvulsant in rats if they were administered chronically (for 1 week).

The effects of chronically administered progesterone and MPA were therefore assessed in several different seizure models. In each model, seizure latency or threshold
- rather than seizure absence or presence - was measured, so that even small changes would be seen. The tests included amygdala kindling, ECS-threshold, MES-threshold, MET, MMT, and pentylenetetrazol-infusion. It was hypothesized that low doses of progestins, chronically administered, would have anticonvulsant effects in these tests.

4.2 Methods

Subjects

In all experiments, intact female Wistar rats were used as subjects. Subjects were 60 days old upon arrival from the breeding farms. They were allowed one week for acclimatization and daily handling before any treatments or tests were performed.

The subjects' stage in the estrous cycle was monitored by daily vaginal smears, as described in the General Methods.

Drug Administration

In all experiments, subjects were randomized into three groups. The first group (the control subjects) was injected with normal saline. The second group was injected with MPA (15mg/kg, 0.2mL, IM, vehicle: saline). This dose of MPA reliably stopped the fertility cycle in subjects, indicating that it was effective. The third group was treated with ¾ inch Silastic® capsules containing progesterone. The Silastic® capsules were implanted subcutaneously in the ruff of the neck under light halothane-induced
anesthesia. This dose of progesterone has produced luteal phase levels of progesterone in ovariectomized rats (Edwards et al., 1999).

After one week of drug treatment seizure testing was initiated. Control subjects were tested during proestrous. Drug-treated subjects who stopped cycling were tested once every four days (in studies where repeated testing was required). Otherwise, subjects were tested during proestrous.

**Testing and Seizure Scoring**

**Experiment 2a: Kindling**
Before drug administration, subjects were amygdala-kindled to 5 stage 5 seizures, using procedures described in the General Methods. Progesterone or MPA were then administered for 1 week. On the 8th day, the threshold for amygdala-kindled generalized convulsions was determined by the half-split method, as described previously. The lowest current that produced generalized seizures (stage 4 or 5) as defined by Racine’s classification was considered the “threshold”.

**Experiment 2b: ECS Threshold**
ECS threshold was measured by the half-split method, as described in the General Methods section. The lowest current required to produce forelimb clonus was considered the threshold.
Experiment 2c: MES Threshold
MES threshold was measured by the half-split method, as described in the General Methods section. The lowest current required to produce hind-limb tonus was considered the “threshold”.

Experiment 2d: MET
The MET test was administered as described in the General Methods section. Subjects were scored for presence or absence of clonic seizures within 30 minutes after injection, and also for the delay to onset of the seizures (latency).

Experiment 2e: MMT
The MMT test was administered as described in the General Methods section. Subjects were scored for presence or absence of tonic (forelimb extension) seizures within 30 minutes after injection, and also for the delay to onset of the seizures (latency).

Experiment 2f: Pentylenetetrazol Infusion
Pentylenetetrazol was administered via the tail vein, as described in the General Methods section. The dose of pentylenetetrazol required to cause forelimb clonous was calculated and was considered to be “threshold”.

82
Data Analysis

In all experiments, the data were analyzed using one-way analysis of variance (ANOVA) or – when they were not normally distributed – the Kruskal-Wallis one-way analysis on ranks.

4.3 Results

Effects on the Estrous Cycle

The estrous cycle was arrested in 84% of subjects that were treated with MPA (N=50). Vaginal smears from subjects that had stopped cycling displayed characteristics of cells from diestrous or metestrous. With progesterone, only 28% of the subjects stopped cycling (N=43).

Experiment 2a: Kindling

As indicated by Figure 8, the mean thresholds (+/- SE) for generalized amygdala-kindled convulsions (GCT) for saline, progesterone and MPA-treated subjects were 63 ± 21, 68 ± 32, and 83 ± 53 μA, respectively. Analysis of variance revealed no significant difference among these means (P=0.941). These data indicate that chronic administration of progestins does not significantly alter the threshold for amygdala-
Figure 8: The Effect of Chronic Progesterone and MPA on the Generalized Convulsive Threshold in Amygdala-Kindled Rats.

The control, progesterone, and MPA groups contain 13, 8, and 6 subjects respectively. The data are presented as mean +/- standard error. No significant difference was found using Kruskal-Wallis one-way analysis on ranks (P=0.941).
kindled convulsions in rats. There was, however, a “trend” towards anticonvulsant activity with both progesterone and, to a greater extent, the MPA treatments.

Experiment 2b: ECS Threshold
As indicated by Figure 9, the mean ECS thresholds (+/- SE) for saline, progesterone and MPA-treated subjects were 25 ± 3, 25 ± 2, and 27 ± 3 mA, respectively. Analysis of variance revealed no significant difference among these means (P=0.140). These data indicate that chronic administration of progestins does not significantly alter the ECS threshold in rats. There was, however, a slight “trend” towards anticonvulsant activity with the MPA treatment.

Experiment 2c: MES Threshold
As indicated by Figure 10, the mean MES thresholds (+/- SE) for saline, progesterone and MPA-treated subjects were 40 ± 6, 42 ± 6, and 42 ± 5 mA, respectively. Analysis of variance revealed no significant difference among these means (P=0.718). These data indicate that chronic administration of progestins does not significantly alter the MES threshold in rats. There was, however, a slight “trend” towards anticonvulsant activity with both the progesterone and the MPA treatments.
ECS Convulsive Threshold

Figure 9: The Effect of Chronic Progesterone and MPA on the Threshold for ECS Seizures.

Each group contains at least 13 subjects. The data are presented as mean +/- standard error. No significant difference was found using the Kruskal-Wallis one-way analysis on ranks (P=0.140).
Figure 10: The Effect of Chronic Progesterone and MPA on the Threshold for MES Seizures.

Each group contains at least 13 subjects. The data are presented as mean +/- standard error. No significant difference was found using the Kruskal-Wallis one-way analysis on ranks (P=0.718).
**Experiment 2d: MET**

All test subjects had a clonic seizure within 30 minutes of pentylentetrazol injection.

As indicated by Figure 11, the mean latencies (+/- SE) to onset of forelimb clonus following pentylentetrazol injection in saline, progesterone and MPA-treated subjects were 457 ± 133, 590 ± 423, and 871± 465 seconds, respectively. Kruskal-Wallis one-way analysis on ranks revealed no significant difference among these means (P=0.082), although the data were approaching significance. These data indicate that chronic administration of progestins does not significantly alter response to the MET stimulus in rats. There was a “trend”, however, for progesterone and, to a greater extent, for MPA to increase latency. Using the Mann-Whitney rank sum test to compare only the saline and MPA groups, the MPA group would have differed significantly from the saline group (P=0.018).

**Experiment 2e: MMT**

Eight out of 9 test subjects in each group had a tonic seizure (forelimb extension) within 30 minutes of pentylentetrazol administration. There was no noteworthy difference in seizure occurrence between two groups.
Figure 11: The Effect of Chronic Progesterone and MPA on Latencies to the Onset of MET Clonic Seizures.

Each group contains at least 8 subjects. The data are presented as mean +/- standard error. No significant difference was found using the Kruskal-Wallis one-way analysis on ranks (P=0.082).
Figure 12: The Effect of Chronic Progesterone and MPA on Latencies to the Onset of MMT Tonic (Forelimb Extension) Seizures.

Each group contains at least 8 subjects. The data are presented as mean +/- standard error. No significant difference was found using the Kruskal-Wallis one-way analysis on ranks (P=0.254).
As indicated by Figure 12, the mean latencies (+/- SE) to onset of forelimb extension following pentylenetetrazol injection in saline, progesterone and MPA-treated subjects were 547 ± 111, 678 ± 374, and 774 ± 323 seconds, respectively. Kruskal-Wallis one-way analysis on ranks revealed no significant difference among these means (P=0.254). These data indicate that chronic administration of progestins does not significantly alter response to the MMT stimulus in rats. There was, however, a “trend” for progesterone and, to a greater extent, MPA to increase latency.

**Experiment 2f: Pentylenetetrazol Infusion**

As indicated by Figure 13, the mean doses (+/- SE) that produced clonic seizures in the pentylenetetrazol infusion test for saline, progesterone and MPA-treated subjects were 52 ± 17, 48 ± 20, and 56 ± 21 mg/kg, respectively. Analysis of variance revealed no significant difference among these means (P=0.666). These data indicate that chronic administration of progestins does not significantly alter the convulsant pentylenetetrazol infusion dose in rats. There was, however, a “trend” towards anticonvulsant activity with MPA treatment.
Figure 13: The Effect of Chronic Progesterone and MPA on the Threshold Dose of Pentylenetetrazol Required to Induce Clonic Seizures (FLC).

The control, progesterone, and MPA groups contain 22, 12, and 13 subjects, respectively. The data are presented as mean +/- standard error. No significant difference was found using the Kruskal-Wallis one-way analysis on ranks (P=0.666)
4.4 Summary

Chronically administered progesterone and, to a greater degree, MPA displayed trends towards anticonvulsant activity in several tests. In no case, however, was the anticonvulsant activity of progesterone and MPA statistically significant. Our hypothesis that low doses of progestins, chronically administered, would have significant anticonvulsant effects was not supported.
Chapter 5

The Anticonvulsant Effects of Progesterone in Adult and 15-day-old Male and Female rats in the MMT and the MES Seizure Models
5 The Anticonvulsant Effects of Progesterone in Adult and 15-day-old Male and Female rats in the MMT and the MES Seizure Models

5.1 Rationale

Experiment 1, the initial dose-response study, was conducted with a 2-hour injection/test interval. This interval was the interval used by Edwards et al. (1999), and was based on the assumption that progesterone’s effects were genomic effects, mediated by the “classic” intracellular receptors. Genomic effects take some time to appear.

Recently, Edwards et al. (submitted) have performed further experiments, which showed anticonvulsant effects of a single dose of progesterone using a 15-minute injection/test interval. In Experiment 3, therefore, the time-response of progesterone was studied. These studies established that 15 minutes is an optimal injection/test interval. Subsequently, dose-response studies were performed using a 15-minute injection/test interval.

The time-response studies were performed in the MMT model. The dose-response studies were performed in both the MMT and the MES models. The MES model was used because it is a standard model of tonic-clonic seizures. The MMT model was used because it allows the evaluation of both tonic and the clonic responses simultaneously.
In experiment 3, studies were done in both female and male adults rats, and in male and female 15-day-old rat pups. The following experiments were performed:

Experiment 3a: the anticonvulsant effects of progesterone in female rats
Experiment 3b: the anticonvulsant effects of progesterone in male rats
Experiment 3c: the anticonvulsant effects of progesterone in female rat pups
Experiment 3d: the anticonvulsant effects of progesterone in male rat pups

These experiments were designed to determine the pharmacology of progesterone in tonic-clonic seizure models, and also to determine whether progesterone is as effective in stopping seizures in males and infants as it is in females. It was hoped that these studies would provide a basis for using progesterone in clinical trials in a broader epileptic population (children and men as well as women). It was hypothesized that progesterone would be as effective in infants and adult males as it is in adult females.

5.2 Methods

Subjects
In experiments 3a and 3b, intact young adult female or male Wistar rats were used as subjects. Subjects were 60 days old upon arrival from the breeding farm. Subjects were allowed one week for acclimatization and daily handling before any treatments or tests were performed.
In experiment 3c and 3d, 15-day-old, Wistar male and female rats were used as subjects. Subjects were 8 days old upon arrival from the breeding farm. They arrived as “made up” litters of 12 with their dam.

**Drug Preparation and Administration**

In all of the experiments, progesterone was prepared with beta-hydroxy cyclodextrin and administered SC in the rough of the neck.

In experiments 3a and 3b, progesterone (various doses) was injected in a volume of 4mL/kg. Pentylenetetrazol (85mg/kg) was prepared in normal saline and administered in a different part of the rough of the neck in a volume of 4.2mL/kg.

In experiments 3c and 3d, progesterone (various doses) was injected in a volume of 10mL/kg. Pentylenetetrazol (150mg/kg) was prepared in normal saline and administered in a different part of the rough of the neck in a volume of 10 mL/kg.

**Testing and Seizure Scoring**

In the MMT test, pentylenetetrazol was administered at 85mg/kg in experiments 3a and 3b and at 150mg/kg in experiment 3c and 3d as described above. Subjects were observed for 30 minutes, and scored as described in the General Methods.
MES was administered according to the standard procedures that were described in the General Methods.

In the time-response studies, a dose of 60mg/kg of progesterone was used, plus the following injection/test intervals: 5 minutes, 15 minutes, 1 hour, 2 hours, 6 hours and 24 hours.

Data Analysis

Time-response and dose-response curves were plotted for both the tonic and clonic elements of the seizures. Quantal curves were constructed by plotting the percentage of animals at each time or dose that were protected against the clonic or tonic seizures. Dose-response curves were fitted with linear regression, and ED$_{50}$'s and maximal responses were calculated.

5.3 Results

5.3.1 Experiment 3a: Adult Female Subjects

Figure 14a shows the time-response curve in adult females for the suppression of tonic and clonic seizures in the MMT model after a single dose of progesterone (60mg/kg). Both tonic (forelimb extension) and clonic components of the seizure were considerably suppressed 15 minutes after treatment. Protection against the clonic component began to disappear after 1 hour. Thereafter, protection against both
Figure 14a,b,c: Anticonvulsant effects of Progesterone in Adult, Female Wistar Rats

The anticonvulsant effects of progesterone on suppression of forelimb clonus (●), forelimb extension (○), and, in figure 14c, hind-limb extension (►). Each point represents data from at least 5 subjects.

Figure 14a: time-response study following a 60mg/kg injection of progesterone in the MMT seizure test (85mg/kg of pentylenetetrazol, SC).

Figure 14b: dose-response study in the MMT seizure test. Progesterone was administered 15 minutes prior to the seizure test.

Figure 14c: dose-response study in the MES seizure test (150mA of current delivered via corneal electrodes). Progesterone was administered 15 minutes prior to the seizure test.
Figure 14a, b, c: adult females
components declined. Curiously, there was major protection against both components of the seizure only 5 minutes after SC progesterone injection.

Based on the time-response study, dose-response tests were carried out using a 15-minute injection/test interval. Figure 14b shows the dose-response curve for the suppression of MMT-induced tonic and clonic seizures in adult females. The ED$_{50}$s for tonic (forelimb extension) and clonic seizures were 14 and 19 mg/kg, respectively. Maximal responses (100% protection) were observed at 80 and 160 mg/kg for tonic and clonic seizures, respectively.

Figure 14c shows the dose-response curve for the suppression of MES-induced tonic and clonic seizures in adult females. The ED$_{50}$ for both tonic (hind-limb extension) and clonic seizures was >320 mg/kg. Maximal response for tonus (40% protection) was observed at 320 mg/kg.

5.3.2 Experiment 3b: Adult Male Subjects

Figure 15a shows the time-response curve in adult males for the suppression of tonic and clonic seizures in the MMT model after a single dose of progesterone (60 mg/kg). Both tonic (forelimb extension) and clonic components of the seizures were drastically suppressed 15 minutes after treatment. Protection against both components
began to disappear after 2 hours. At 5 minutes, there was complete protection against the tonic seizure, but only partial protection against the clonic seizure.

Based on the time-response study, and also for consistency with the experiments on female rats, dose-response tests were carried out using a 15-minute injection/test interval. Figure 15b shows the dose-response curve for the suppression of MMT-induced tonic and clonic seizures. The ED$_{50}$s for tonic (forelimb extension) and clonic seizures were 23 and 35 mg/kg respectively. Maximal responses (100% protection) were observed at 80 mg/kg for both tonic and clonic seizures.

Figure 15c shows the dose-response curve for the suppression of MES-induced tonic and clonic seizures. The ED$_{50}$s for tonic (hind-limb extension) and clonic seizures were 220 and >320 mg/kg respectively. The maximal response for tonic hind-limb extension (100%) was reached at 320 mg/kg.

5.3.3 Experiment 3c: Immature Female Subjects

Figure 16a shows the time-response curve in immature females for the suppression of tonic and clonic seizures in the MMT model after a single dose of progesterone (60 mg/kg). Both the tonic (forelimb extension) and clonic components of the seizure were considerably suppressed 15 minutes after treatment. Protection against
Figure 15a,b,c: Anticonvulsant effects of Progesterone in Adult, Male Wistar Rats

The anticonvulsant effects of progesterone on suppression of forelimb clonus (●), forelimb extension (○), and, in figure 15c, hind-limb extension (▲). Each point represents data from at least 5 subjects.

Figure 15a: time-response study following a 60mg/kg injection of progesterone in the MMT seizure test (85mg/kg of pentylenetetrazol, SC).

Figure 15b: dose-response study in the MMT seizure test. Progesterone was administered 15 minutes prior to the seizure test.

Figure 15c: dose-response study in the MES seizure test (150mA of current delivered via corneal electrodes). Progesterone was administered 15 minutes prior to the seizure test.
Figure 15a,b,c: adult males
both components began to disappear after 2 hours. Even at 5 minutes, there was major protection against both the clonic and the tonic components of the seizures.

Based on the time-response study, dose-response tests were carried out using a 15-minute injection/test interval. Figure 16b shows the dose-response curve for the suppression of MMT-induced tonic and clonic seizures. The ED₅₀s for tonic (forelimb extension) and clonic seizures were 15 and 28 mg/kg respectively. Maximal responses (100% protection) were observed at 80 and 160 mg/kg for tonic and clonic seizures, respectively.

Figure 16c shows the dose-response curve for the suppression of MES-induced tonic and clonic seizures. The ED₅₀s for tonic (hind-limb extension) and clonic seizures were 23 and 160 mg/kg respectively. Maximal responses (100% protection) were observed at 320 mg/kg for both tonic and clonic seizures.

5.3.4 Experiment 3d: Immature Male Subjects

Figure 17a shows the time-response curve in immature males for the suppression of tonic and clonic seizures in the MMT model after a single dose of progesterone (60 mg/kg). Both tonic (forelimb extension) and clonic components of the seizures were considerably suppressed 15 minutes after treatment. Protection against the tonic and clonic components began to disappear after 2 hours and 1 hour, respectively. Even at 5
The anticonvulsant effects of progesterone on suppression of forelimb clonus (●), forelimb extension (○), and, in figure 16c, hind-limb extension (►). Each point represents data from at least 8 subjects.

Figure 16a: time-response study following a 60mg/kg injection of progesterone in the MMT seizure test (150mg/kg of pentylenetetrazol, SC).

Figure 16b: dose-response study in the MMT seizure test. Progesterone was administered 15 minutes prior to the seizure test.

Figure 16c: dose-response study in the MES seizure test (150mA of current delivered via corneal electrodes). Progesterone was administered 15 minutes prior to the seizure test.
Figure 16a,b,c: 15-day-old female rat pups
Figure 17a, b, c: Anticonvulsant effects of Progesterone in 15-Day-old, Male Wistar Rats

The anticonvulsant effects of progesterone on suppression of forelimb clonus (●), forelimb extension (○), and, in figure 17c, hind-limb extension (▲). Each point represents data from at least 8 subjects. Figure 17a: time-response study following a 60mg/kg injection of progesterone in the MMT seizure test (150mg/kg of pentylenetetrazol, SC). Figure 17b: dose-response study in the MMT seizure test. Progesterone was administered 15 minutes prior to the seizure test. Figure 17c: dose-response study in the MES seizure test (150mA of current delivered via corneal electrodes). Progesterone was administered 15 minutes prior to the seizure test.
Figure 17a,b,c: 15-day-old male rat pups
minutes there was important protection against the tonic seizures, and partial protection against the clonic seizures.

Based on the time-response study, dose-response tests were carried out using a 15-minute injection/test interval. Figure 17b shows the dose-response curve for the suppression of MMT-induced tonic and clonic seizures. The ED$_{50}$s for tonic and clonic seizures were 12 and 85 mg/kg, respectively. Maximal responses (100% protection) were observed at 80 and 160 mg/kg for tonic and clonic seizures, respectively.

Figure 17c shows the dose-response curve for the suppression of MES-induced tonic and clonic seizures. The ED$_{50}$s for tonic (hind-limb extension) and clonic seizures were 8 and 160 mg/kg, respectively. Maximal responses (100% protection) were observed at 20 and 320 mg/kg for tonic and clonic seizures, respectively.
Table 4  
The anticonvulsant ED50’s of progesterone for the suppression of MMT / MES seizures

*Tonic seizures were defined as forelimb extension in the MMT model, and hind-limb extension in the MES model.

<table>
<thead>
<tr>
<th>Seizure Test</th>
<th>Age</th>
<th>Gender</th>
<th>ED50 (mg/kg) (Tonic*)</th>
<th>ED50 (mg/kg) (Clonic)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMT</td>
<td>Adult</td>
<td>Female</td>
<td>14</td>
<td>19</td>
</tr>
<tr>
<td>MMT</td>
<td>Adult</td>
<td>Male</td>
<td>23</td>
<td>35</td>
</tr>
<tr>
<td>MMT</td>
<td>Pup</td>
<td>Female</td>
<td>15</td>
<td>28</td>
</tr>
<tr>
<td>MMT</td>
<td>Pup</td>
<td>Male</td>
<td>12</td>
<td>95</td>
</tr>
<tr>
<td>MES</td>
<td>Adult</td>
<td>Female</td>
<td>&gt;320</td>
<td>&gt;320</td>
</tr>
<tr>
<td>MES</td>
<td>Adult</td>
<td>Male</td>
<td>220</td>
<td>&gt;320</td>
</tr>
<tr>
<td>MES</td>
<td>Pup</td>
<td>Female</td>
<td>23</td>
<td>160</td>
</tr>
<tr>
<td>MES</td>
<td>Pup</td>
<td>Male</td>
<td>8</td>
<td>260</td>
</tr>
</tbody>
</table>
5.4 Summary

The results of Experiment 3 are summarized in Table 4. Progesterone is an anticonvulsant in both female and male rats. Progesterone is an anticonvulsant in both female and male rat pups. The ED₅₀s in the MMT model are far lower than in the MES model.

The original hypothesis was supported that progesterone is anticonvulsant in males and in infants. These data are novel and warrant further study in clinical trials.

In addition, the very rapid onsets of progesterone's anticonvulsant actions call into question the previous assumptions about the genomic nature of progesterone's anticonvulsant effects. Preliminary studies of progesterone's mechanism of action were performed in Experiment 4.
Chapter 6:
Mechanistic Studies of the Anticonvulsant Effects of Progesterone
Mechanistic Studies of the Anticonvulsant Effects of Progesterone

6.1 Rationale

It seems probable that the anticonvulsant effects of progesterone are mediated by one of three different mechanisms: 1) by its active metabolite, allopregnanolone; 2) by binding to its "classic" (intracellular) receptors; or 3) by binding to the newly discovered cell surface receptors: Experiment 4 was designed as an initial investigation of these mechanisms.

Most investigators believe that allopregnanolone, progesterone's GABA-A-acting metabolite, is responsible for its anticonvulsant effects (General Introduction). We could not test this hypothesis by directly antagonizing GABA-A receptors, because all known GABA-A antagonists are proconvulsant. Therefore, we blocked the conversion of progesterone to allopregnanolone by using indomethacin. Indomethacin binds to, and blocks the action of, 5α-reductase, first of the enzymes involved in the conversion of progesterone to allopregnanolone.

The initial hypothesis of this thesis had been that progesterone worked through the classic (intracellular) progesterone receptor. RU486 (Mefipristone), an antagonist of the classic progesterone receptor, was used to test this hypothesis.
The onset of progesterone’s anticonvulsant effects in Experiment 3 seems too rapid to be explained either by conversion to allopregnanolone or by binding to the intracellular (genomic) receptors. It was hypothesized, therefore, that progesterone’s “fast” anticonvulsant effects were mediated by binding to the cell surface receptor. The expectation was that neither indomethacin nor RU486 would block progesterone’s anticonvulsant effects. The MMT model was used in these tests.

6.2 Methods

Subjects

Intact, young adult, female Wistar rats were used as subjects. Subjects were 60 days old upon arrival from the breeding farm. They were allowed one week for acclimatization and daily handling before any treatments or tests were performed. After one week, subjects were randomly sorted into the following eight treatment groups:

Experiment 4a (Indomethesin)

1) Vehicle          + Vehicle          + MMT
2) Vehicle          + P4              + MMT
3) Indomethacin     + Vehicle          + MMT
4) Indomethacin     + P4              + MMT

Experiment 4b (RU486)

5) Vehicle          + Vehicle          + MMT
6) Vehicle          + P4              + MMT
7) RU486 + Vehicle + MMT
8) RU486 + P4 + MMT

Drugs and Drug Administration

Progesterone (100mg/kg) was dissolved with beta-hydroxy cyclodextrin to a concentration of 20mg/kg. It was administered SC in the rough of the neck in a volume of 4mL/kg. This dose is above the ED_{100} (Experiment 3).

After 15 minutes, pentylenetetrazol (85mg/kg) – prepared in normal saline at a concentration of 21 mg/mL and injected in a volume of 4mL/kg – was administered in a different part of the rough of the neck in a volume of 4mL/kg. This dose produces maximal seizures.

Indomethacin (150 mg/kg) was prepared as a suspension, using normal saline, at a concentration of 37mg/mL. It was administered IP in a volume of 4mL/kg, fifteen minutes before injecting progesterone. This dose of indomethacin is above doses used previously to block 5-alpha reductase.
RU486 was prepared as a suspension, using cyclo-hydroxy cyclodextrin, in a concentration of 5mg/mL. It was administered IP, at a dose of 20 mg/Kg in a volume of 4mL/kg, 15 minutes before injecting progesterone. This dose of RU486 is well above doses used previously to block the progesterone receptor.

Testing and Seizure Scoring

Seven days after arrival from the breeding farm, testing was initiated. Subjects were preinjected with either RU486 or indomethacin. Fifteen minutes later, progesterone was administered and, 15 minutes after that, pentylenetetrazol was administered. Subjects were scored for the presence or absence of tonic seizures within 30 minutes after injection.

Data Analysis

In both experiments, the Chi Square test was used to analyze the data.

6.3 Results

6.3.1 Experiment 4a: Indomethacin
Figure 18 shows the effect of pre-treatment with indomethacin on the anticonvulant effects of a single high dose of progesterone. These data show that: 1) pentylenetetrazol was an effective convulsant agent (vehicle+vehicle group), 2) progesterone at 100mg/kg was an effective anticonvulsant (vehicle+progesterone group), 3) indomethacin did not interfere with the convulsant action of pentylenetetrazol (indomethacin+vehicle group), and 4) indomethacin did not block the anticonvulsant action of progesterone (indomethacin+progesterone group). These data are statistically significant using the Chi Square test (P ≤ 0.001).

6.3.2 Experiment 4b: RU486

Figure 19 shows the effect of pre-treatment with RU486 on the anticonvulant effects of a single high dose of progesterone. These data show that 1) pentylenetetrazol was an effective convulsant agent (vehicle+vehicle group), 2) progesterone was an effective anticonvulsant (vehicle+progesterone group), 3) RU486 did not interfere with the convulsant action of pentylenetetrazol (RU486+vehicle group), and 4) RU486 did not block the anticonvulsant action of progesterone (RU486+progesterone group). These data are statistically significant using the Chi Square test (P ≤ 0.001)

6.3 Summary
These data suggest that the “rapid” anticonvulsant effects of progesterone are not mediated by the classic progesterone receptor or by the GABA-A-binding metabolite of progesterone, allopregnanolone. They suggest that progesterone’s “rapid” anticonvulsant effects are mediated by the newly discovered cell surface receptors.
Anticonvulsant effect of progesterone in the presence or absence of indomethacin

Figure 18: The Influence of Indomethacin (150 mg/kg, IP) on the Anticonvulsant Effects of Progesterone (100 mg/kg).

Seizures were triggered by pentylenetetrazol. Subjects were young adult female rats. Each point represents data from 10 rats. (*) indicates a significant difference from the "vehicle+vehicle" controls, using the Chi Square test (P<0.001).
Anticonvulsant effect of progesterone in the presence or absence of RU486

Figure 19: The Influence of RU486 (20 mg/kg, IP) on the Anticonvulsant Effects of Progesterone (100 mg/kg).

Seizures were triggered by pentylenetetrazol. Subjects were young adult female rats. Each point represents data from 10 rats. (*) indicates a significant difference from the "vehicle+vehicle" controls, using the Chi Square test (P<0.001)
Chapter 7:

Discussion
1. Discussion

7.1 The Kindling Data: Experiment 1

Experiment 1 was designed to test the anticonvulsant effects of progesterone in dose-response studies involving the kindling (amygdala) model. Since Experiment 1 attempted to replicate a single-dose study by Edwards et al. (1999), an injection/test interval of 2 hours was used. The hypothesis was that progesterone would be anticonvulsant at low doses. It was thought that the low-dose anticonvulsant effects would be “genomic” effects, and that they might be seen in female, but not male, rats.

Anticonvulsant effects were observed only at high doses in Experiment 1 ($ED_{50} \geq 30\text{mg/kg}$). These findings are in agreement with Mohammad et al.’s (1998) findings. Mohammad et al. (1998), working in amygdala-kindled male rats, found seizure suppression with $ED_{50}$s of greater than 60mg/kg. The results of Experiment 1, however, do not agree with the findings of Edwards et al. (1999), since low dose effects were not observed in either males or females. Edwards et al. (1999) found that a low dose of progesterone (5mg/kg) suppressed seizures in 60% of female subjects.
Since progesterone was administered two hours before testing, high concentrations of allopregnanolone, but not of the parent compound (progesterone) would have been present at the time of seizure testing. The data from Experiment 1 appear to support the hypothesis that the anticonvulsant effects of progesterone are mediated by allopregnanolone. They appear to refute the original hypothesis that low doses of progesterone, acting through genomic mechanisms, have anticonvulsant effects.

Although the animal data – from Experiment 1 and Mohammad's (1998) – appear to show considerable seizure suppression only at high doses, the clinical data (Introduction) indicate that progesterone can suppress seizures at low doses. Therefore, low-dose effects were further investigated. Some features of the technique, which might have “masked” the low-dose effect of progesterone, were considered, and appropriate experiments were performed: 1) Pharmacokinetic effects were ruled out by altering the route of administration and the vehicle. Neither changing the route nor the vehicle revealed a low dose effect. 2) The role of estrogen in synergizing progesterone’s anticonvulsant effect was explored by using estrogen-primed (in ovariectomized rats) and intact (as opposed to ovariectomized) subjects. Since subsets of progesterone receptors are “inducible” by estrogen (Introduction) and the initial preparation used ovariectomized rats, it was thought that these “inducible” receptors might mediate a low-dose anticonvulsant effect of progesterone. Neither estrogen “priming”, however, nor the use of intact subjects revealed a low dose anticonvulsant response to progesterone. 3) As a positive control, dose-response studies were preformed with phenobarbital in ovariectomized and estrogen “primed” subjects. These studies replicated Albright and
Burnham's findings (1980) in male rats, and indicated that anticonvulsant effects could be found in the paradigm used in the present study.

7.2 The "Chronic, Low-Dose" Data: Experiment 2

In a further search for low-dose effects, Experiment 2 was designed to test the anticonvulsant action of chronically administered, low-dose progesterone and MPA. This treatment regimen imitates clinical practice more closely than the acute treatments used in Experiment 1. The hypothesis was that low-dose of progesterone and MPA administered chronically would be anticonvulsant. It was assumed again that the anticonvulsant effects would be "genomic" effects.

Chronic (7 day), low-dose administration of progesterone (Silastic® capsules resulting in luteal phase levels) or MPA (15mg/kg, IM), however, failed to show a significant anticonvulsant action in a variety of seizure models. These findings are in agreement with Holmes and Weber (1984) who reported that repeated administration of low doses of progesterone (5mg/kg) had no effect on kindling parameters. These findings are in disagreement with Edwards et al. (1999) who reported that chronic low-dose progesterone (Silastic® capsules) retarded kindling rates and raised seizure thresholds. It appears then that chronic, treatment with progesterone does not produce noteworthy anticonvulsant effects at low doses. These data are in agreement with Stitt and Kinnard (1968) who found that chronic low-doses of progesterone (5mg/kg) and MPA (10mg/kg) did not alter MES seizure thresholds. These data are also in agreement with Woolley et
al. (1962) who found that chronic progesterone (5mg/kg) only altered MES seizure thresholds by 5%. These data refute the original hypothesis. It should be noted, however, that trends were seen in several models. These represented the first suggestion of low-dose effects that were seen.

7.3 The Influence of gender and Age: Studies at 15-Minute Injection/Test Interval

Experiment 3 was undertaken after Edwards et al. (submitted) reported anticonvulsant effects of progesterone in 15-day-old rat pups using a 15-minute injection/test interval and a dose of 20mg/kg. These data suggested that Experiments 1 had failed to show low dose effects because the injection/test interval had been too long, and Experiment 2 had failed to show low dose effects because the dose had been too low.

Time-response studies were therefore performed in adults and pups. These were designed to measure the time of onset and offset of progesterone’s anticonvulsant actions. Anticonvulsant actions were seen at 5 minutes after SC injection. These peaked by 15 minutes, and declined after 2 hours.

Subsequent to the time-response studies, dose-response studies were performed in adult and 15-day-old female and male rats in the MMT model. It was hypothesized that progesterone would be an effective anticonvulsant in the female and male adults and in 15-day-old rat pups. This was found to be true in the MMT seizure test, where relatively
low dose effects were found (for tonic forelimb extension), but not in the MES seizure test, where only high dose effects were found, at least in adults.

Comparing the effects of progesterone in the different MMT groups, few differences are seen. The ED$_{50}$s for tonic forelimb extension are similar in adult females and 15-day-old rat pups (12-14 mg/kg). They are somewhat higher in adult males (30mg/kg) but still relatively low. These data are consistent with the kindling data (Experiment 1), where male testosterone-replaced subjects had higher ED$_{50}$s than female subjects. The MMT data are a new addition to the literature.

Comparing the effects of progesterone in the different MES groups, ED$_{50}$s for tonic hind-limb extension are much higher in adults (>320 mg/kg in females, 240mg/kg in males) than in 15-day-old subjects (24mg/kg in females, 7mg/kg in males). It is interesting that progesterone is not an effective anticonvulsant against adult MES seizures, but it is quite effective against infant MES seizures. This may reflect the difference in seizure thresholds between adult and 15-day-old subjects – immature subjects having much higher thresholds. The MES stimulus may be closer to threshold in infants than adults. Consequently, a lower anticonvulsant dose of progesterone is required to raise thresholds above the seizure threshold.

In the MES model, the data regarding the immature subjects are a new addition to the literature. The data regarding the adult subjects are consistent with Craig (1966) and Kokate et al. (1998), who found high ED$_{50}$s for progesterone in the MES model using
adult mice (300mg/kg and 250mg/kg, respectively). These data are also consistent with Stitt and Kinnard (1968), who found that a 70 mg/kg of progesterone had no effect on MES seizure threshold in adult female rats.

It is unclear why progesterone was a more effective anticonvulsant in the MMT seizure test in adults than in the MES seizure test. It is possible, however, that in the MMT test the epileptogenic stimulus is close to threshold than in the MES test. The MES stimulus is 5 times threshold (Experiment 2), whereas the MMT stimulus is possibly just above threshold, since some rats fail to seize. This would suggest that progesterone raises seizure thresholds by a small amount.

The time-course studies provided some surprising findings, which suggest a possible novel mechanism of action for progesterone. Progesterone was effective as early as five minutes after administration. It must be remembered that MMT seizures have a latency of 5 to 10 minutes, so it would be fair to say that effects were seen at 10 to 15 minutes after injection. Still, this is a very rapid response. These findings suggested that progesterone’s anticonvulsant action was not mediated by the gene transcription through the classical PR. Genomic effects do not occur this fast. They further suggested that the parent compound, and not the active metabolites, might mediate progesterone’s anticonvulsant effect. The time-course data led to Experiment 4.
7.4 Studies of Mechanism: Experiment 4

Experiment 4 was designed to test whether, at an early time after injection (15 minutes), progesterone's metabolites were involved in the anticonvulsant action of progesterone. It was hypothesized that they were not. Indomethacin was used to block the 5α-reductase-mediated conversion of progesterone to metabolites such as allopregnanolone. It was hypothesized that progesterone would be an effective anticonvulsant in the presence of indomethacin. This was found to be true. These findings are in disagreement with Kokate et al. (1998), who found that finasteride – another inhibitor of 5-alpha reductase – blocked the anticonvulsant effects of progesterone. This conflict will be discussed below.

Experiment 4 was also designed to test whether genomic effects via the "classic" receptors participated in progesterone's anticonvulsant action. RU486 (20mg/kg) was used to block the classical progesterone receptor. It was hypothesized that RU486 would not block progesterone's anticonvulsant action. This was found to be true. This finding is consistent with the previous findings of Mohammad et al. (1998), who also found that RU486 does not block progesterone's anticonvulsant effects. It appears, then, that the progesterone's anticonvulsant action does not involve the classic progesterone receptor. This is consistent with the initial hypothesis.
7.5 How Does Progesterone Stop Seizures? : The Role of Allopregnanolone

Herzog has argued that the clinical anticonvulant actions of progesterone are completely mediated by its GABA-modulating metabolite, allopregnanolone. A number of animal researchers agree with this point of view (eg. Mohammad et al., Kokate et al., Landgren et al., and Frye et al.) Two facts, however, have always suggested that this is not the case: 1) Matson's Clinical study (1985) found that MPA was effective in treating catamenial seizures. MPA is not metabolized to any major degree to a GABA-A receptor-binding compound like allopregnanolone. 2) Relatively low doses of progesterone are used clinically. Herzog's progesterone treatment achieves normal luteal phase levels of 5-25ng/mL (Herzog, 1986). This results in allopregnanolone concentrations lower than those necessary to produce its anticonvulsant effects(Kokate et al., 1994;Kokate et al., 1996).

Data from present experiments provide new and direct evidence that non-GABA-mediated anticonvulsant effects exist for progesterone. In Experiment 4, blockade of the conversion of progesterone to allopregnanolone had no effect on progesterone's anticonvulsant actions. This implies that progesterone has major anticonvulsant effects that are entirely independent of GABA.

These data are not in complete agreement with the past studies of Kokate et al. (1998). There are only two commercially available 5α-reductase inhibitors, finasteride
(Proscar®) and indomethacin (Indocid®). Finasteride has been used by other investigators in the epilepsy field to determine the mechanism of action of progesterone. Kokate et al. (1998) used high doses of finasteride in conjunction with progesterone, and found that it completely blocked progesterone’s anticonvulsant actions. Hence, Kokate concluded that progesterone’s anticonvulsant action is entirely due to its metabolite, allopregnanolone. Recent studies (Edwards et al., personal communication), however, have shown that finasteride is itself a convulsant, and that high doses of finasteride cause seizures in both mature and immature rats (unpublished data). Therefore, this excludes the use of finasteride for mechanistic studies in seizure models. The finding, by Kokate, that finasteride blocks the anticonvulsant effects of progesterone could be due to finasteride’s proconvulsant effects, rather than its inhibitory effects on 5α-reductase. It seems unlikely, therefore, that allopregnanolone plays a major role either in the clinical, or in the “fast” effects seen in the present study.

7.6 How Does Progesterone Stop Seizures? : Non-GABA-Mediated Actions

What are the possible non-GABA-modulating mechanisms of progesterone’s “fast” anticonvulsant action? Four possible mechanisms may be considered: 1) non-specific effects, 2) binding to the intracellular receptor, or 3) non-GABA-acting metabolites, and 4) binding to the membrane receptor.
1) Non-Specific Effects

Non-specific effects refer to alterations in neuronal excitability due to changes in membrane fluidity, or membrane expansion. In other words, might progesterone be acting as an anesthetic? If this were an important mechanism of progesterone’s anticonvulsant action, then it would be expected that cholesterol, which has a similar chemical structure, would be an equally good anticonvulsant. Experiments on cholesterol have not shown it to be an effective anticonvulsant at similar doses (Likhodi et al., unpublished data).

2) Binding to the Intracellular Receptor

The assumption that guided the original hypothesis of this thesis was that progesterone was acting through the “classic” intracellular receptors. The rapid onset of progesterone’s anticonvulsant actions in Experiment 3 made this dubious, and the fact that RU486 in Experiment 4 failed to block progesterone’s anticonvulsant action seems to rule it out. This indicates that the classic progesterone receptor is not involved in progesterone’s anticonvulsant actions.

3) Other Metabolites

As well as being metabolized to allopregnanolone, progesterone is also metabolized to 20-alpha dihydroprogesterone and 11-deoxycorticosterone (Figure 1). There is no evidence that 20-alpha dehydroprogesterone has any anticonvulsant effects. 11-deoxycorticosterone, however, is further broken down to GABA-A-binding metabolites and does have GABA-mediated anticonvulsant effects. Recently, Edwards et
al. (unpublished) have shown that 11-deoxycorticosterone has significant anticonvulsant properties even in the presence of finasteride. This contrasts with the anticonvulsant effects of progesterone, which can be blocked by finasteride (Kokate et al., 1998). This implies that progesterone’s and 11-deoxycorticosterone’s non-GABA-mediated effects are different. Therefore, it is highly unlikely that progesterone’s anticonvulsant effects are mediated via 11-deoxycorticosterone.

4) Binding to the Membrane Receptor

The most likely candidate for the non-GABA-mediated “fast” anticonvulsant effects of progesterone is the membrane progesterone receptor. The RU486 (Experiment 4) data support this mechanism. RU486 blocks the intracellular progesterone receptor but has a very low affinity for the membrane progesterone receptor.

The MPA data are also consistent with this hypothesis. MPA binds the intracellular, but not the cell surface receptor.

Assuming that progesterone’s anticonvulsant action is mediated by its membrane receptor, what downstream mechanisms could be involved? This is not clear as yet. The primary known effect of the membrane progesterone receptor is to increase calcium currents, which should be procovulsant (Introduction) in the brain. These findings, however, are from sperm data. There is no evidence as yet, that the progesterone membrane receptors enhance calcium currents in the brain.
There is currently a lack of comprehensive information on the electrophysiological effects of progesterone membrane receptors in the brain. Further research will be required. It seems probable, however, that progesterone's "fast" anticonvulsant effects are mediated by binding to cell surface progesterone receptors.

7.7 How Does Progesterone Stop Seizures? Threshold Rises

What is the effect of progesterone on the "systems" level? The fact that progesterone was effective against MMT seizures, but not against MES seizures (in adults), suggests that progesterone raises seizure thresholds, but that the threshold rises are not large.

Previous studies have also shown that small changes in seizure thresholds can have clinical significance. The ketogenic diet, for instance, raises seizure thresholds minutely in animal seizure models by only about 20% (Thavendiranathan et al., 2000), yet it is an effective treatment for intractable childhood seizures. Small rises in threshold can be important if they occur in patients that resist the standard anticonvulsant drugs.

7.8 How Does Progesterone Stop Seizures: Clinical Actions
The present work has shown "fast" anticonvulsant effects of progesterone, unrelated to allogregnanolone and possibly mediated by membrane receptors. Do these play a role in progesterone's clinical actions? While they could play an important role (below), it seems unlikely that they can account for the effects seen clinically by Herzog and others.

Clinical studies establish progesterone concentrations within the normal physiological range. Even 5mg/kg would produce supra-physiological concentrations, and the doses used in Experiment 3 (ED50's were 10-15 mg/kg.) are certainly supra-physiological.

Thus, while we have discovered new anticonvulsant actions of progesterone, they are probably unrelated to the effects seen in clinical studies. Those effects probably relate to progesterone's contraceptive and anti-estrogen effects.

1) Contraceptive effects

The contraceptive-related anticonvulsant effects of progesterone relate to its ability to eliminate the seizure triggers in catamenial exacerbation – the seizure triggers being estrogen and progesterone withdrawal. Progesterone does this by stopping the production of endogenous hormones through negative feedback to the hypothalamus and the anterior pituitary (Introduction). The progesterone intracellular receptor may be involved in this process. This mechanism would require prolonged exposure to progesterone.
2) Anti-estrogen Effects

Progesterone’s anti-estrogen effects may also be related to its actions on the classic progesterone receptors (Introduction). This mechanism involves the ability of PR-A to act as a transcriptional inhibitor of other hormone receptors, including the estrogen receptors. Together, these mechanisms may explain progesterone’s low dose clinical effects. This hypothesis is termed the “contraceptive” hypothesis.

The “contraceptive hypothesis” of the anticonvulsant actions of progesterone – outlined above – would deal with all three patterns of catamenial epilepsy: Pattern 1) exacerbation at the time of ovulation, Pattern 2) exacerbation during the entire luteal phase, and Pattern 3) exacerbation during the premenstrual period. Progestins (used as contraceptives) stop the production of sex hormones through negative feedback in the hypothalamus and the ovaries. Hence, a patient who uses progestins as contraceptives: 1) would not have an estrogen surge which is associated with ovulation, 2) would not have elevated levels of estrogen which are associated with the luteal phase, and 3) would not have the progesterone withdrawal which is associated with the perimenstrual period.

Bauer’s clinical studies (1992) support the hypotheses that the anticonvulsive properties of progestins are linked to their contraceptive mechanisms of action. He used a synthetic GnRH analogue to suppress the fertility cycle in intractable epileptic patients with catamenial exacerbation. Using this treatment, he was able to reduce the frequency
or abolish seizures to an equal or greater degree as in the studies, which have used progestins.

In summary, progesterone may stop seizures in three ways. At truly low doses, in the clinic, it may have “contraceptive” effects. At very high doses, in animal studies, it may have “slow” anticonvulsant effects related to its sedative metabolite, allopregnanolone. In addition, as we have shown in the present work, at moderate doses, progesterone has “fast” anticonvulsant effects, possibly related to binding to the cell surface receptor.

The “fast effects” of progesterone constitute a novel finding. It may provide a new target, and a new mechanism of action, for anticonvulsant drug development.

7.9 Clinical Relevance of the Present Study

To date, only a small number of female patients suffering from catamenial seizures have benefited from progesterone therapy. The present study found major moderate-dose anticonvulsant actions of progesterone in adult male as well as female subjects (Experiment 3). Similarly, major anticonvulsant effects were seen in immature subjects (Experiment 3). These “fast”, moderate-dose effects were not associated with sedation. These findings imply that progesterone might be used clinically in males, infants and non-catamenial females. A series of expanded clinical trials are in order.
Future studies must investigate the mechanisms of action of progesterone, and also the nature of catamenial and intractable seizures:

1) To investigate the “fast” effects of progesterone, specific agonist and antagonists against the membrane progesterone receptor should be tested. Currently, these compounds exist, although they are not available commercially. This work should be carried on in whole animals, as well as in slice preparations. An alternative to specific agonists is BSA-conjugated progesterone. This compound does not cross cell membranes and its activity is limited to membrane receptors. Since BSA-conjugated progesterone also does not cross the blood-brain-barrier, it should be used in slices or administered ICV via an implanted cannula in whole animals.

2) To show more conclusively that the “fast” effects of progesterone are not mediated by its GABA-modulating metabolite, plasma levels of progesterone and allopregnanolone should be measured. Hormone levels should be measured in a time response study, using the same parameters as Experiment 3. Hormone levels should also be measured in the presence or absence of indomethacin.
3) In the kindling experiments, dose-response studies were performed two hours after the administration of progesterone. These studies should be repeated at a test interval of fifteen minutes. This would show whether the “fast” effects are also present in the kindling model, a model of complex partial seizures.

4) One of the anticonvulsant mechanisms of progesterone may be antiestrogenic effect. This can be tested easily and directly using antiestrogens such as roloxifene. These should be tested as anticonvulsants.

5) The intractable nature of seizures with catamenial exacerbation may relate to the role that estrogen plays in complex partial seizures. In Experiment 1, Estrogen treatment, alone, lowered seizure thresholds at the (amygdala-kindled) seizure focus. This finding that estrogen is proconvulsant is in agreement with all previously published data. The fact that seizure thresholds drop at the seizure focus, however, explains why the catamenial exacerbation is often associated with complex partial seizures. Estrogen lowers seizure thresholds focally (in limbic structures) and also globally by a small degree (less than 30%). While this would not be important for the treatment of generalized seizures, it has radical consequences for complex partial seizures. Albright and Burnham (1980) showed that AED’s raise seizure thresholds globally, but not in limbic structures. Hence, an endogenous substance (estrogen) that lowers limbic seizure thresholds by as much as 30% could cause seizures despite the presence of therapeutic doses of AED’s. In fact, toxic doses of AED’s would be required to return limbic seizure thresholds back to normal.
The role that estrogen plays in complex partial seizures, therefore, can be studied in the following series of experiments. First dose-response studies should be performed on the effect of estrogen on after-discharge threshold in amygdala and cortical implanted subjects (before and after kindling). Next, the effect of AED’s and antiestrogens on raising focal and generalized seizure thresholds in estrogen-pretreated, amygdala and cortical kindled subjects should be studied. If there is a difference in response between cortical and amygdala kindled subjects (as hypothesized), then the mechanism of action should be studied in slice preparations, which compare the effect of estrogen (on current fluxes for instance) on tissue from the cortex and the amygdala.

6) The finding that estrogen lowers seizure thresholds at the seizure focus also has significance for the role that glia play in the development of complex-partial seizures. There are no estrogen receptors in the neurons of the baso-lateral amygdala (Table 1) - the site of the seizure focus – in the normal rat. Garcia-Segura’s group (1999), however, found that in response to injury activated glia express aromatase (Appendix 2). This response is irrespective of the site of injury. Hence, it is likely that at the site of the seizure focus, estrogen is stimulating activated glia rather than neurons to lower the seizure threshold.

This theory can be verified by studying the effect of activation and inactivation of astrocytes on kindling thresholds. Alpha-aminoadipate, a compound that selectively destroys astrocytes, can be used at the site of kindling focus to test this hypothesis.
Appendix 1:

AEDs
Pharmacological intervention in epilepsy began with the use of potassium bromide by Locock in 1857 (Friedlander, 1986b).

In 1912, Hauptman introduced phenobarbital as the first organic antiseizure compound (cited by Woodbury and Fingl, 1975). Ticku and Olsen discovered that phenobarbital binds to the barbiturate site on the gamma-aminobutyric acid (GABA) receptor, and enhances GABA-mediated inhibitory pathways (Ticku and Olsen, 1978). Phenobarbital is no longer used as a first line therapy due to its severe sedative side effects. It is still used however, in poly-pharmacy and, in some cases, in the treatment of tonic-clonic and partial seizures (Lerman-Sagie and Lerman, 1999; Yukawa, 2000).

Meritt and Putnam introduced phenytoin in 1938 (Glazko, 1986). The discovery of phenytoin was the result of a conscious effort to find a phenobarbital-like drug with less sedative side effects. Many variants of the phenobarbital molecule were generated and tested. Phenytoin was the compound discovered. Phenytoin also marked an important advancement in the development of anticonvulsant drugs, since it was the first marketed antiseizure drug to be developed using an animal model of epilepsy (Friedlander, 1986a). Willow and Catterall later discovered that phenytoin exerts its effect by blocking voltage gated Na⁺ channels (Willow and Catterall, 1982). Phenytoin is used primarily in the treatment of tonic-clonic and partial seizures (Perucca, 1996).
Phenobarbital, and phenytoin are not effective in the treatment of absence seizures (Murphy and Delanty, 2000). In 1945, Lennox found that trimethadione - a variant of phenobarbital - was selectively effective against absence seizures. It was ineffective against tonic-clonic or partial seizures. In 1963, it was found that ethosuximide – another variant of Phenobarbital - was effective in the treatment of absence seizures (Chen et al. 1963). Ethosuximide replaced trimethadione due to less severe side effects. Coulter has shown that ethosuximide (and trimethadione) act through inhibition of T-type voltage gated calcium channels in the thalamus (Coulter et al., 1989; Davies, 1995). This finding was possible because of the work of Rowgoski who discovered how neuronal firing in the thalamus is affected by T-type calcium channels (Suzuki and Rogawski, 1989).

Benzodiazepines were introduced in the 1970's. They include clonazepam, clobazam, and diazepam. They are effective against a broad range of seizures including partial, tonic-clonic and absence seizures (Krall et al., 1978). Mohler and Okada discovered the benzodiazepine receptor, which was later found to be a site on the GABA-A-related chloride channel (Mohler and Okada, 1977). Benzodiazepines increase GABAergic inhibition by binding to the benzodiazepine site of the GABA-A receptor (Doble, 1999). By tradition, clonazepam and clobazam are used in the chronic treatment of seizures, whereas diazepam is administered i.v. to stop recurrent seizures (‘status epilepticus’).
Two important drugs introduced in the 1970's were carbamazepine and valproate. Carbamazepine, has a similar mechanism of action to phenytoin (Macdonald and Kelly, 1995), but it has fewer side effects than phenytoin. It has therefore, become more widely used in the treatment of tonic-clonic and partial seizures (Perucca, 1996).

Valproic acid was discovered accidentally when it was used as a solvent for potential AED. It has a broad range of activity, including activity against partial, tonic-clonic, and absence seizures (Perucca, 1996). This is possibly due to valproate’s multiple mechanisms of action. Valproic acid both increases GABAergic inhibition and inhibits NMDA-mediated cell firing (Loscher, 1993).

A number of new AED’s have entered clinical PR-Activity in the past decade. These are called “second generation” AED’s (Loscher, 1998). Vigabatrin and tiagabine, enhance GABA-ergic inhibition. They are effective for the treatment of partial seizures and tonic-clonic seizures. Vigabatrin increases the levels of the neurotransmitter GABA by covalently binding to and inhibiting GABA transaminase, GABA’s catalytic enzyme (Graves and Leppik, 1993). Tiagabine hydrochloride increases synaptic GABA levels by blocking the re-uptake mechanism of GABA (Bourgeois, 1998).

Gabapentin and topiramate are “second generation” AED’s with multiple mechanisms of action. They are effective for partial, tonic-clonic and absence seizures. Gabapentin has also been used as an add-on therapy in cases of intractable complex-partial seizures (Marson et al., 2000). Gabapentin’s mechanisms of action include raising
GABA levels in the brain and decreasing glutamate levels (McLean, 1999). It is unclear which of gabapentine’s mechanisms of action is primarily responsible for its anticonvulsant effects. Topiramate blocks sodium channels (Shank et al., 2000).

Lamotrigine acts mainly through sodium channel blockade. It also inhibits glutamate release (Coulter, 1997). It is effective against partial, tonic-clonic, and absence seizures. It also protects against some instances of drop attacks, which are a part of the Lennox-Gastaut syndrome (Perucca, 1999).
Appendix 2:

Neurosteroids – Three Important Metabolic Enzymes
Appendix 2: Neurosteroids – Three Important Metabolic Enzymes

A2.1 Aromatase

Aromatase converts testosterone into estrogen. Aromatase is normally found only in the neurons of the limbic structures such as the amygdala, the hippocampus, and the hypothalamus. After brain injury however, astrocytes begin to express aromatase regardless of the location of the injury (Brenner et al., ). This strongly supports the notion that estrogen is involved in brain repair, and helps explain the positive effects of this hormone in the treatment of neurodegenerative diseases such as Alzheimer’s (Oriowo et al., 1980).

Aromatase distribution in the rat brain changes in three distinct, ontological, phases (Oriowo et al., 1980). Aromatase is expressed in moderate levels in the following structures between GD16 and PND2 only: anterior medial preoptic nucleus (aMPN), the strial part of the preoptic area (stPA), and the rostral portion of the medial preoptic nucleus (rMPN). In a second set of structures aromatase appears at GD16, and reaches maximum levels between GD18 and PND2. Aromatase expression then declines gradually to adult levels. Expression in these second set of structures forms a continuum that extends from the caudal parts of the lateral periphery of the medial preoptic nucleus (clMPN) to the principal nucleus of the bed nucleus of the stria terminalis (PR-BNST) and on further to the posterodorsal part of the medial amygdaloid nucleus (pDMAmg). In
a third group of neurons aromatase appears only after PND14. These neurons are in the lateral septal nucleus (iLS), the oval nucleus of the bed nucleus of the stria terminalis (ovBNST), and the central amygdaloid nucleus (CAmg). These findings by Shinoda (1994) were in complete agreement with Lauber and Lichtestiger who also characterized the brain distribution of aromatase simultaneously (Oriowo et al., 1980). Using a different technique, Maclusky’s group found that in addition to the high levels of aromatase in the hypothalamus-preoptic area and the amygdala, there are lower levels in the hippocampus, midbrain, and the cingulated cortex (Oriowo et al., 1980).

The spatial as well as the temporal distribution of aromatase in the brain suggests that it plays an important role in the sexual differentiation of the brain. Ontologically, masculinization of the brain, or lack of it, is determined by sex hormones. Mammalian brains are ‘female’ by default. It is the presence of estrogen that “masculinizes” the brain. This is possible because of aromatase (McEwen et al., 1997): the enzyme that converts testosterone to estrogen. Aromatase distribution and expression however, is very similar between male and females (Oriowo et al., 1980). The only difference seems to be that males express higher levels of the enzyme (Lauber et al., 1997; Oriowo et al., 1980). It is primarily the pre and postnatal surge in testosterone in males (and lack thereof in females) that is converted to estrogen via aromatase and thereby masculinizes the brain (Shinoda, 1994). Maternal estrogen does not affect the fetal brain because of the expression of high concentrations of feto/neonatal estrogen binding protein (FEBP) that keep free-estrogen levels very low. FEBP binds estrogens with high affinity, but it does not sequester androgens (Korneyev et al., 1993).
Subcellular localization of aromatase by electron microscopy has revealed the presence of this enzyme on the surface of vesicles in presynaptic buttons (Medlock et al., 1991). This finding lends support to theories on the actions of estrogen at surface receptors modulating membrane potentials (Leyendecker et al., 1975).

Two studies have looked at aromatase expression and activity in brain samples from human patients with epilepsy (Oriowo et al., 1980). Expression and activity of aromatase in human brains is consistent with data obtained from the rat. The authors of these studies suggest that enhanced aromatase activity in epileptic patients may increase their risk for having seizures. The present studies however, fail to prove this point since proper controls are lacking.

A2.2 5-alpha reductase

5-alpha reductase metabolizes a number of hormones, including progesterone. 5-alpha reductase is ubiquitously distributed in the brain in both neurons and glia (Leyendecker et al., 1975). The enzyme activity however is not uniform in the brain. Melcangi’s group has shown two seemingly contradictory results with respect to 5-alpha reductase activity. They showed that neurons have greater enzyme activity than glia (Reddy and Kulkarni, 1998). They also showed that 5-alpha reductase activity is greater in white matter structures than in the cerebral cortex (Kokate et al., 1999). They
fail to explain this discrepancy in their results, however, the latter finding has been reproduced and investigated in subsequent experiments (Gee et al., 1988; Korneyev et al., 1993; Medlock et al., 1992).

The finding that 5-alpha reductase activity is greater in white matter led Melcangi’s group to speculate that this enzyme is involved in the process of myelin formation. They found support for this hypothesis by showing that 5-alpha reductase is present in myelin sheaths, while axons are devoid of the enzyme (Medlock et al., 1992). They also showed that dihyroprogesterone, the 5-alpha reduced metabolite of progesterone that binds to the progesterone receptor, induces gene expression of peripheral myelin protein zero (P0) (Kokate et al., 1999). This metabolite however is only part of the mechanism regulating myelination since other metabolites of progesterone also regulate myelin related proteins. Baulieu’s group simultaneously confirmed Melcangi’s findings (and came to the same conclusions) by showing that in addition to its effects on Schwann cells, progesterone also increases the expression of myelin-specific proteins in oligodendrocytes (Oriowo et al., 1980).

Evidence from ontological expression and activity of 5-alpha reductase also supports its role myelin formation and maintenance. In an early experiment, Melcangi’s group found that 5-alpha reductase activity in purified myelin from rats was highest in the third week of life (Melcangi et al., 1988). Lauber and Lichtensteiger preformed more detailed studies on the ontogeny of 5-alpha reductase type 1 and they found three distinct patterns of its mRNA expression (Lauber and Lichtensteiger, 1996). 1) In the embryonic
stage, on GD12-18, 5-alpha reductase is expressed in the germinal and ventricular zones.

2) During the late fetal and early postnatal development 5-alpha reductase levels gradually decrease in the ventricular zone, but are expressed in differentiating regions of the brain including the cortical plate, the thalamus, the cerebellum, and the pyramidal cell layer of the hippocampus with the subiculum. 3) From PND15 and into adulthood lower levels of the enzyme are detected primarily in white matter structure. This last finding is in agreement with Melcangi’s findings and hypothesis. The distribution of 5-alpha reductase in pre and early postnatal rat however, suggests a role in proliferation and differentiation (Lauber and Lichtensteiger, 1996).

A second isoform 5-alpha reductase has been found (Russell and Wilson, 1994). It is called type 2. 5-alpha reductase type 2 has a much higher affinity for various substrates than the type 1 isoform, however it is active only in a very narrow PH range around 5. The type 2 isoform has a different ontological profile from the type 1 enzyme. It is absent at GD14. It is detected at GD18. It reaches its peak levels at PND2, and then decreases to extremely low levels or becomes absent altogether (Poletti et al., 1998). In adult rats the type 2 enzyme is detected primarily in the hypothalamas (Poletti and Martini, 1999). Unlike 5-alpha reductase type 1, the type 2 isoform gene is highly inducible by testosterone, but only in cells derived from the hypothalamus. This is intriguing because both isoforms have a glucocorticoid response element in their promoter regions. Differences in presence or absence of cofactors might explain differential responses to testosterone. The correlation between the levels of 5-alpha reductase type 2 during the ‘critical period’ of sexual differentiation and it’s pattern of
inducability by testosterone has led Poletti to hypothesizes that the type 2 isoform may be involved in sexual differentiation. Their studies to date however, have been in male rats only (Poletti et al., 1998). None-the-less there is strong support for this idea from the observation that a genetically defective type 2 enzymes in the male human results in pseudohermaphroditism (Imperato-McGinley et al., 1974; Katz et al., 1995).

Recently Kellogg and Frye showed that the activity of 5-alpha reductase is different in infants and adults (Kellogg and Frye, 1999). In the fetal rat brain (from the last five days of gestation) the levels of progesterone metabolites are twenty fold higher than the levels of progesterone, whereas the levels of testosterone metabolites are ten fold lower than the levels of testosterone. In adults however, the main substrates for 5-alpha reductase seem to change. The conversion of progesterone to its metabolites becomes more inefficient, but the levels of testosterone 5-alpha reduced metabolites are three to ten folds higher than the levels of the parent hormone.

A2.3 3-alpha hydroxysteroid dehydrogenase (3-alpha HSD)

3-alpha HSD metabolizes a number of hormones, including 5-alpha reduced progesterone. 3-alpha HSD is not found in neurons (Melcangi et al., 1993). 3-alpha HSD is found primarily in type 1 astrocytes and to a lesser degree in oligodendrocytes (Melcangi et al., 1994). All regions of the rat brain express 3-alpha HSD. The highest levels of 3-alpha HSD however, are found in the olfactory bulb (Cheng
et al., 1994a). It is curious that the olfactory bulb also contains the highest concentration of interneurons in the brain. The distribution of 3-alpha HSD is consistent with the role of 3-alpha modified hormones in modulating the activity and genomic expression of GABA-a receptors (Belelli et al., 1990; Smith et al., 1998a).

The promoter region of 3-alpha HSD contains response elements for estrogen, glucocorticoids and progesterone (Penning et al., 1997).

3-alpha HSD has a substrate affinity in the low micro molar range for 5-alpha reduced hormones. It has specific activity that is a 100 fold higher than 5-alpha reductase (Penning et al., 1985). This indicates that in vivo, 5-alpha reductase is the rate limiting step in producing 5-alpha,3-alpha modified steroids.

3-alpha HSD is responsible for giving testosterone and progesterone metabolites GABA-modulating activity. There are however, important interspecies differences in the activity of this and other enzymes in the brain. Costa’s group established the levels of various metabolites of progesterone in the brains of rats, mice and monkeys (Korneyev et al., 1993). In the rat the major metabolites are 5-alpha dihydroprogesterone, and 3-alpha,5-alpha tetrahydroprogesterone. In the mouse the major metabolites are 5-alpha dihydroprogesterone, and 20-alpha dihydroprogesterone. In the monkey the main metabolite is 20-alpha dihydroprogesterone.
Appendix 3:

The Role of Sex Hormones in Some Important Aspects of Neuronal Plasticity
A number of behavioral changes that occur during the estrous cycle, in pregnancy, at puberty or at menopause have provided insights into the role of sex hormones on brain plasticity. Figure 2 demonstrates the variation in levels of progesterone and estrogen in the estrous cycles of human and rat respectively.

Premenstrual syndrome is correlated temporally to progesterone withdrawal. Smith’s group suggests that premenstrual irritability is due to the effect of progesterone withdrawal on the genomic expression of the alpha-4 subunit of the GABA-a receptor (Smith et al., 1998a). The differential expression of various GABA-a subunits alters the electrophysiological properties of the GABA-a complex. GABA-a complexes containing alpha-4 subunits have decreased chloride currents. A brain rich in alpha-4 subunits is more susceptible to seizures (Smith et al., 1998a) and is insensitive to the potentiating effect of benzodiazepines (Reddy and Rogawski, 2000; Smith et al., 1998c). Smith suggests that postpartum depression and postmenopausal dysphoria also share the same mechanism as premenstrual syndrome.
Tiredness is linked to elevated levels of progesterone. This phenomenon may be apparent in the late luteal phase; however, it is much more pronounced in pregnancy where progesterone levels increase to more than ten times luteal levels (Behrenz and Monga, 1999). There is a close relationship between plasma levels of progesterone and allopregnanolone (Concas et al., 1998). Tiredness during pregnancy is due to the agonistic affects of allopregnanolone on the GABA-a receptor (Rupprecht and Holsboer, 1999). The general sedative effects of progesterone can manifest itself in depression as experienced by some women during pregnancy or during hormonal therapy (Herzog, 1986a).

The behavioural change that is of particular interest to this thesis is the catamenial pattern of seizures. If pathological changes in the brain are an extention of neuronal placicity (McEachern and Shaw, 1999), then catamenial exacerbation too is a form of neuronal plasticity in response to hormones. In Catamenial epilepsy seizure frequency is increased during the estrous cycle at times of high estrogen levels or with progesterone withdrawal, and it seems to decrease at times of high progesterone levels (Herzog et al., 1997b).

Rat behavior in response to hormones is often similar to human behavior. There is a noteworthy exception. In the human female the height of sexual receptivity generally occurs at ovulation (Singer and Singer, 1972; Stanislaw and Rice, 1988). This corresponds to a peak in estrogen levels. In rats, sexual receptivity also occurs at ovulation; however, it is not primarily mediated by estrogen. This event occurs on estrous at the height of the
progesterone peak (Ichikawa et al., 1972). Estrogen priming in proestrous however, is a necessary prelude for the lordosis behavior (Freeman et al., 1976).

A3.2 Neuronal Plasticity and the Fertility Cycle: Cellular Changes

It is possible to think of epilepsy as a pathological form of neuronal plasticity. In this paradigm plasticity is a continuum encompassing development of neuronal circuitry, learning, memory, and pathological disorders of the CNS (McEachern and Shaw, 1999). Hence a number of cellular and molecular markers of neuronal plasticity can be used to assess the extent of changes in the brain in response to various stimuli. Sex hormones induce a number of cellular and molecular changes that are associated with neuronal plasticity.

LTP and LDP are standard measurements of synaptic plasticity. LTP is very simply a semi-permanent enhancement in synaptic strength while LDP describes the inverse process. Estrogen enhances long-term potentiation (LTP) and attenuates long-term depression (LTD) (Good et al., 1999; Warren et al., 1995). This effect is mediated by NMDA receptors (Foy et al., 1999). The effect of progesterone on these parameters is not clear. Allopregnanolone however, decreases LTP (Dubrovsky et al., 1993). LTP and LDP are also correlated with seizure activity. Electrical and chemical kindling enhance LTP (Ben Ari and Gho, 1988; Sutula and Steward, 1986). Likewise, LTP speeds the kindling process (Sutula and Steward, 1987).
In rats increased neurogenesis is observed during proestrus (Tauboll and Lindstrom, 1993). Neurogenesis is a natural process that occurs at a basal rate in the dentate gyrus and around the third ventricle (Kuhn and Svendsen, 1999). Many of the new neurons that are produced die soon after through apoptosis. Estrogen is an anti-apoptotic agent (Garcia-Segura et al., 2001), and it increases the number of new neurons by increasing cell survival and also by increasing the rate of production (Tanapat et al., 1999). Progesterone’s effect on neurogenesis is unclear.

New dentate granule neurons have a greater capacity for long-term potentiation (Wang et al., 2000). They also have less GABA-ergic inhibition than older dentate granule neurons (Wang et al., 2000). Therefore, enhanced neurogenesis potentially enhances hippocampal excitability. Gould has proposed that neurogenesis is a potential mechanism through which hormones affect spatial learning and memory (Gould et al., 1999). Recently, abnormal neurogenesis has been linked to both the onset and recovery from depression (Jacobs et al., 2000). Also, various methods of inducing seizures have been shown to enhance neurogenesis (Parent et al., 1998; Scott et al., 2000).

Sex hormones affect spine density of the pyramidal neurons of the hippocampus (Woolley and McEwen, 1993). Spines are outgrowths of dendrites onto which afferent axons synapse. Woolley’s group found that estrogen increases spine density by activating NMDA receptors (Woolley and McEwen, 1994). Hence increased spine density is associated with increased neuronal excitability. In the rat estrous cycle,
spine density is greatest in proestrous, corresponding to an estrogen peak, and drops sharply in estrous corresponding to a progesterone peak (Woolley and McEwen, 1994).

Hormonal regulation of mossy fiber sprouting is similar to the regulation of pyramidal cell spine densities. Mossy fibers are axons of dentate granule cells that synapse onto the dendrites of pyramidal cells in CA3 region of the hippocampus (Sutula et al., 1992). Estrogen enhances sprouting while the effects of progesterone have not been examined so far (Teter et al., 1999). Also, various forms of inducing seizures have been shown to enhance mossy fiber sprouting (Sutula et al., 1992).

Recently Garcia-Segura proposed that estrogen increases synaptic excitability by altering synaptic architecture (Garcia-Segura et al., 1999). This process involves the interaction between glia and neurons (Mor et al., 1999). On the afternoon of proestrous estrogen activates astroglia by increasing the synthesis of GFAP. Activated astroglia ensheathe the soma of glutamatergic neurons in their vicinity. In doing so they temporarily force the disinhibition of axonosomatic synapses by interneurons.

A3.3 Neuronal Plasticity and the Fertility cycle: Some Molecular Changes

A3.3.1 GABA
GABA is the primary inhibitory amino acid in the brain. GABA plays an important role in neuronal plasticity by regulating the expression of its receptors or receptor subunits. Two alpha, two beta, and one gamma subunit make up the GABA receptor complex and each subunit is present in a multitude of isoforms (Mohler et al., 1996). Abnormalities in GABA-ergic response underlie the GABA hypothesis of epilepsy. Loscher’s group did not see any long-term changes in the number of GABA-ergic neurons in the hippocampus after kindling (Lehmann et al., 1996). Reductions however, were seen in the ipsilateral piriform cortex (Lehmann et al., 1998). Also, regardless of the number of GABA-ergic neurons, kindling causes a long-lasting decrease in inhibitory effect of GABA in the hippocampus (Kamphuis et al., 1991). GABA receptor subunits are altered in various seizure models including kindling (Follesa et al., 1999; Kokaia et al., 1994; Poulter et al., 1999).

Estrogen’s effects on the GABA complex are still clouded in the literature. Canonaco has argued that estrogen decreases GABA receptor levels in the brain (Canonaco et al., 1993). Herbison on the other hand has argued that in areas of the brain containing estrogen receptors, estrogen significantly increases the levels of some GABA receptor subunits (Herbison and Fenelon, 1995). Herbison’s work is in agreement with Perez’s findings that estrogen changes the function of GABA-a receptors by increasing the binding of GABA agonists in specific regions of the brain (Perez et al., 1988). This enhancement of GABA-ergic activity however, does not increase protection against convulsants (Perez et al., 1988).
Progesterone’s effects on GABA receptors are primarily indirect and involve progesterone’s tetra-hydroxy metabolite, also called allopregnanolone (Concas et al., 1999). Allopregnanolone binds to the alpha subunit of the GABA-a receptor with a hundred fold greater affinity than barbiturates (Gee et al., 1988). Allopregnanolone enhances the effects of GABA and can alter subunit expression as well. Progesterone withdrawal increases the formation of the alpha-4 subunit of the GABA-a receptor (Smith et al., 1998b). This makes the complex less responsive to agonists of the alpha subunit.

A3.3.2 Glutamate

Glutamate is the primary excitatory amino acid in the brain. It plays an important role in neuronal plasticity via the AMPA, kainite, and NMDA receptors. Abnormalities in glutamatergic response underlie the glutamate hypothesis of epilepsy.

Wooley and McEwen have shown that estrogen enhances certain excitatory glutamergic pathways. Estrogen increases NMDA agonist binding directly by regulating the agonist binding site (Weiland et al., 1997). Estrogen also increases the number of synapses containing glutamatergic receptors (Woolley and McEwen, 1994). These axonodendritic synapses are made on dendritic spines. The estrogen-induced spine formation in the hippocampus during proestrus contain elevated levels of NMDA-1 receptors, but they lack AMPA receptors (Woolley, 1998).
Progesterone does not alter the expression or bind directly to glutamergic receptors. It may however, inhibit NMDA receptors indirectly through it’s action on the sigma receptor (Monnet et al., 1995). Sigma receptor upon agonist binding potentiates neuronal response to NMDA (Bergeron et al., 1999). Progesterone is a Sigma receptor antagonist. Progesterone’s effect on NMDA receptors is probably more apparent through cross-talk with estrogen receptors. Woolley showed that initially, progesterone acts synergistically with estrogen to enhance CA1 dendritic spines containing NMDA receptors (Woolley and McEwen, 1993). Eighteen hours after progesterone treatment however, spine density is significantly decreased.

A3.3.3 Connexin

Gap junctions mediate a non-synaptic mechanism of cell-to-cell communication by permitting direct electrical coupling between cells (Rozental et al., 2000b). Gap junctions are comprised of a large family of connexins. Six connexins form a hemichannel complex called a connexon at the cell surface (Dermietzel, 1998). Connexons aggregate together into plaques. Disulfide bonds adjoin connexons from plaques on adjacent cells (Foote et al., 1998; Manjunath et al., 1987). Gap junctions are involved in neuronal plasticity and they are regulated by sex hormones (Perez et al., 1990). Estrogen, alone or in combination with progesterone, increases connexin expression (Lye et al., 1993; Shinohara et al., 2000). Progesterone withdrawal also increases connexin expression (Micevych et al., 1996). The combination of mechanical or electrical stimulation and estrogen synergistically increase gap junction mRNA.
expression. While electrical stimulation alone, or progesterone without estrogen
however, do not increase connexin expression (Edwards and Maclusky, unpublished
data).

Connexin 43 is the first isoform to be expressed in the brain (Chang and Balice-
Gordon, 2000). In the rat it is expressed at GD12, peaks at PND21 and levels remain
high into adulthood. It is primarily a glial gap junction although it is also found in some
neurons such as the CA1 pyramidal neurons (Rozental et al., 2000a). Neuronal connexins
such as connexin 32 and 36 are expressed most prominently in late gestation and drops to
adult levels in the first few weeks of life (Belliveau and Naus, 1995; Rozental et al.,
2000c). An exception is the neuronal connexin 26, which is primarily expressed in the
developing brain.

Outside the brain, gap junctions have a wide range of physiological functions.
Connexin 43 is responsible for the synchronous contractions of the heart. Gap junctions
also synchronize labor contractions. In the brain, they mediate high frequency
oscillations (100-200 Hz) in the hippocampus (Draguhn et al., 1998; Draguhn et al., 2000).
These high frequency oscillations seem to involve pyramidal neurons interconnected by
axoaxonal gap junctions (Traub et al., 1999). Gap junctions also mediate slow calcium
waves among astrocytes (Giaume and Venance, 1998; Rottingen and Iversen, 2000).
Temporal expression of gap junctions suggests that they may also play a role in neuronal
migration (Naus and Bani-Yaghoub, 1998). Hence, gap junctions may provide a general
mechanism through which excitatory or inhibitory pathways operate in a synchronized fashion.

There is some evidence that gap junctions may be responsible for synchrony of epileptic seizures. Carlen's group showed that in absence of synaptic transmission, gap junctions mediate epileptiform activity in hippocampal slices (Carlen et al., 2000; Perez Velazquez and Carlen, 2000). Ironically, in animal seizure models, connexin expression is decreased or remains unchanged (Elisevich et al., 1997a; Elisevich et al., 1998; Khurigel and Ivy, 1996). Likewise, hippocampal tissue from human epileptic patients does not show an increase in connexin expression (Elisevich et al., 1997b). Chang however, showed that following axonal injury there is increased gap junction coupling without an increase in expression (Chang et al., 2000).

A3.3.4 c-fos

Sex hormones alter the expression of number of early response genes, such as c-fos. Both estrogen and progesterone enhance c-fos expression (Auger and Blaustein, 1997). c-fos expression is one of the hallmarks of neuronal plasticity and it is commonly used as a molecular marker of seizure activity (Andre et al., 1998; Applegate et al., 1995; Dragunow et al., 1988; Samoriski et al., 1998; Simler et al., 1999). There is some evidence that c-fos is a convulsant agent and is involved in the spread of seizures in the kainic acid seizure model (Panegyres and Hughes, 1997). On the other hand, c-fos seems to be anticonvulsive in the kindling model (Rocha and Kaufman, 1998).
Another family of early response genes is the neurotrophic factors. Neurotrophic factors are an important part of the maintenance and repair mechanism of neurons and glia (Mackay-Sima and Chuahb, 2000). Estrogen alters the expression of number of neurotrophic factors, such as brain-derived neurotrophic factor (BDNF). The response appears as an increase or decrease in expression depending on brain location or time interval after application of estrogen (Gibbs, 1999; Murphy and Segal, 2000). Progesterone can act in synergy with estrogen to enhance BDNF expression (Gibbs, 1999). Murphy’s group has shown that BDNF prevents estrogen–induced dendritic spine formation in hippocampal neurons (Murphy et al., 1998). Furthermore, progesterone prevents spine formation through a non-BDNF mechanism (Murphy and Segal, 2000). Consistent with Murphy’s findings, BDNF decreases seizure susceptibility in the kindling model (Osehobo et al., 1999; Reibel et al., 2000). Ironically, BDNF enhances LTP (Lu and Chow, 1999).
Reference List


Canonica, M., Tavolaro, R., Maggi, A., 1993. Steroid hormones and receptors of the GABAA supramolecular complex. II. Progesterone and estrogen inhibitory effects on the chloride ion channel receptor in different forebrain areas of the female rat. Neuroendocrinology 57, 974-984.


Friedlander, W.J., 1986b. Who was 'the father of bromide treatment of epilepsy'? Arch Neurol 43, 505-507.


Murphy, D.D., Segal, M., 2000. Progesterone prevents estradiol-induced dendritic spine formation in cultured hippocampal neurons [In Process Citation]. Neuroendocrinology 72, 133-143.


172


Willow, M., Catterall, W.A., 1982. Inhibition of binding of [3H]batrachotoxinin A 20-alpha-benzoate to sodium channels by the anticonvulsant drugs diphenylhydantoin and carbamazepine. Mol Pharmacol 22, 627-635.


