

Progesterone and Its Metabolites: Anticonvulsant and Behavioral Studies

By

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Abstract

Progesterone is a well-known anticonvulsant neurosteroid. Progesterone's inhibitory effects in the brain have long been attributed to its secondary metabolite, 3 α ,5 α -tetrahydroprogesterone (THP, also known as allopregnanolone), a powerful positive modulator of GABA_A receptors. Behavioral and electrophysiological studies of progesterone and its metabolites were conducted for this thesis.

Behavioral Experiments

Recent studies in our laboratory suggested that the primary progesterone metabolite 5 α -dihydroprogesterone (DHP) might have anticonvulsant effects in amygdala-kindled rats. Simple measures of sedation and ataxia were minimal in kindled rats treated with anticonvulsant doses DHP and THP. We therefore hypothesized that anticonvulsant doses of DHP and THP (5-10 mg/kg) would not cause psychotoxicity.

Anticonvulsant doses of DHP and THP were tested in the elevated plus maze, the forced swim test, open field test, and the Morris water maze, models of anxiety, depression, locomotion, and learning and memory, respectively. No psychotoxicity was found the forced swim test, the open field test or the elevated plus maze. Minimal psychotoxicity was observed in the Morris water maze.

Electrophysiology Experiments

Pilot studies in our laboratory had also indicated that progesterone might have anticonvulsant effects in mice independent of its metabolites. We hypothesized that progesterone had a non-GABAergic, non-THP-mediated mechanism of anticonvulsant action.

We first conducted studies in hippocampal kindled mice using a range of doses of progesterone (10-160 mg/kg), DHP (5, 10 mg/kg), THP (1-30 mg/kg), progesterone (100 mg/kg) with finasteride (50 mg/kg), as well as finasteride alone (50 mg/kg), the anticonvulsant drugs carbamazepine (50 mg/kg) and midazolam (2 mg/kg). DHP was not anticonvulsant, whereas THP was anticonvulsant at sedative doses. Surprisingly, high doses of progesterone were anticonvulsant when metabolism to DHP and THP was inhibited with finasteride. Focal seizures were not prevented, but seizure spread and severity were attenuated.

Subsequent studies were done in the entorhinal slice preparation in the presence of 4-aminopyridine and the GABA_A receptor antagonist picrotoxin. We found that progesterone, in the presence and absence of finasteride, was antiepileptiform. This result confirmed that progesterone had, at least in part, a non-GABAergic mechanism of antiepileptiform action independent of progesterone's metabolism to THP. These are novel findings.

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Table of Contents

Abstract.....	ii
Acknowledgements	iv
Table of Contents.....	v
Published Papers.....	xii
Submitted Papers	xii
Paper In Preparation	xii
1st Author Published Abstracts	xiii
Poster presentations	xiii
List of Figures.....	xv
List of Tables	xvii
List of Abbreviations	xviii
Chapter 1: Introduction.....	1
1.1 Epilepsy	2
1.1.1 Definitions.....	2
1.1.2 Epilepsy and the Electroencephalogram (EEG)	6
1.1.3 The Epidemiology and Onset of Epilepsy.....	8
1.1.4 Catamenial Epilepsy: Endogenous Hormones Affect Seizure Susceptibility in Women	10
1.2 Comorbidities of Epilepsy	13
1.2.1 Psychiatric Comorbidities in Epilepsy.....	13

1.2.2 Reproductive/Hormonal Comorbidities of Epilepsy	14
1.2.3 Death and Sudden Unexpected Death in Epilepsy (SUDEP)	16
1.2.4 The Burden of Epilepsy	17
1.3 Causes and Classifications of Epilepsy	18
1.3.1 Structural/Metabolic Epilepsies (formerly “Symptomatic Epilepsies”)	18
1.3.2 Genetic Epilepsies (formerly “Idiopathic Epilepsies”)	19
1.3.3 Epilepsies of Unknown Cause (formerly “Cryptogenic Epilepsies”)	20
1.4 Types of Seizures	21
1.4.1 Focal Seizures (formerly Simple and Complex “Partial Seizures”)	21
1.4.2 Generalized Seizures	23
1.5 Therapy for Epilepsy	24
1.5.1 Pharmacotherapy	24
1.5.2 Drug Mechanisms: Drugs That Modulate Ion Channels	25
1.5.3 Drug Mechanisms: Drugs That Modulate Synaptic Function	30
1.5.4 Limitations of Pharmacotherapy	35
1.5.5 Non-Pharmacological Therapies for Epilepsy	36
1.5.6 The Need for New Pharmacotherapies	37
1.5.7 The Electrical Kindling Model of Epilepsy & Drug Discovery	39
1.6 Steroid Hormones	42
1.6.1 Hormonal Steroids	43
1.6.2 Neurosteroids	43
1.6.3 Neuroactive Steroids	44
1.6.4 Regulation of Neuroactive Steroids	45
1.6.5 Neuroactive Steroids and Neural Excitation	45
1.7 Progesterone and Its Metabolites	48
1.7.1 Progesterone	48

1.7.2 Progesterone Metabolites.....	50
1.7.3 5 α -dihydroprogesterone (DHP).....	51
1.7.4 5 α ,3 α -tetrahydroprogesterone (THP).....	51
1.7.5 The Progesterone Metabolizing Enzymes <i>5α-Reductase (5α-R)</i> and <i>3α-hydroxysteroid oxidoreductase (3α-HSOR)</i>	54
1.7.6 Blockade of <i>5α-reductase</i> : Finasteride	55
1.8 Progesterone Receptors	58
1.8.1 Nuclear Progesterone Receptors	58
1.8.2 Non-Nuclear Progesterone Receptors.....	59
1.9 The GABA_A System.....	61
1.10 Other Potential Targets of Progesterone in the CNS.....	63
1.11 Previous Work on Progesterone, its Metabolites, and Analogs as Anticonvulsant Compounds.....	63
1.11.1 Animal Studies.....	63
1.11.2 Human Studies	66
1.12 Objectives	68
1.12.1 General Objectives	68
1.12.2 Objectives of the Behavioral Studies	68
1.12.3 Objectives of the Studies in Hippocampal-Kindled Subjects.....	69
1.12.4 Objectives of Studies in Entorhinal Slices	69
Chapter 2: Behavioral Studies of Progesterone Metabolites.....	70
2.1 Introduction	71
2.2 General Methods.....	72
2.2.1 Subjects	72
2.2.2 Drugs	73

2.2.3 Handling Procedure.....	74
2.2.4 Statistics.....	74
2.2.5 Forced Swim Test (FST) and Open Field Test (OFT).....	74
2.2.6 Morris Water Maze (MWM)	76
2.2.7 Elevated Plus Maze (EPM)	80
2.3 Results.....	81
2.3.1 The Forced Swim and Open Field Tests.....	81
2.3.2 The Morris Water Maze	87
2.3.3 The Elevated Plus Maze	109
2.4 Chapter Discussion	117
2.4.1 Forced Swim and Open Field Test.....	117
2.4.2 Morris Water Maze	119
2.5.3 Elevated Plus Maze	123
2.5 Summary	125
2.6 Limitations	125
Chapter 3: Electrophysiological Studies In Vivo	128
3.1 Introduction	129
3.2 Methods	131
3.2.1 Animals	131
3.2.2 Drugs	131
3.2.3 Electrode Construction.....	132
3.2.4 Surgery for Electrode Implantation	135
3.2.5 Electrical Stimulation.....	137
3.2.6 Hippocampal Kindling.....	137
3.2.7 Recordings and Measurements	138

3.2.8 Behavioral Assessments.....	140
3.2.9 Confirming Electrode Placement: Brain Histology and Electrophysiological Assessments	140
3.2.10 Statistical Analysis.....	141
3.3 Results.....	144
3.3.1 Progesterone and THP – But Not DHP – Reduced Hippocampal ADs and Motor Seizures	144
3.3.2 Anticonvulsant Effects of High Dosage Progesterone Were Not Diminished by Finasteride Pretreatment.....	150
3.3.3 Carbamazepine Produced Anticonvulsive Effects Similar to Progesterone With Finasteride Pretreatment, Whereas Midazolam Did Not	154
3.4 Chapter Discussion	156
3.4.1 Effects of Progesterone	156
3.4.2 Effects of DHP	156
3.4.3 Effects of THP	157
3.4.4 Effects of Progesterone in the Presence of Finasteride	158
3.5 Originality	159
3.6 Limitations	161
3.7 Conclusion	162
Chapter 4: Electrophysiological Studies In Vitro.....	163
4.1 Introduction	164
4.2 Methods	165
4.2.1 Overview	165
4.2.2 Brain slice preparation and extracellular recordings.....	166
4.2.3 Drugs	166

4.2.4 Examination of epileptiform field potentials in vitro	167
4.2.5 Statistical analysis	167
4.3 Results: Progesterone Decreased Entorhinal Epileptic Potentials in the Presence of a GABA _A Antagonist With and Without Finasteride Pretreatment	168
4.3 Major Findings	172
4.4 Originality	174
Chapter 5: General Discussion	176
5.1 Overview	177
5.2 Behavioral Studies	178
5.2.1 Overview	178
5.2.2 Results	180
5.2.3 Behavioral Studies: Limitations	181
5.2.4 Behavioural Studies: Summary	183
5.3 Electrophysiological Studies	184
5.3.1 Electrophysiology In Vivo: Overview (Chapter 3).....	184
5.3.2 Progesterone, DHP and THP: Hippocampal ADs and Behavioral Motor Seizures	185
5.3.3 Mice with Recording Electrodes in the Hippocampus and the Motor Cortex: Progesterone and Finasteride	187
5.3.4 Mice with Recording Electrodes in the Hippocampus and the Motor Cortex: Midazolam Resembles THP, and Carbamazepine Resembles Progesterone with Finasteride	188
5.3.5 Electrophysiology In Vitro: Overview (Chapter 4)	189
5.3.6 In vitro results: Progesterone Retains its Anticonvulsant Effects in the Presence of Picrotoxin and Finasteride	189

5.3.7 Electrophysiology Experiments: Originality	190
5.4 Disparities Between Past and Present Kindling Studies.....	191
5.4.1 Neurosteroids and Their Receptors Differ in Mice and Rats	191
5.4.2 Kindling the Hippocampus	191
5.5 Potential Mechanisms for Progesterone's Antiepileptiform Effects.....	192
5.5.1 THP-Mediated GABAergic Inhibition	192
5.5.2 Action on Ion Channels	193
5.5.3 Action on Sigma Receptors.....	193
5.6 Clinical Meaning	195
5.6.1 Clinical Meaning: Behavioral Studies.....	195
5.6.2 Clinical Meaning: Electrophysiological Studies	195
5.7 Future Studies	195
5.7.1 Future Behavioral Studies	196
5.7.2 Future Electrophysiology Studies In Vivo.....	197
5.7.3 Future Studies In Vitro	198
References	199
Appendix 1: Forced Swim Test and Open Field, Submitted	235
Appendix 2: Elevated Plus Maze Paper, Submitted.....	254
Appendix 3: Novel Surgical Methods, Accepted	273
Appendix 4: Electrophysiological Studies In Vivo and In Vitro, Accepted	282

Published Papers

Novel anticonvulsant effects of progesterone in a mouse model of electrical kindling

Melanie A. Jeffrey, Min Lang, Jonathan Gane, Edwin Chow, Chiping Wu, Liang Zhang. Neuroscience (2014). 257(17):65-75. doi: 10.1016/j.neuroscience.2013.1074

A reliable method for intracranial electrode implantation and chronic electrical stimulation in the mouse brain

Melanie Jeffrey, Min Lang, Jonathan Gane, Chiping Wu, W.M. Burnham, Liang Zhang. BMC Neuroscience (2013), 14:82 (open access). doi: 10.1186/1471-2202-14-82

Acute administration of docosahexaenoic acid increases resistance to pentylenetetrazol-induced seizures in rats

Ameer Y. Taha, Melanie A. Jeffrey, Nadine M.Y. Taha, Saimir Bala, W. M. Burnham. Epilepsy & Behavior, 2010, 17: 336-343

Comparative study of five antiepileptic drugs on a translational cognitive measure in the rat: relationship to antiepileptic property

Guy A. Higgins, Nathalie Breysse, Elijus Undzys, D. Richard Derksen, Melanie Jeffrey, Brian W. Scott, Tao Xin, Corinne Roucard, K. Bressand, A Depaulis, W.M. Burnham. Psychopharmacology, 2010, 207: 513-527

Submitted Papers

Anticonvulsant doses of progesterone metabolites are not toxic in the forced swim or open field tests

Melanie A. Jeffrey, Deborah Lonsdale, W. M. Burnham. Submitted to Epilepsy Research.

Progesterone Metabolites are not psychotoxic in the elevated plus maze

Melanie Jeffrey, Deborah Lonsdale, Deepali Bhatta, Sneha Patel, W.M. Burnham. Submitted to Behavioral Brain Research, rejected, resubmission planned.

Paper In Preparation

Anticonvulsant doses of progesterone metabolites are not toxic in the Morris water maze

Melanie A. Jeffrey, Deborah Lonsdale, W.M. Burnham

1st Author Published Abstracts

Anticonvulsant doses of 5 α -DHP: behavioral toxicity results and possible mechanisms of action. 2010 annual meeting of the Eastern Association of Electroencephalographers, Bromont, Quebec. Clinical Neurophysiology 122(2011):e4. doi:10.1016/j.clinph.20103.043

Anticonvulsant doses of 5-alpha-dihydroprogesterone and allopregnanolone: Behavioral toxicity results in male and female rats. 2011 annual meeting of the Eastern Association of Electroencephalographers, New York, NY. Clinical Neurophysiology 124(2013):e5. doi:10.1016/j.clinph.2012.08.032

1) Hormones, Epilepsy, and Autonomy, and 2) Comprehensive Epilepsy Care in Canada. Principles of Autonomous Neurodynamics, 2011 annual meeting of the Society for Autonomous Neurodynamics, www.utoronto.ca/sand/PAND2011

The Effects Of Progesterone, DHP, And THP On Spontaneous Interictal Discharges In CA3 Electrically Kindled Mice. 2013 Annual meeting of the Eastern Association of Electroencephalographers, New York, NY. Clinical Neurophysiology, doi:10.1016/j.clinph.2013.09.018

Novel anticonvulsant effects of supraphysiological doses of progesterone in hippocampal-kindled mice. 2014 Annual meeting of the Eastern Association of Electroencephalographers, Bromont, Quebec. Clinical Neurophysiology, in press.

Poster presentations

Effects of progesterone on entorhinal epileptiform activities *in vitro*. Society for Neuroscience, New Orleans, October 16, 2012. Poster 656.25/N10.

Differential effects of neurosteroids and anticonvulsant drugs on drug-refractory seizures and spontaneous epileptiform discharges in the mouse. Canadian Association for Neuroscience, Toronto, May 22, 2013 Poster I-C-73.

Progesterone and allopregnanolone may have different mechanisms of anticonvulsant action. International Epilepsy Congress, Montreal, June 24, 2013, poster p-558.

List of Figures

Figure	Page
1.1 Interictal spikes	4
1.2 Hormone levels and seizure susceptibility in catamenial epilepsy	5
1.3 Normal human EEG	6
1.4 EEG traces showing interictal spikes and a generalized seizure	9
1.5 Steroid form and numbering	42
1.6 Metabolic pathway of progesterone	48
1.7 Finasteride	56
2.1 Male immobility ratio in the forced swim test	83
2.2 Female immobility ratio in the forced swim test	83
2.3 Male horizontal activity in the open field test	84
2.4 Male vertical activity in the open field test	84
2.5 Female horizontal activity in the open field test	85
2.6 Female vertical activity in the open field test	85
2.7 Acquisition of memory, males	88
2.8 Acquisition of memory, females	88
2.9 Learning a new task, males	89
2.10 Learning a new task, females	89
2.11 Working memory, latency, males	90
2.12 Working memory, latency, females	90
2.13 Working memory, finds platform, males	91
2.14 Working memory, finds platform, females	91
2.15 Reference memory: Crossings, males	92
2.16 Reference memory: Crossings, females	92
2.17 Reference memory: Direct swim, males	93
2.18 Reference memory: Direct swim, females	93
2.19 Drug effects on male velocity day 1	97
2.20 Drug effects on female velocity day 1	97
2.21 Drug effects on male velocity day 4	98
2.22 Drug effects on male velocity day 4	98
2.23 Drug effects on male velocity day 2	99
2.24 Drug effects on female velocity day 2	99
2.25 Drug effects on male velocity day 3	100
2.26 Drug effects on male velocity day 3	100
2.27 Drug effects on male velocity day 5 trials 1-4	101

2.28	Drug effects on female velocity day 5 trials 1-4	101
2.29	Spatial memory, males, day 5 trials 1-4	103
2.30	Spatial memory, females, day 5 trials 1-4	103
2.31	Spatial memory, males, day 5 trials 1-2	104
2.32	Spatial memory, females, day 5 trials 1-2	104
2.33	Spatial memory, males, day 5 trials 3-4	105
2.34	Spatial memory, females, day 5 trials 3-4	105
2.35	Percent time in open arms, males	110
2.36	Percent time in open arms, females	110
2.37	Percent time in closed arms, males	111
2.38	Percent time in closed arms, females	111
2.39	Open arm entries, males	112
2.40	Open arm entries, females	112
2.41	Closed arm entries, males	113
2.42	Closed arm entries, females	113
2.43	Total arm entries, males	114
2.44	Total arm entries, females	114
3.1	Illustration of connecting pins and electrodes	136
3.2	Histological and electrophysiological verification of implanted electrodes	145
3.3	Effects of progesterone on hippocampal ADs and motor seizures	147
3.4	Effects of DHP on hippocampal ADs and motor seizures	149
3.5	Effects of THP on hippocampal ADs and motor seizures	151
3.6	Effects of progesterone with finasteride pretreatment on hippocampal ADs and motor seizures	153
3.7	Effects of progesterone with finasteride pretreatment on hippocampal/cortical afterdischarges and motor seizures	155
3.8	Effects of midazolam and carbamazepine on hippocampal/cortical afterdischarges and motor seizures	157
4.1	In vitro experimental design	169
4.2	In vitro protocol for progesterone treatment alone	173
4.3	0.1 μM progesterone does not affect waveform properties	174
4.4	1.0 μM progesterone significantly affects waveform properties	175
4.5	Progesterone retains its anticonvulsant effects in the presence of the <i>5α-reductase</i> enzyme inhibitor finasteride	177
4.6	Waveform quantification	178

List of Tables

Table		Page
1	Summary of results in the FST and OFT	86
2	Summary of results in the MWM	106-108
3	Summary of results in the EPM	115-116

List of Abbreviations

3 α HSOR *3 α -Hydroxysteroid oxidoreductase*

5 α R *5 α -Reductase*

4-AP 4-Aminopyridine

7TMPR 7-Transmembrane progesterone receptor

ACSF Artificial cerebrospinal fluid

AD Afterdischarge

AKRC1 Aldo keto reductase “C” family 1

AMPA α -amino-3-hydroxy-5-methyl-4-isoxazole propionate

ANOVA Analysis of variance

BCE Before common era

CA Cornu ammonis

cAMP Cyclic adenosine monophosphate

CNS Central nervous system

DHP 5 α -dihydroprogesterone

DMSO Dimethyl sulfoxide

EEG Electroencephalogram

EPM Elevated plus maze

EPSP	Excitatory post-synaptic potentials
------	-------------------------------------

FST	Forced swim test
-----	------------------

GABA	γ -aminobutyric acid
------	-----------------------------

GPCR	G-protein coupled receptor
------	----------------------------

HPA	Hypothalamic-pituitary-adrenal (axis)
-----	---------------------------------------

HSD	Honest significant difference
-----	-------------------------------

HVA	High voltage activated
-----	------------------------

i.p.	Intraperitoneal
------	-----------------

IZAg	Inner zone antigen
------	--------------------

LHRH	Luteinizing hormone releasing hormone
------	---------------------------------------

M	Mean
---	------

MAPR	Membrane associated progesterone receptor
------	---

MES	Maximal electroshock
-----	----------------------

MPA	Medroxyprogesterone acetate
-----	-----------------------------

mPR	Membrane progesterone receptor
-----	--------------------------------

MRI	Magnetic resonance imaging
-----	----------------------------

MWM	Morris watermaze
-----	------------------

NMDA	N-methyl-D-aspartate
------	----------------------

OFT	Open field test
-----	-----------------

PTX	Picrotoxin
-----	------------

PAQR	Progestin and adipoQ receptors
------	--------------------------------

PGRMC1	Progesterone receptor membrane component 1
PNS	Peripheral nervous system
PXR/SXR	Pregnane xenobiotic receptor/steroid xenobiotic receptor
scPTZ	Subcutaneous pentylenetetrazol
SD	Standard deviation
SE	Standard error
siRNA	Short interfering RNA
SUDEP	Sudden unexpected death in epilepsy
THP	3 α ,5 α -tetrahydroprogesterone, also known as allopregnanolone
VEH	Vehicle
WHO	World health organization

Chapter 1: Introduction

1.1 Epilepsy

1.1.1 Definitions

“Epilepsy” is one of the most common of the serious neurological disorders. It is characterized by spontaneous, recurrent seizures (World Health Organization (WHO), 2005; Burnham, 2007). There are a number of epilepsy disorders. It has been proposed that “epilepsies” would be a more correct descriptor (Burnham, 2007; Engel, 2013).

“Epileptic seizures” are periods of self-sustained, but usually self-limiting, hyperexcitation in the central nervous system (CNS; Burnham, 2007; Berg et al, 2010; Berg & Scheffer, 2011). They consist of abnormal, excessive, hypersynchronous neuronal activity in the brain that can be measured by the electroencephalogram (EEG). Seizures do not always indicate epilepsy (Smithson & Walker, 2012), such as when infants and young children have febrile seizures (caused by fever) or when seizures occur during alcohol withdrawal (Engel, 2006). Psychogenic seizures, which are non-epileptic seizures, are associated with a history of childhood physical and sexual abuse, particularly in women. Psychotherapy can be effective, but anticonvulsant drugs generally are not (Griffith & Szaflarski, 2010).

“Epileptogenesis” is the process of a normal brain becoming one that has spontaneous, recurrent seizures. It is associated with structural and functional changes to the CNS (Scharfman, 2007; Hargus et al, 2011), including the neuroendocrine systems (Pack, 2010).

“Electrical kindling” of the limbic structures models focal seizures with secondary generalization (Goddard et al, 1969). A stimulating electrode is implanted in the amygdala or hippocampus, and delivers repeated, often daily electrical stimulation. This stimulated site is referred to as the “seizure focus.” With daily stimulation, focal seizures progressively become more and more generalized until a convulsive seizure occurs. When fully kindled, the animals are permanently predisposed to generalized seizures induced by mild electrical stimulation of the seizure focus (Racine, 1972a). This model is responsive to most known anticonvulsant drugs, except those used in the treatment of absence seizures (Löscher, 2011 throughout). Electrical kindling is detailed in section 1.5.7 of this thesis.

“Interictal spikes” are typically seen in epileptic brains between seizures. They are brief paroxysmal electrographic discharges that manifest as isolated spikes on the EEG (McCormack & Contreras, 2001). They are much more common in epileptic than non-epileptic brains (Staley et al, 2011). Although the significance of interictal spikes has been debated for decades, it is generally recognized that they are strongly associated with epileptogenic processes in animals (Leung, 1988; Staley et al, 2011) and occur between seizures in most persons with epilepsy (Wendling et al, 2012). **Fig 1.1** shows interictal spikes in the seizure focus in our animal model of seizures in vivo.

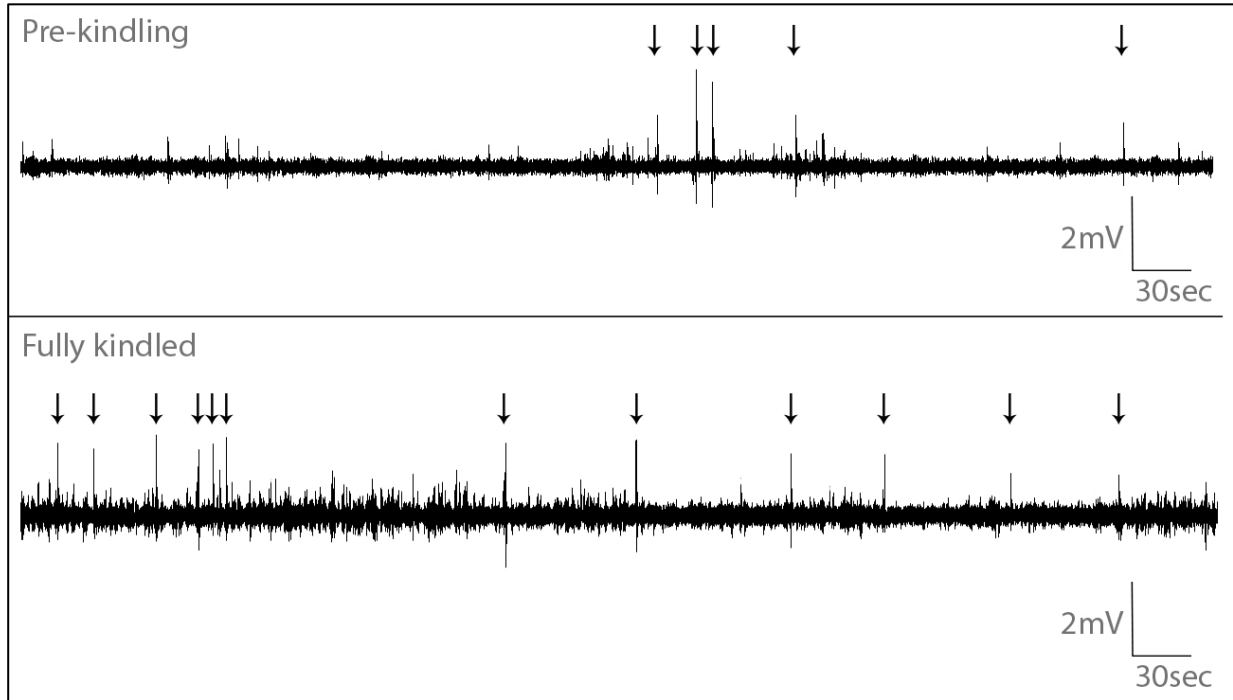


Figure 1.1 Interictal spikes in the hippocampus of mice increase in frequency with kindling. Representative trace showing interictal spikes for a single animal pre- and post-kindling. Top trace, pre-kindling. Bottom trace: Fully kindled. The above recordings were made when animals were immobile.

“Drug resistant” epilepsy is a term for epilepsy where there is not complete seizure control despite the use of two or more well tolerated and appropriate anticonvulsant drugs (Kwan et al, 2010; Beleza, 2009; Engel, 2013). Drug resistant epilepsy is sometimes called “intractable” or “drug-refractory.”

“Catamenial epilepsy” is generally defined as an increase in seizure frequency during 1] the perimenstrual (C1) or periovulatory (C2) phases in normal ovulatory menstrual cycles, or 2] the luteal phase (C3) in anovulatory menstrual cycles (**Fig 1.2**). The increased seizure frequency occurs in the phases of the menstrual cycle when progesterone levels are low relative to estrogen (Smith &

Woolley, 2004; Foldvary-Schaefer et al, 2004; Reddy, 2009; Verrotti et al, 2010).

Catamenial epilepsy is detailed in section 1.1.4 of this thesis.

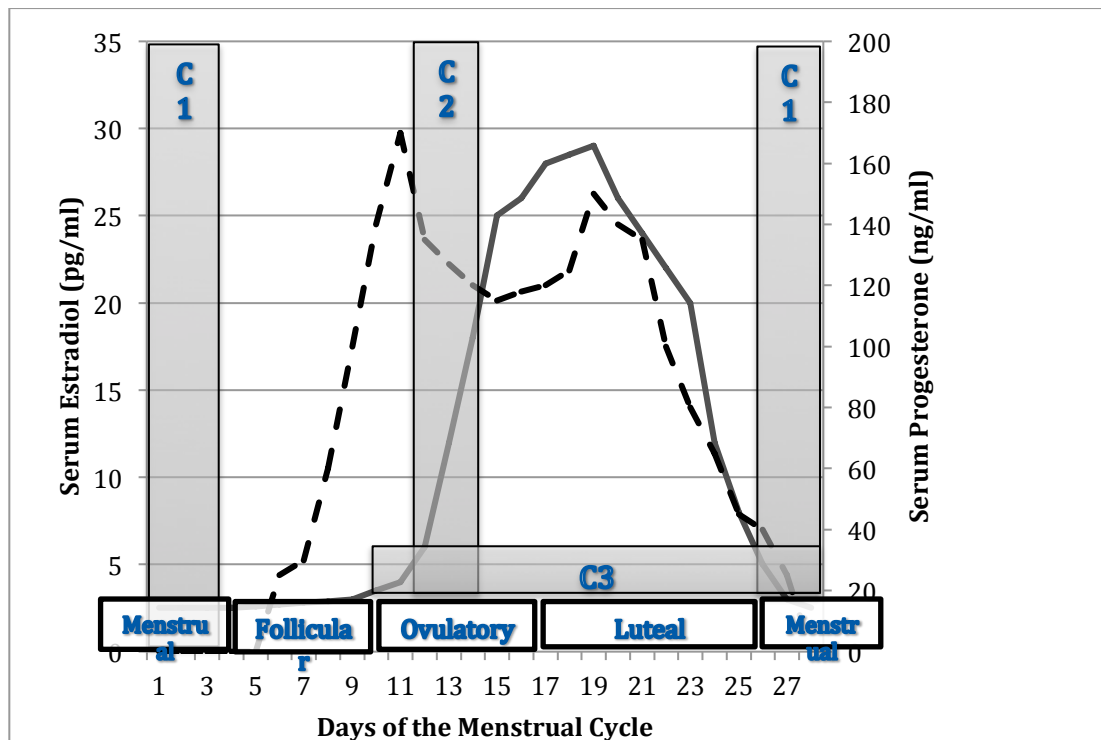


Figure 1.2 Hormone levels and seizure susceptibility in catamenial epilepsy. X-axis indicates days of the menstrual cycle; y-axis (left) serum estradiol levels; y-axis (right) serum progesterone levels. Black dashed line indicates estradiol (pg/ml). Grey solid line indicates progesterone in ovulatory cycles (ng/ml). Bars indicate periods of seizure susceptibility. C1, perimenstrual type; C2, perioovulatory type; C3, inadequate luteal type. In the inadequate luteal type of catamenial seizure susceptibility, serum progesterone levels are unusually low throughout the entire luteal phase (not shown). Data values used are from Reddy, 2009.

1.1.2 Epilepsy and the Electroencephalogram (EEG)

The electroencephalogram (EEG) is a diagnostic test for epilepsy in humans that is also widely used in epilepsy research. In humans, the EEG is done with gross electrodes, which are affixed to the scalp. Wilder Penfield and Herbert Jasper used brain stimulation and the EEG to begin a new era of diagnostics and seizure surgery in Montreal in the 1930s (WHO, 2005 throughout). The EEG remains a standard diagnostic tool for clinicians today, and is still a crucial tool for epilepsy research. Unlike magnetic resonance imaging scans (MRIs) and other recent non-invasive imaging techniques, EEGs are inexpensive and easily accessible (WHO, 2005, throughout). The y-axis of the resultant recording represents voltage from particular recording areas, and the x-axis represents time. In a normal waking EEG (**Fig 1.3**), most activity is low voltage and desynchronized (excepting artifacts, such as eye-blinking).

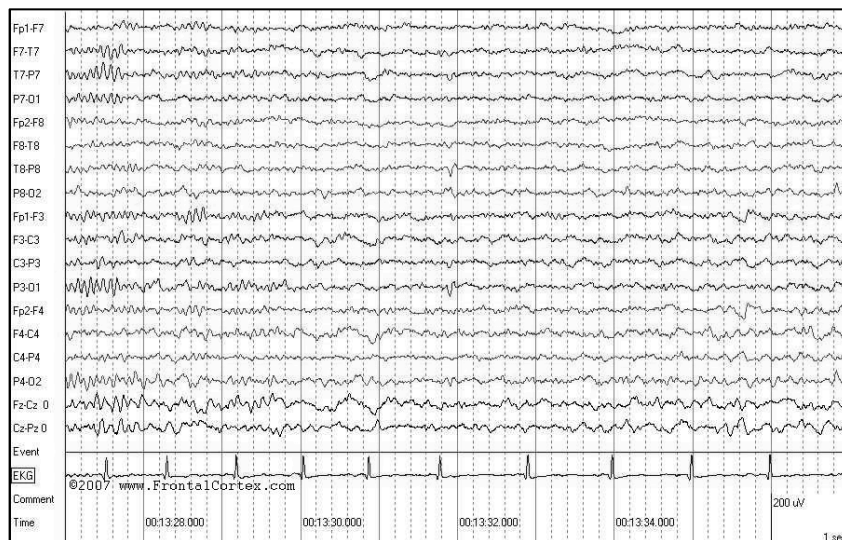


Figure 1.3 Normal waking human EEG. Note that the activity is low-voltage and largely desynchronized. Retrieved from www.frontalcortex.com, used with permission.

Many epileptic seizures cause clearly abnormal EEG patterns (**Fig 1.4B**). Even when the patient is not actively seizing, EEGs of epileptic patients can show interictal EEG “spikes,” a marker of hyperexcitability that may give insight into where a seizure originates (**Fig 1.4A**; McCormack & Contreras, 2001). Correctly reading and interpreting EEGs, however, requires considerable expertise in seizure disorders and other neurological conditions (Tzallas et al, 2012).

Variables used in the classification of EEG activity include frequency (Hz), voltage (V), morphology (waveform shape), synchrony, and periodicity. Seizures on the EEG are characterized by increased frequency, increased peak voltage, increased synchrony within and/or between recording electrodes, and particular periodicity patterns, depending on the seizure type recorded (Pedley, 2008; Tzallas et al, 2012).

Each line of activity on the EEG represents the summated, synchronous activity of thousands or millions of neurons spatially oriented toward a particular recording electrode on the scalp (Tzallas et al, 2012). Scalp surface EEG recordings, however, do not provide information about the role subcortical structures may play in seizure onset and generalization (Pedley, 2008). When patients are potential candidates for surgery, EEG electrodes are temporarily surgically implanted in the brain to identify brain areas suitable for surgery (Pedley, 2008).

With animal research in electrical kindling (section 1.5.7), EEG recording electrodes can be inserted into different areas of the brain to record focal seizures and their generalization in vivo (Burnham et al, 2005; McIntyre, 2006; chapter 3). EEG recordings can also be made in slices to record activity in a “field” or

population of neurons in vitro (Buzsáki et al, 2012; chapter 4). Although none were conducted for this thesis, single cell recordings are also possible in a patch-clamp preparation, recording electrical currents that flow across a single cell membrane (Belelli & Lambert, 2005; Buzsáki et al, 2012).

1.1.3 The Epidemiology and Onset of Epilepsy

All mammals, including humans, can have seizures. Seizures and epilepsy have likely been with humankind since time immemorial (Kwan & Sander, 2004). Human seizures have been recorded since early Babylonian times (1000 BCE; Banerjee et al, 2008; Engel & Pedley, 2008). Seizures and epilepsy have also traditionally been associated with spiritual causes, suspicion and stigma worldwide (Kale, 1997; WHO 2005; Engel, 2013; Viteva, 2013).

Epilepsy is a universal disorder, affecting both sexes in all cultures and socioeconomic groups. Between 50 and 65 million persons worldwide have epilepsy (WHO, 2005; Thurman et al, 2011). Epilepsy is more common than Parkinson's disease, autism, cerebral palsy and multiple sclerosis combined (Engel, 2013). Eighty percent of all epilepsy cases are in resource-poor countries. This is likely due to increased chances of head trauma, infection, and the lack of access to treatment (WHO, 2005).

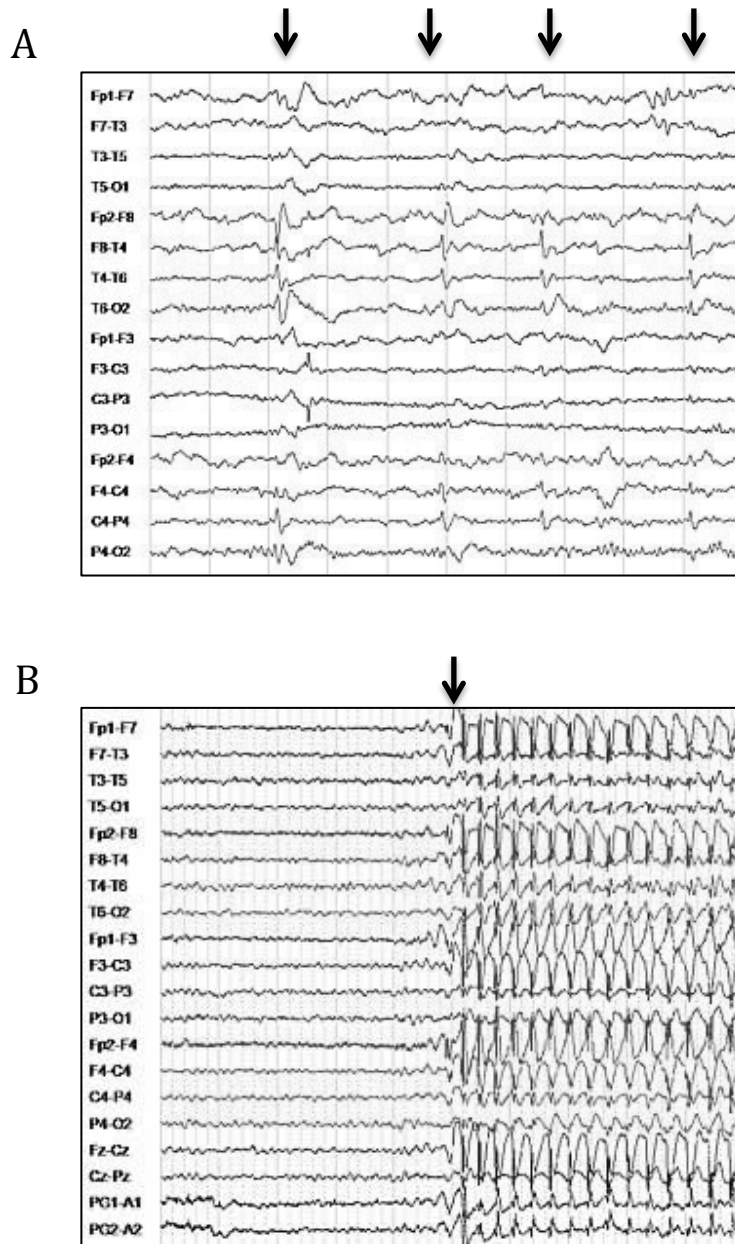


Figure 1.4 EEG traces showing focal interictal spiking and a generalized seizure. *A*, focal spikes (arrows) in the temporal lobe suggest a seizure focus; ***B*,** primarily generalized seizure (beginning at arrow). Jan 2014, Used with permission from: http://www.thebarrow.org/Neurological_Services/Epilepsy/204351

Epidemiological studies in North America suggest that about 3% of persons will be diagnosed with epilepsy at some point in their lifetime (Westbrook, 2012). It is estimated that 30 to 40% of persons with epilepsy will have seizures that are refractory to treatment (Kwan & Sander, 2004). Currently, the point prevalence of epilepsy in Canada is approximately 300,000 persons. Canadians with epilepsy often have low incomes, are unemployed, and/or have low education levels (Tellez-Zenteno et al, 2004). Persons with epilepsy also have a higher mortality rate than the general population. Suicide, increased risk of accidental death (i.e., drowning), brain tumors and cerebrovascular disease are all common causes of death in persons with epilepsy (Lhatoo et al, 2008).

The onset of epilepsy may occur at any age, but it most commonly occurs during childhood or old age (Burnham, 2007; Berg et al, 2010; Smithson & Walker, 2012). Ages of seizure onset and/or offset may accompany endogenous hormonal changes, such as puberty (El-Khayat et al, 2008; Frye, 2010; Smith, 2012) or menopause (Folvary-Schaefer et al, 2004; Harden, 2008). Geriatric-onset epilepsy is a relatively recent phenomenon. As human longevity has increased in recent years, so has the prevalence of geriatric-onset seizures (Duncan et al, 2006; Smithson & Walker, 2012).

1.1.4 Catamenial Epilepsy: Endogenous Hormones Affect Seizure Susceptibility in Women

When women's seizures cluster at certain points in the menstrual cycle, their epilepsy phenotype is known as catamenial epilepsy (Maguire et al, 2005;

Herzog, 2008a; Herzog & Fowler, 2008; Reddy, 2009; Kim et al, 2010; Verrotti et al, 2010; Pack, 2010; Finocchi & Ferrari, 2011). The cyclical occurrence of epileptic seizures in women has been noted for centuries. “Catamenial” is derived from the Greek word “katamenios,” which means “monthly” (Newmark & Penry, 1980). “Circalunar” is another term used less frequently (Quigg et al, 2008).

It is hypothesized that 30-60% of women with epilepsy have catamenial epilepsy (Reddy, 2009). Catamenial epilepsy is a form of epilepsy that has particular relevance to this thesis. As mentioned above, catamenial epilepsy is generally defined as an increase in seizure frequency during 1] the perimenstrual (C1) or periovulatory (C2) phases in normal ovulatory menstrual cycles, or 2] the luteal phase (C3) in anovulatory menstrual cycles (**Fig 1.2**), when estradiol levels are high compared to progesterone levels (Herzog, 2008a; Quigg et al, 2008; Reddy, 2009). In both cases, seizures tend to occur when levels of estrogen (excitatory) are high, and levels of progesterone (inhibitory) are low or dropping (Pack, 2010). Brain substrates susceptible to seizures are very sensitive to electrophysiological and structural changes caused by these fluctuating neurosteroids (Herzog & Fowler, 2005; 2008). Neurosteroids are detailed in section 1.6 of this thesis.

Many women with catamenial seizures have lower endogenous progesterone levels than controls, and higher estrogen to progesterone ratios in their cycles overall compared to age-matched relatives without epilepsy (El-Khayat et al, 2008). These neuroendocrinological abnormalities are hypothesized to contribute to the physical and psychiatric comorbidities in women with epilepsy (Pack et al, 2011).

Women with drug-refractory, focal and/or temporal lobe epilepsy are particularly likely to have catamenial epilepsy (Kalinin & Zheleznova, 2007; Reddy, 2009; Verrotti et al, 2010). Their catamenial phenotype (ovulatory, anovulatory) and seizure pattern vary with the site of the seizure focus (Quigg et al, 2008). Interestingly, in a model of temporal lobe epilepsy in rats, laterality of the kindled amygdala influences which reproductive disorders present in female animals (Hum et al, 2009).

Currently, standard anticonvulsant drugs such as lamotrigine and the benzodiazepines are used to treat catamenial epilepsy. Since hormone levels affect seizure susceptibility, it is hypothesized that hormone-based therapies might be effective for women with catamenial epilepsy (Foldvary-Schaefer et al, 2004; Reddy, 2009; Reddy & Rogawski, 2009; Steven & Harden 2011; Pack et al, 2011). The two types of neuroendocrine treatment currently used are: 1] hormonal suppression, and 2] cyclic progestogen supplementation. Suppression of ovarian hormones with agents such as the progesterone analog medroxyprogesterone acetate or ganadotropin-releasing hormone reduces the variability of levels of progesterone and estradiol levels in a woman's menstrual cycle, reducing the risk of high estrogen to progesterone ratios. Cyclic progestin supplementation can also be used to decrease the ratio of estradiol to progesterone during low progesterone phases of the menstrual cycle (Pack et al, 2011).

1.2 Comorbidities of Epilepsy

Epilepsy is associated with a number of other disorders, both physical and psychiatric (Gaitatzis et al, 2004; Pinna et al, 2006; Tellez-Zenteno et al, 2007; Zaccara, 2009; Manni & Terzhaghi, 2010; Adelöw et al, 2012). Pharmacological treatments for the comorbidities of epilepsy are complicated by pharmacokinetic interactions with anticonvulsant medications (Griffin & Mellon, 1999; Salzberg & Vajda, 2001).

Of relevance to this thesis, the comorbidities of epilepsy are sometimes related to changes in the endocrine system (Toledano & Gil-Nagel, 2008). Quantitative evidence of the physical and the psychiatric comorbidities of epilepsy, especially with respect to neuroendocrine function, has increased in recent years (Morrell et al, 2005; Pennell, 2009; Pack et al, 2011; Zorumski et al, 2013). These reproductive and psychiatric disorders reflect, at least in part, the hormonal dysfunction common in epilepsy (Lambert, 2001; Pack et al, 2011).

1.2.1 Psychiatric Comorbidities in Epilepsy

Psychiatric comorbidities in persons with epilepsy are much more common than in persons without epilepsy (Harden & Goldstein, 2002; Gaitatzis et al, 2004; Tellez-Zenteno et al, 2007; Titlic et al 2009; Mani & Terzhagi, 2010; Jones & O'Brien, 2013). Recent research suggests a bidirectional relationship between epilepsy and psychiatric disorders, with each contributing to the other both in humans (Adelöw et al, 2012; Hesdorffer et al, 2012) and in animal models (Epps et al, 2012; Jones & O'Brien, 2013).

Suicidal ideation, depression, anxiety, psychoses, bipolar disorder and mania are common psychiatric comorbidities of epilepsy, and particularly of temporal lobe epilepsy (Schmitz et al, 1999; Fuller-Tomson & Brennenstuhl, 2009, Kwon et al, 2011; Pack et al, 2011; Hesdorffer et al, 2012). Of these, depression and anxiety are most common (Salzberg & Vajda, 2001; Harden, 2002a; Gaitatzis et al, 2004; Titlic et al, 2009; Zaccara, 2009). Depression in persons with epilepsy, however, is often not diagnosed or treated in Canada (Fuller-Tomson & Brennenstuhl, 2009).

The relationship between anxiety and seizures has been recognized for centuries, and is hypothesized to be the result of interactions between seizures, neuroendocrine alterations, and amygdala function (Goldstein & Harden, 2000; Harden, 2002a; Gaitatzis et al, 2004). The associations between stress, epilepsy and psychiatric disorders are under investigation (Jones & O'Brien, 2013). The role of neurosteroidogenesis in anxiety and depression is also under investigation (Schüle et al, 2014). Cognition can also be altered in persons with epilepsy (Elger et al, 2004), which further impacts locus of control, relationships, and depression (Sperling et al, 2008; Kobau et al, 2010).

1.2.2 Reproductive/Hormonal Comorbidities of Epilepsy

Men and women with epilepsy have unusually high levels of neuroendocrine and reproductive dysfunction, including infertility in both sexes (Harden, 2002b; Harden, 2003; Herzog, 2002, 2006, 2008b; Herzog et al, 2005; Gaitatzis et al, 2004; Pack et al, 2011). Altered hypothalamic-pituitary-adrenal

(HPA) axis function is related to psychiatric comorbidities (Phillips et al, 2006) as well as reproductive disorders (Pennel, 2009). The specific alteration in HPA function is specific to the seizure type experienced (Morrell & Montouris, 2004).

Ovulatory dysfunction is common in women with epilepsy, including anovulatory menstrual cycles, which are associated with increased risk of seizures (Herzog, 2006; Verrotti et al, 2010). Polycystic ovarian syndrome, hirsutism, problems with libido and fertility, and decreased bone mineral density are also common in women with epilepsy (Harden, 2005a; 2005b; Morrell et al, 2005). All of the above are related to hormone levels (El-Khayat et al, 2008; Pennel, 2009). Common obstetric risks for women with epilepsy, despite their anticonvulsant drug status, include preeclampsia and/or eclampsia, preterm delivery, spontaneous abortion, placental abruption, and small birth weight (Kaplan et al, 2007). More research into the optimal clinical management of pregnancy in epilepsy is needed (Harden, 2007; Vadja et al, 2008; Meador et al, 2008; Vajda et al, 2008; Harden et al, 2008).

In men with epilepsy, a population-based study has shown high rates of erectile dysfunction (Keller et al, 2012). Men with epilepsy can also have hypogonadism and low testosterone levels, affecting testicular function (Harden & MacLusky, 2004; Pack et al, 2011). Men with temporal lobe epilepsies in particular tend to have lower testosterone levels than men with other epilepsies (Isojärvi et al, 2004). Sexual dysfunction in men with epilepsy is significantly correlated with depression, anxiety and psychological distress (Talbot et al, 2008). Anticonvulsant

drug choice can also affect men's hormonal milieu and sexual function (Herzog & Fowler, 2008).

These comorbidities in men and women probably relate to the effects of repeated seizures, since repeated seizures in animal models of epilepsy have been shown to cause reproductive dysfunction in male and female rats (Edwards, 1998; Edwards et al, 1999a; 1999b; Edwards et al, 2000b; Hum & Burnham, 2007; Hum et al, 2009). The adverse reproductive effects of repeated seizures in humans may be mediated by subsequent changes to the neuroendocrine systems (Herzog, 2008b; Pack, 2010; Pack et al, 2011). The reproductive disorders of epilepsy reflect, at least in part, HPA-mediated hormonal dysfunction (Lambert, 2001; Herzog, 2002; El-Khayat et al 2003; 2008; Morrell & Montouris, 2004; Herzog, 2008a; 2008b; Pennell, 2009; Pack et al, 2011). Anticonvulsant drugs can further affect reproductive and neuroendocrine function (Isojärvi et al, 2004; 2005).

1.2.3 Death and Sudden Unexpected Death in Epilepsy (SUDEP)

Sudden Unexpected Death in Epilepsy (SUDEP) is hypothesized to contribute to the high rates of mortality in persons with epilepsy. It is a leading cause of death in persons with uncontrolled seizures, and is usually found in persons with epilepsy refractory to the available treatment options (Tomson et al, 2008). Untreated seizures, frequent generalized tonic-clonic seizures, sudden withdrawal of medication or polytherapy, living and/or sleeping alone, and young adulthood are all risk factors for SUDEP (Tomson et al, 2008; Hesdorffer et al, 2011). SUDEP usually occurs shortly after a complex partial or generalized tonic-

clonic seizure. It is not known why a particular seizure may be fatal compared to other seizures. Otherwise, causal factors are unknown, and are under active investigation (Tomson et al, 2008; Hesdorffer et al, 2011).

1.2.4 The Burden of Epilepsy

Quality of life for persons with epilepsy is affected by uncontrolled seizures, and, often, by the drugs used to treat them (Harden et al, 2007; Bootsma et al, 2009; Quintas et al, 2012). Persons with epilepsy have lower socioeconomic status, family function and support overall as compared to other chronically ill persons (Jennum et al, 2011; Begley et al, 2011; Quintas et al, 2012). Social stigma can also compromise the quality of life for persons with epilepsy (Theodore et al, 2006; Viteva, 2013).

Uncontrolled seizures are also costly for health care systems (Giatatzis et al, 2004; Theodore et al, 2006). Persons with epilepsy, especially those with uncontrolled seizures, are very high users of health care resources (Wiebe et al, 1999; Begley et al, 2011). Despite a much lower prevalence, epilepsy has the same worldwide burden of disease as breast cancer in women, and lung cancer in men (Kale, 1997; WHO, 2005). In combination, uncontrolled seizures, the drugs used to treat them (Toledano & Gil-Nagel, 2008; Bootsma et al, 2009), and the comorbidities of epilepsy (Giatatzis et al, 2004) make life very difficult for persons with uncontrolled epilepsy (WHO, 2005; Quintas et al, 2012). Thus, epilepsy is costly for the persons who suffer from it, and for the health care system that treats them.

1.3 Causes and Classifications of Epilepsy

The epilepsy classifications have recently been revised. Three primary types of epilepsy are now recognized, based on current knowledge of etiologies: structural/metabolic, genetic, and unknown cause (Berg et al, 2010; Thurman et al 2011). The distinctions between these three types of epilepsy may fade as our knowledge of the underlying mechanisms of epilepsy and gene-environment interactions in epilepsy improves (Berg & Cross, 2012). Due to varying epidemiological methodologies, it is difficult to estimate the prevalence of each epilepsy type.

1.3.1 Structural/Metabolic Epilepsies (formerly “Symptomatic Epilepsies”)

The causes of the structural/metabolic epilepsies can be genetic in origin, but are defined by observable structural/metabolic abnormalities. Structural/metabolic epilepsy rates, to our knowledge, have not been reported since the revised classification. A recent epidemiological study in France noted 46.4-48.1% of pharmaco-resistant epilepsies were “symptomatic” as per the previous epilepsy classification (Picot et al, 2008).

Structural injuries that may cause structural/metabolic epilepsies include birth injury, brain infection, stroke, traumatic brain injury, tumor, or scarring (Berg et al, 2010). Meningiomas are more prevalent in older adults, whereas brain tumors are more prevalent in young adults (Gaitatzis et al, 2004).

Examples of structural/metabolic etiologies not due to injury include neurocutaneous syndromes, such as tuberous sclerosis, and malformations of cortical development, such as focal cortical dysplasia or lissencephaly (Berg & Cross, 2012). Structural/metabolic epilepsies often resist pharmacological treatment as compared to genetic epilepsies without structural abnormalities (Burnham, 2007; Berg et al 2010). Surgery can be an option for some persons with structural/metabolic epilepsies (Jones & Andermann, 2000; Duchowny et al, 2008).

1.3.2 Genetic Epilepsies (formerly “Idiopathic Epilepsies”)

Genetic epilepsies are hypothesized to account for 20-30% of all epilepsies, but estimates vary widely due to non-standardized epidemiological studies (Tan & Berkovic, 2010; Kao & Rao, 2012). Genetic epilepsies generally do not involve obvious brain pathology, which is how they are distinguished from the structural/metabolic epilepsies (Berg et al, 2010; Thurman et al, 2011). Recent evidence has shown that many of the epilepsies previously characterized as “idiopathic” are actually genetic epilepsies (Berg et al, 2010). Genetic epilepsies are more amenable to pharmacological treatment than structural/metabolic epilepsies. They often present as epilepsy syndromes, with a common time of onset, offset, a specific set of symptoms, and possibly a typical drug response (Rogawski & Löscher, 2004; Steinlein, 2008; Berg et al, 2010; Pitkänen & Lukasiuk, 2011; Kao & Rao, 2012). Examples of the genetic syndromes associated with generalized epilepsies (and the drugs used to treat them) include: myoclonic epilepsy in infants or

juveniles (clonazepam, valproic acid); childhood absence epilepsy (ethosuximide, valproic acid); and epilepsy with tonic-clonic seizures (topiramate, lamotrigine, zonisamide, levetiracetam; Kao & Rao, 2012).

While some of the genetic epilepsies are linked to single genes, most genetic epilepsies are multifactorial, with complex inheritance (Ottman & Winawer, 2008; Kasperavičiūtė et al, 2010; Kao & Rao, 2012). Known epilepsy-associated genes often cause channelopathies (Kearney et al, 2001; Chen et al, 2003; Mulley et al, 2005; Heron et al, 2007; Lowenstein, 2008; Reid et al, 2009; Striano & Zara, 2011; Tan et al, 2013). Recently, patterns of multiple single nucleotide polymorphisms in ion channel genes that result in epilepsy have been recognized (Klassen et al, 2011; Tan et al, 2013). Examples of genes that harbor known epilepsy mutations include the following: potassium channel genes *KCNQ2*, *KCNQ3*, *KCNMA1*, and *KCNA1*; sodium channel genes *SCN2A*, *SCN1A*, and *SCN1B*; the T-type calcium channel gene *CACNA1H* and GABA_A receptor genes *GABRA1*, *GABRA3*, and *GABRA2* (Klassen et al, 2011; Striano & Zara, 2011; Cherepanova et al, 2013).

1.3.3 Epilepsies of Unknown Cause (formerly “Cryptogenic Epilepsies”)

This term describes epilepsies in which the underlying cause is as yet unknown. It is meant to be a neutral term, which will disappear as knowledge progresses (Berg et al, 2010). All epilepsies will likely be discovered to have structural/metabolic and/or genetic causes, including gene-environment interactions (Ottman & Winawer, 2008).

1.4 Types of Seizures

Epileptic seizures manifest when the balance of excitatory and inhibitory signaling in the brain is disturbed. Most seizures are self-limiting, apart from status epilepticus, which is a potentially life-threatening condition that is marked by prolonged or repeating seizures (Engel, 2006; Scharfman, 2007; Burnham, 2007).

Different types of seizures and/or epilepsy require different therapy. Correct identification of seizure type(s), therefore, is necessary so that doctors can choose rational pharmacological and non-pharmacological therapies (Burnham, 2007).

1.4.1 Focal Seizures (formerly Simple and Complex “Partial Seizures”)

Regardless of epilepsy classification, approximately 60% of seizures in Western countries are focal seizures (Neligan et al, 2012). Focal seizures are limited to a particular brain network in one hemisphere, at least at their onset. The network can be very localized or very broad (Berg & Scheffer, 2011). This onset region is referred to as the “seizure focus,” and is identified by epileptic spiking in localized areas of the EEG recording (Burnham, 2007; Berg et al 2010; Berg & Scheffer, 2011).

Both subcortical and cortical structures can generate focal seizures (Berg et al, 2010). Depending on which area of the brain is involved in the seizure focus, different motor and sensory symptoms may occur (Engel, 2006). Focal seizures may spread through the brain to become bilaterally generalized seizures, known as “secondarily generalized seizures,” with tonic, clonic, or tonic-clonic convulsive

features (Burnham, 2007). Focal seizures are categorized according to the degree of impairment experienced during the seizure (Berg et al, 2010).

1.4.1.1 Focal Seizures with Psychic or Sensory Manifestations (Auras)

Some focal seizures are known as auras. They involve subjective psychic and/or sensory phenomena only, without impairment of consciousness or altered awareness (Berg et al, 2010; Thurman et al, 2011). Symptoms can include inexplicable feelings, strong and unusual emotions, sensory illusions, derealization, déjà vu or jamais vu, and difficulty speaking (WHO 2005; Blume et al, 2001). The subject will usually remember the seizure, unlike dyscognitive focal and generalized seizures (Burnham et al, 2007; Berg & Scheffer, 2011). When they precede a partially generalized or generalized seizure, an aura may be perceived as a “warning” that a seizure might occur (Blume et al, 2001; Burnham, 2007).

1.4.1.2 Focal Seizures With Motor Manifestations (formerly “Simple Partial” motor seizures)

These focal seizures were previously known as “simple partial,” “focal cortical” or “autonomic” depending on how the seizures manifested (Burnham, 2007; Berg et al, 2010). These focal seizures that do not involve impairment of consciousness, but may involve observable autonomic or sensory motor signs.

1.4.1.3 “Dyscognitive” Focal Seizures With Cognitive Impairment (formerly “Complex Partial” Seizures)

Dyscognitive focal seizures do involve impairment of consciousness and/or awareness, including effects on perception, attention, emotion, memory and

executive function (Blume et al, 2001). The subject may display automatisms; lip smacking, unusual vocal sounds, blank staring, and confused responses can be symptoms (Blume et al, 2001; Berg et al, 2010). This seizure type is the most common type in adults, and is often refractory to pharmacotherapy (Burnham, 2007; Picot et al, 2008; Mitchell et al, 2012).

1.4.2 Generalized Seizures

Generalized seizures involve both hemispheres of the brain, rapidly engaging varying bilateral neural networks, both cortical and subcortical (Berg et al, 2010). Generalized seizures may be primarily generalized from the time of their onset, or secondarily generalized from a focal seizure (Burnham, 2007).

Consciousness is lost, and there is no memory for the period of the seizure (Berg et al, 2010; Berg & Scheffer, 2011). These seizures may have subtle symptoms, as in absence epilepsy, or dramatic symptoms, as in tonic-clonic epilepsy.

1.4.2.1 Tonic-Clonic Generalized Seizures (Formerly "Grand Mal" Seizures)

Tonic-clonic generalized seizures involve unconsciousness with convulsions. They are less than five minutes in duration. Tonic phases of the convulsions are characterized by muscle contractions lasting seconds to minutes, whereas the clonic phases are characterized by sudden involuntary muscle jerks (Blume et al, 2001; Burnham, 2007). As shown in **Fig 1.4B**, an EEG recorded during a tonic-clonic seizure shows constant, rapid spiking in all areas of the brain.

1.4.2.2 Absence Generalized Seizures (Formerly Petit Mal Seizures)

Absence generalized seizures involve unconsciousness with a blank stare or fluttering eyelids. Absence seizures are less than thirty seconds long. During an absence seizure, the EEG shows a 3 per second spike and wave pattern (Burnham, 2007; Stefan et al, 2008). These generalized seizures do not involve convulsions.

1.5 Therapy for Epilepsy

1.5.1 Pharmacotherapy

There are a variety of treatments for epilepsy, with pharmacotherapy being the most common. There are over twenty drugs available for the treatment of epilepsy today (Bialer, 2012). Anticonvulsant medication choice is mediated by many patient-specific factors. These factors include age, sex, seizure type(s), concomitant medication, comorbidities and childbearing potential (Perucca & Tomson, 2011). Seventy percent of adults with epilepsy can be treated successfully with the available anticonvulsant medications (Löscher, 2011; Mitchell et al, 2012; Mula, 2013). Despite the variety of anticonvulsant drugs available today, however, many persons with epilepsy do not achieve complete seizure control (Bialer et al, 2010), especially for seizures of focal onset (Nasreddine et al, 2010).

All pharmacotherapies for the epilepsies are drugs that act on the CNS to promote inhibitory or suppress excitatory signaling processes. Anticonvulsant medications may act to enhance synaptic or extrasynaptic inhibition, and/or to suppress synaptic excitation, and/or to modulate voltage-gated ion channels to

prevent seizure activity (Krall et al, 1978; Rogawski & Löscher, 2004). Since these drugs have diverse effects, more than one target may be involved in individual treatments. The mechanisms of anticonvulsant drug action are complex and likely complementary (Löscher, 2011).

Early drugs (bromides, barbiturates, benzodiazepines, carbamazepine, valproate) were discovered serendipitously (Bialer, 2012). Today, however, drug/compound optimization is based on rational principles, such as the structure of a known receptor target and structural analogs of chemicals of interest (Weaver & Sankar, 2008). Since many targets for anticonvulsant therapy remain to be discovered and characterized, the pharmacology of epilepsy will be a major field of study for many years to come (Löscher, 2011; Bialer 2012; Banerjee & Sharma, 2012). Progesterone's many targets in the CNS, for example, are areas of active investigation (Stevens & Harden, 2011; Zorumski et al, 2012; King, 2013).

1.5.2 Drug Mechanisms: Drugs That Modulate Ion Channels

Ion channels are one of the primary targets for anticonvulsant drugs (Meldrum, 2008; French & Ben-Menachem, 2008). The present drugs probably work in a non-specific fashion, but genetic studies are revealing many ion channel mutations in animal and human epilepsies. Future drugs may target specific channelopathies (Kullmann & Schorge, 2008).

1.5.2.1 Voltage-Gated Sodium Channels

Voltage-gated sodium channels contribute to the rising phase of the action potential, and the propagation of action potentials along neurons. Voltage-gated sodium channels go through an inactivated phase during the refractory period between action potentials. If the channels' inactivation phase is lengthened, the trains of high-frequency action potentials seen in an epileptic discharge are less likely to occur, which has an anticonvulsant effect (Meldrum, 2008). Many of our current anticonvulsant drugs act on voltage-gated sodium channels. They are effective against both partial and generalized seizures. They include lamotrigine, carbamazepine, oxcarbazepine, and phenytoin, mephenytoin, ethosuximide, and possibly felbamate, topiramate, and valproate (Rogawski & Löscher, 2004; Bialer 2012). These drugs are hypothesized to bind to the inactivated phase of the voltage-gated sodium channel, prolonging the inactivated state. This does not affect normal excitatory and inhibitory synaptic activity, since it does not prevent the generation of normal action potentials, but it does prevent the unchecked excitation seen during seizures (Rogawski & Löscher, 2004; Burnham, 2007).

1.5.2.2 Voltage-Gated Calcium Channels

Voltage-gated calcium channels are located in the presynaptic membrane and also on the cell bodies of neurons. Voltage-dependent calcium channels are activated when the membrane reaches a threshold depolarization voltage (the specific threshold voltage depends on the channel subtype, below).

High voltage-activated (HVA) calcium channels primarily release neurotransmitters (Cain & Snutch, 2012). Low voltage-activated (LVA) calcium

channels regulate neurotransmission in a pacemaker-like fashion in the thalamus (Rogawski & Löscher, 2004). Mutations in both LVA and HVA channel genetics are associated with epilepsy in humans and animal models. Gain of function of voltage-gated calcium channels occurs in several in vivo models of chronic seizures (Cain & Snutch, 2012).

1.5.2.3 High Voltage-Activated (HVA) Calcium Channels

HVA calcium channels include subtypes L, R, N, and P/Q. Their “high” voltage of activation is approximately -40 mV (Cain & Snutch, 2012). Since the resting potential of most cell membranes is -70 mV, a substantial (~30 mV) depolarization of the cell from its resting potential is needed to activate HVA calcium channels. This means that voltage-gated sodium channels, which have a voltage of activation of -50 mV, are likely to open before the HVA calcium channel threshold of activation is reached.

HVA channels regulate pre-synaptic calcium entry and neurotransmitter release, a process known as “action-secretion coupling” (Lança, 2007).

When activated by a sufficiently depolarized membrane, pre-synaptic calcium channels open to allow intracellular calcium to enter the pre-synaptic bouton from the synaptic cleft. Vesicles containing transmitter substances are then released into the synaptic cleft and diffuse to post-synaptic receptors, facilitating excitatory neurotransmission (MacKay, 2003). Inhibition of HVA calcium channels, therefore, can suppress excitatory neurotransmission (Rogawski & Löscher, 2004).

Drugs affecting HVA calcium channels are used to treat partial and generalized seizures. It is thought that these drugs differentially decrease excitatory

transmitter release in a way that limits excitatory synaptic responses (Rogawski & Löscher, 2004). This can prevent the runaway excitatory neurotransmission seen during seizures. HVA calcium channel drugs include gabapentin and pregabalin, both of which are thought to bind to the $\alpha 2\delta$ -subunits of HVA calcium channels (Rogawski & Löscher, 2004). It has also been suggested that phenobarbital and felbamate may bind to HVA calcium channels (Meldrum, 2008), although phenobarbital produces most of its anticonvulsant potency by enhancing GABAergic inhibition (below). Lamotrigine and levetiracetam also have effects on HVA calcium channels at clinically relevant concentrations (Lukyanetz et al, 2002; Rogawski & Löscher, 2004). Lamotrigine blocks N- and P/Q-type HVA calcium channels, and levetiracetam primarily blocks N-type channels (Rogawski & Löscher, 2004).

1.5.2.4 Low Voltage-Activated (LVA) Calcium Channels

LVA calcium channels include the T-type channel. This LVA channel is hypothesized to maintain a balance of normal neuronal firing, particularly in the thalamus, by contributing to thalamic oscillatory responses (Cain & Snutch, 2012). When activated, T-type calcium channels generate a series of low threshold calcium spikes, which subsequently trigger sodium channel-mediated action potentials (Rogawski & Löscher, 2004). The T-type calcium channel's voltage of activation is more negative than HVA channels, approximately -60 mV (Cain & Snutch, 2012). Since the resting potential of neuronal membranes is approximately -70 mV (MacKay, 2003), T-type calcium channels are activated by a very small increase in intracellular current with respect to extracellular current. Abnormal T-type calcium channel function can dysregulate the action potential pacemaker

function of the channel, resulting in hypersynchronous activity in thalamocortical circuits. This can result in a generalized absence seizure (Rogawski & Löscher, 2004; Meldrum, 2008; Budde et al, 2006; Cain & Snutch, 2012).

Ethosuximide, trimethadione and zonisamide inhibit LVA T-type calcium channels without effects on other calcium channels (Zhang et al, 1996).

Ethosuximide is currently the drug of choice for absence seizures, but not other seizure types. Valproic acid, zonisamide and lamotrigine may have effects on LVA T-type calcium channels as well as other mechanisms, which may explain their efficacy in treating refractory absence seizures, as well as other seizure types (Rogawski & Löscher, 2004).

1.5.2.5 Potassium Channels

In a depolarized neuron, potassium channels open to return the cell to its resting potential (Dichter & Pollard, 2006). Most potassium channels open to release potassium from intracellular stores to the extracellular space, which causes an outward potassium current, repolarizing the membrane (MacKay, 2003).

Potassium channel subtypes are voltage activated, calcium activated, inwardly rectifying, and slow current leak (Rogawski & Löscher, 2004). Extracellular calcium also forms a complex with calmodulin to open calcium activated potassium channels. Potassium channel genes can be mutated in inherited human and animal models of epilepsy (Cooper, 2012).

The potassium channel superfamily is considered a promising target for anticonvulsant drugs. A few of the newer anticonvulsant drugs may prolong

potassium channel open time to decrease excitability, including retigabine (Rogawski & Löscher, 2004) and topiramate (Dichter & Pollard, 2006).

1.5.3 Drug Mechanisms: Drugs That Modulate Synaptic Function

In the CNS, glutamate is the primary excitatory neurotransmitter and γ -aminobutyric acid (GABA) is the primary inhibitory neurotransmitter.

Anticonvulsant drugs can decrease synaptic glutamatergic activity, or enhance GABAergic activity, to prevent synchronization and propagation of neural discharges (Rogawski & Löscher, 2004; Dichter & Pollard, 2006).

1.5.3.1 GABA modulation

The GABA receptors mediate most inhibitory neurotransmission in the CNS (MacKay, 2003). GABA_A receptors are ionotropic and form post-synaptic chloride channels. GABA_B receptors are metabotropic, and are coupled to ion channels via G-proteins (Han et al, 2012). Imbalances in either can disrupt normal inhibitory signaling in the brain, causing seizures and/or epilepsy.

GABA_A receptor pharmacology is mediated by the receptor's subunit conformation. The most common pentameric subunit conformation of the GABA_A receptor in the CNS is 2 α subunits, 2 β subunits, and 1 subunit that is either γ , δ or ϵ . Mutations in the $\alpha 1$ and $\gamma 2$ subunits in particular are associated with a number of epilepsy syndromes (Rogawski & Löscher, 2004; Cherubini, 2012). GABA_A receptor subunit composition determines whether the GABA_A receptor will affect "phasic" or "tonic" inhibition.

GABA_A receptors mediating phasic inhibition are located in the synapse and are activated by GABA released in vesicular quanta (Belelli et al, 2006). GABA_A receptors that mediate phasic inhibition almost always contain a $\gamma 2$ subunit. They also tend to contain two $\alpha 1$, $\alpha 2$ or $\alpha 3$ subunits, and one $\beta 2$ or $\beta 3$ subunit. When activated by GABA, the synaptic GABA_A receptors produce a transient (“phasic”) inhibitory post-synaptic current (Belelli & Lambert, 2005; Belelli et al, 2009; Cherubini, 2012). Tonic inhibition, by contrast, influences overall excitability in the brain, or an inhibitory “tone.” The GABA_A receptors that mediate this “tonic” inhibition are located peri- or extrasynaptically. They are very sensitive to low GABA concentrations, and are not easily desensitized. Hence, they are able to respond strongly to low concentrations of ligand that spillover from the synapse. Extrasynaptic receptors GABA_A receptors that incorporate the $\alpha 1$ or $\alpha 4$ subunit and the δ -subunit are particularly sensitive to modulation by neurosteroids at physiological levels, whereas synaptic receptors are not (Belelli et al, 2006). In models of non-absence seizures, tonic inhibitory current is increased. This is hypothesized to be a compensatory mechanism for loss of phasic inhibition in these models (Walker & Kullman, 2012).

The blockade or antagonism of GABA_A receptors is a common feature of several acute chemoconvulsant models of epilepsy in vivo and in vitro. Blockers of the GABA_A receptor used experimentally include pentylenetetrazol, bicuculline, picrotoxin, and β -carboline (Velíšek, 2006; Budde et al, 2006; Dichter & Pollard, 2006).

There are a variety of binding sites on the GABA_A receptor/channel. GABA itself binds to the α and β subunits of the GABA_A receptor. Benzodiazepines bind to a site between the α - and γ -subunits, and enhance the affinity of GABA_A receptors for GABA, increasing their channel open time (Cherubini, 2012). Barbiturates are also positive allosteric modulators of GABA_A receptors that can increase the probability of channel open time (Rogawski & Löscher, 2004). There are also other anticonvulsant binding sites that have not yet been identified, including a neurosteroid binding site (Cherubini, 2012). Neurosteroids enhance inhibitory transmission by allosterically modulating GABA_A receptors at lower concentrations, and directly activating the receptors at higher concentrations (Belelli & Lambert, 2005). The exact binding sites for inhibitory neurosteroids are unknown, but they are distinctive from the benzodiazepine and barbiturate binding sites, and are associated with particular subunits synaptically and extra-synaptically (Cherubini, 2012).

A number of anticonvulsant drugs also enhance GABAergic inhibition by a variety of mechanisms. These drugs are generally effective against tonic-clonic and some partial seizures, but are not effective against absence seizures (Rogawski & Löscher, 2004). Some GABAergic drugs bind to the GABA_A-related chloride channel itself. The benzodiazepines (ie, clobazam, clonazepam, diazepam) and barbiturates (ie, primidone, phenobarbital) are both positive allosteric modulators of GABA_A-related chloride flux (Dichter & Pollard, 2006). Their clinical utility is limited by sedation (Rogawski & Löscher, 2004). Other anticonvulsant drugs raise GABA levels in the synapse. Tiagabine prevents GABA reuptake, prolonging

GABAergic effects (Bialer, 2012). Felbamate and topiramate may also be positive allosteric modulators of GABA_A receptors (Rogawski & Löscher, 2004). Gabapentin may increase GABA turnover in addition to its blocking effects on HVA calcium channels (Rogawski & Löscher, 2004). Vigabatrin enhances GABA levels by irreversibly inhibiting GABA *transaminase*, a GABA-metabolizing enzyme, which has synaptic effects on GABA_B receptors (Rogawski & Löscher, 2004).

GABA_B receptors are metabotropic and slower acting, and thus mediate inhibitory responses that are slower and longer lasting. They have particular relevance for absence seizures (Han et al, 2012). GABA_B receptors activate enzymes to produce dynamic effects in adenylyl cyclase/cyclic adenosine monophosphate (cAMP)-dependent signaling cascades. Some GABA_B receptors are expressed on the pre-synaptic terminal of glutamatergic neurons. It is hypothesized that they inhibit glutamate release, and hence, increase pre-synaptic inhibition. Peri-synaptic GABA_B receptors can act as GABA auto-receptors by responding to levels of GABA “spillover” into the synaptic cleft (Han et al, 2012). Presumably, these pre-synaptic effects are mediated by G protein effects on P/Q-type and N-type calcium channels.

GABA_B receptors play a special role in absence seizures, as noted above. In normal thalamocortical circuitry, GABA_B receptors and LVA (T-type) calcium channels play a role in maintaining the appropriate oscillatory “pacemaker” function. In the thalamocortical circuitry of absence seizure phenotypes, the GABA_B receptor mediates inhibition that results in the de-inactivation of LVA calcium channels, and subsequently, increased excitatory calcium signaling (Han et al, 2012). In general, GABA_B receptor antagonists suppress absence seizures in animal

models, whereas GABA_B agonists exacerbate them. Antagonists of GABA_B receptors are anticonvulsant for absence seizures, but not other seizure types (Han et al, 2012). To date, GABA_B receptor antagonists are not yet available for clinical use as anticonvulsant drugs.

1.5.3.2 Glutamate Modulation

Glutamate receptors are essential to rapid excitatory signaling in the CNS. They include N-methyl-D-aspartate (NMDA) receptors and the non-NMDA α -amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) and kainate receptors. The presynaptic release of glutamate during excitatory neurotransmission activates post-synaptic glutamate receptors to produce excitatory post-synaptic potentials (EPSPs). When enough of these EPSPs have summated, they can produce an action potential. Glutamate receptors, therefore, are critical to normal excitatory neurotransmission in the CNS (Dingledine, 2012).

The AMPA and kainate glutamate receptors open ligand-gated sodium channels. They produce short duration EPSPs. NMDA receptors are particularly important in the production of long-lasting EPSPs. They are also permeable to calcium ions as well as sodium ions, unlike kainate and AMPA receptors. At the resting membrane potential, NMDA receptors cannot be activated, even if they have been bound by glutamate and its cofactor, glycine. This is because the channel is blocked by a magnesium ion at the resting membrane potential. When the NMDA receptor is bound to glutamate and glycine, and the cell membrane has depolarized, the magnesium block is relieved. NMDA receptors then become permeable to

calcium and sodium ion influx and potassium ion efflux. This initiates field potentials, which contribute to action potentials (Dingledine, 2012).

Antagonism of glutamate receptors is a proposed mechanism of some anticonvulsant drugs, including felbamate (NMDA receptors) and topiramate (AMPA and kainate receptors). Perampanel, a recently introduced anticonvulsant drug, is thought to block AMPA receptors (Mula, 2013). Recent evidence has shown that barbiturates may also block AMPA/kainate receptors, reducing excitatory neurotransmission, in addition to their well-known role as GABAergic anticonvulsants (Rogawski & Löscher, 2004; Mula, 2013). Compounds that activate glutamate receptors, such as kainic acid, are proconvulsant (Rogawski & Löscher, 2004; Mula, 2013).

1.5.4 Limitations of Pharmacotherapy

Although pharmacotherapy is the most common therapy for epilepsy, not all seizures are drug responsive. Drug-refractory seizures occur in about one third of adults with epilepsy, even those with access to the best medications (Theodore et al, 2006; Burnham, 2007; Beleza, 2009; Mitchell et al, 2012). It is the failure of pharmacotherapy that leads to the use of other therapies, such as surgery, diet and vagal stimulation (discussed here), as well as the search for better drugs (discussed below).

1.5.5 Non-Pharmacological Therapies for Epilepsy

1.5.5.1 Surgery

Surgery involves the removal of the focus or “seizure onset zone.” This can drastically improve the lives of persons with drug-resistant epilepsy, especially temporal lobe epilepsy (Zupanc et al, 2010). Surgery is only an option, however, when a clear seizure focus can be identified in an area amenable to resection. Unfortunately, access to seizure surgery is limited even in wealthy countries, and is nearly non-existent in poor countries (WHO, 2005).

1.5.5.2 Dietary therapies

Dietary therapies are effective in some children and in a limited number of adults with epilepsy (Lee & Kossoff, 2011; Lutas & Yellen, 2013). Several of these dietary therapies cause metabolic “ketosis,” reducing neuronal excitability by an unknown mechanism. The classic “ketogenic diet” was the first diet used to treat children with drug-refractory seizures. The diet removes almost all carbohydrates in the child’s diet and replaces them with fats. The ketogenic diet is very strict. It requires serious commitment, and is often intolerable (Lutas & Yellen, 2013, throughout). A medium-chain triglyceride diet allows slightly more protein and carbohydrates relative to fat. More recently, a “modified Atkins diet” and a “low-glycemic-index diet” have also been used. They do not require weighing of food, and are more palatable, although they resemble the classic ketogenic diet in that they require low carbohydrates and a high fat content. Unlike the classic ketogenic diet, they do not restrict calories or protein (Lee & Kossoff, 2011, throughout).

The exact mechanism(s) of these diets is unclear. Since they can be effective in children that fail to achieve seizure control with available anticonvulsant medications, the mechanism is hypothesized to differ from those of the known anticonvulsant drugs. Proposed mechanisms have included altered glutamate metabolism, inhibition of glycolysis, anticonvulsant effects of ketones, and activation of potassium channels. Serious long- and short-term side effects are common in all dietary therapies (Lee & Kossoff, 2011; Lutas & Yellen, 2013, throughout).

1.5.5.3 Vagal Nerve Stimulation

Vagal nerve stimulation can be effective, but is usually a treatment of last resort (Beekwilder & Beems, 2010). A programmable, battery-operated signal generator, the “stimulator,” is surgically implanted in the left chest muscle under the skin. The stimulator delivers an intermittent pulse of electricity to the left vagus nerve in the neck. This electrical pulse then affects the central nervous system via the vagus nerve. The mechanism of action is not clear (WHO, 2005; Beekwilder & Beems, 2010).

1.5.6 The Need for New Pharmacotherapies

As noted above, despite the number of anticonvulsants available, ~30% of persons with epilepsy have seizures that cannot be treated by the existing drugs (Kwan et al, 2010; Bialer et al, 2012). In addition to being ineffective in many patients, the existing anticonvulsant medications also have serious adverse side

effects (Mula & Sander, 2007; Gaitatzis & Sander, 2013). Persons with epilepsy perceive these adverse effects to be as damaging as uncontrolled seizures to their quality of life (Toledano & Gil-Nagel, 2008). Common long-term side effects of anticonvulsant drugs include adverse effects on body weight, appearance, reproductive health and sexual function, bone health, and thyroid function in men and women (Herzog, 2008b; Meador et al, 2008). Less common long-term side effects include peripheral neuropathies and cerebellar atrophy (Pack & Gidal, 2008; Gaitatzis & Sander, 2013). Even when drugs successfully control seizures, non-compliance with pharmacotherapy is common due to these side effects (Bootsma et al, 2009). Since most persons with epilepsy must take medication for a lifetime, there is a great need for pharmacological treatments with fewer adverse long-term side effects.

Additionally, many anticonvulsant drugs are teratogens (Yerby et al, 2008; Gaily & Meador, 2008; Gaitatzis & Sander, 2013). Altered neuroendocrine status in women with seizures can interfere with normal fetal development (Pennel, 2009; Brunton & Russell, 2008; Harden & Sethi, 2008). Neurocognitive and behavioral disorders in infants born to mothers with epilepsy are also common (Holmes et al, 2007). Some anticonvulsant drugs have a higher risk of teratogenic effects than others. Polytherapy is particularly associated with adverse outcomes (Harden & Sethi, 2008; Meador et al, 2008; Sukumaran et al, 2010).

Drug side effects are particularly prominent in patients with intractable epilepsy who are placed on polytherapy. Pharmacological polytherapy is often unsuccessful in improving seizure control; adding drugs often greatly increases

drug side effects, but yields only a modest increase in seizure protection (Bourgeois & Gilliam, 2008; Bootsma et al, 2009, throughout).

1.5.7 The Electrical Kindling Model of Epilepsy & Drug Discovery

In 1969, Goddard et al were the first to report that daily electrical stimulation of limbic structures could eventually cause motor seizures. They hypothesized that daily electrical stimulation to limbic structures could permanently reorganize function in brain (Goddard et al, 1969). Racine later introduced electrophysiological recording to the model and investigated changes in kindled animals' electrically induced electroencephalographic discharges (Racine 1972a), as well as the development of the behavioral/motor seizures (Racine, 1972b), and potential mechanisms (Racine, 1972c). This became known as electrical kindling. It is a model that can be used to develop new pharmacotherapies for drug-resistant focal seizures that may secondarily generalize (Albright & Burnham, 1980). In this thesis, the kindling model was used to investigate anticonvulsant properties of progesterone and its metabolites (chapter 3).

Electrical kindling delivers electrical current via a “stimulating electrode” surgically implanted in the brain. The site of stimulation is referred to as the seizure “focus.” The site of stimulation and/or recording is usually in the amygdala or hippocampus, although other sites are also used. The stimulating electrode is used to deliver mild electrical stimulation to the focus, and the recording electrode(s) records excitability in a local population of neurons on the

EEG. The seizure-like event recorded on the EEG is referred to as the afterdischarge (AD; Burnham et al, 2005; McIntyre, 2006).

The convulsions that are the behavioral manifestation of the kindled seizure are referred to as “behavioral” or “motor” seizures. They are assessed using a standardized scale: stage 0, no response; stage 1, chewing; stage 2, chewing and head nodding; stage 3, forelimb clonus; stage 4, rearing; and stage 5, rearing and falling (Racine, 1972b). When animals have had a number of consecutive stage 5 seizures, they are considered fully kindled. Currently, 3 consecutive stage five seizures are typically used as the criterion for kindling mice (Reddy et al, 2012), whereas 30 stage five seizures are more typical for kindling rats (Albright & Burnham, 1980).

With initial kindling stimuli, at low intensities, there are not behavioral seizures or ADs. With repeated stimulation, however, simple biphasic AD spikes appear. With repeated stimulations these ADs lengthen and become more complex as the frequency and amplitude of spikes increase (Racine, 1972a, b). After 10-15 ADs have been elicited in rats, or 5-10 ADs have been elicited in mice, behavioral seizures appear and the stages progressively increase (Racine, 1972a). Subsequently, an ascending series is performed to determine each animal’s AD threshold, defined as the lowest voltage that evokes an AD. After they are fully kindled, animals are permanently predisposed to seizures (Goddard et al, 1969).

Focal seizures can be induced from many structures, including the cortex. Although there are many types of focal seizures in the kindling model, there is only one type of generalized seizure, which suggests a convergent mechanism

between the focus and the motor seizure substrate (Burnham et al, 1981). Racine (1972c) hypothesized that the mechanism of motor seizure development involved the strengthening of connections between the limbic structures and the “motor” structures in the brain stem that control skeletal motor responses (Racine, 1972c).

This “centrencephalic” mechanism of seizure generalization hypothesizes that, upon stimulation of the kindled seizure focus, the resultant AD propagates to a convergent point in the upper brain stem, presumably the rostral reticular formation. The reticular formation of the upper brain stem in particular has widespread connections to limbic structures and the neocortex in both hemispheres, making it an ideal structure to mediate seizure spread within and between hemispheres of the brain (Burnham et al, 1981, throughout).

Racine observed that behavioral seizures often begin with a delay (latency) of up to 30 seconds following the start of ADs recorded from limbic areas (Racine, 1972b). In our own kindling studies we observed a delay between the manifestation of the motor seizure and the beginning of the AD in the motor cortex (Jeffrey et al, 2014). This might indicate that the motor seizure generalization from the hippocampal focus is mediated by a structure other than the motor cortex, possibly the reticular formation (Burnham et al, 1981).

Technically, electrical kindling offers a number of advantages for pharmacological experiments. Specific injection-test latencies are possible since the seizures are evoked by electrical stimulation. Repeated experiments using different drugs and/or doses and/or test-injection latencies are possible using the same animals (each animal can thus serve as its own control, strengthening statistical

analyses). Electrographic as well as motor seizures can be studied, and they can be examined with specialized software (pClamp, IBM) that allows sophisticated analyses.

1.6 Steroid Hormones

Steroids are organic compounds with 20 carbon atoms bonded together in a distinctive manner to form four fused cycloalkane rings (**Fig 1.5**).

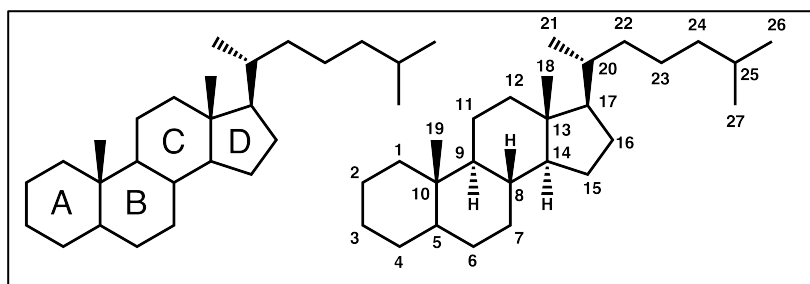


Figure 1.5. Steroid form (left) and carbon numbering system (right).
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Steroid hormones affect social, emotional, cognitive and physical functioning (King, 2013). In addition, it is well known that steroid hormones directly affect the excitability of the brain (Rhodes et al, 2004; Pennel, 2009; Frye, 2010; King, 2013). Steroids are a particular focus in this thesis, since it has been suggested that the progestins may be developed into a new class of anticonvulsant drugs (Reddy, 2009; Pack et al, 2011). Steroids will be discussed as hormonal steroids, neurosteroids, and neuroactive steroids. Mineralcorticoids,

glucocorticoids, and androgens will not be discussed in this thesis. Estrogens and progestagens, in particular, will be discussed (below).

1.6.1 Hormonal Steroids

Hormonal steroids are those produced by the endocrine system in the reproductive organs and adrenal glands (Melcangi et al, 2008). The hormonal steroids include estrogen and progesterone. Apart from their well-known roles in reproduction, these hormonal steroids have significant effects on brain function and excitability, and, hence, seizure activity (Rhodes et al, 2004; Pennell, 2009).

Hormonal steroids are lipophilic and enter the brain, unlike their pre-cursor cholesterol (Schumacher et al, 1996; Mellon et al, 2001; Do Rego et al, 2009).

Progesterone in particular, as a hormonal steroid, is produced in the ovaries and placenta, and in the mammary glands during lactation. It is also produced in the testes and adrenal glands in smaller amounts (Pennell, 2009). Progesterone has complex and wide-ranging functions in the periphery, including participating in the cyclic regulation of the menses, sperm production and motility, and embryonic development (Do Rego et al, 2009).

1.6.2 Neurosteroids

Neurosteroids are steroids produced de novo in the brain and spinal cord. Progesterone, estrogen and testosterone are all neurosteroids as well as hormonal steroids (Do Rego et al, 2009). Brain levels of neurosteroids can be up to fifty times higher than levels in plasma; thus, brain steroid levels do not reflect

plasma steroid levels and vice versa (Do Rego et al, 2009; King, 2013). Levels of neurosteroids, their metabolizing enzymes, and various receptors are specific to different regions and cell types in the brain (Do Rego et al, 2009; King, 2013). Effects of neurosteroids can thus vary in different brain areas and are dependent on the enzymes and receptors present in the target cells (Saalman et al, 2007; King, 2013).

Neurosteroids are produced in both neurons and glia. Although neurosteroids do have genomic effects, effects at membrane-associated signaling pathways are suspected to underlie many of the neurosteroid effects that regulate neural functioning (Schumacher et al, 1996; Melcangi et al, 2008). Physiological plasma and brain levels of neurosteroids range from 10-300 nM (Belelli et al, 2009; Melcangi et al, 2013).

1.6.3 Neuroactive Steroids

Neuroactive steroids are neurosteroids that interact with receptors in the CNS or the peripheral nervous system (PNS). This class of steroids includes both the gonadal sex steroids produced peripherally, and the neurosteroids produced in the CNS and PNS (King, 2013).

Neuroactive steroids regulate many aspects of neurophysiology and behavior (Do Rego et al, 2009). They have effects on both the structure and the function of the CNS (Foy et al, 2010). These include effects on endocrine regulation, affect, cognition, and many sorts of behavior (Pack et al, 2011; King, 2013). Neuroactive steroids also have profound effects on brain excitability, and, hence,

seizure activity (Rhodes et al, 2004; Herzog & Fowler, 2005; Pennell, 2009; Verotti et al, 2010). Neuroactive steroid targets are also considered promising candidates for the rational design of novel antidepressant and/or anxiolytic drugs (Schüle et al, 2011).

1.6.4 Regulation of Neuroactive Steroids

A complex interaction between peripheral steroids, neurosteroids and their many receptors regulates the gonadal and the neurological levels of these compounds (Melcangi et al, 2008; Pack, 2010; King, 2013). In addition, both seizures and anticonvulsant medications can alter steroid profiles in the hypothalamus and pituitary, which in turn can regulate neuroactive steroid levels in the rest of the brain, and vice versa (Pennell, 2009). The hypothalamus regulates neuroendocrine function, including neurosteroid levels, by regulating hormones of the pituitary gland. The hypothalamus also has direct connections to convergent circuitry from the limbic forebrain (hippocampus, amygdala, septum, cingulate cortex), the neocortex, and the cerebellum (Felten & Jozefowicz, 2004). Complex feedback systems exist between steroid hormone levels in the brain and the release of neuroactive steroids from the HPA axis (Pennell, 2009).

1.6.5 Neuroactive Steroids and Neural Excitation

Neuroactive steroids have profound effects on overall brain excitability and thus play complex roles with respect to seizures, epilepsy and anticonvulsant drugs (Verrotti et al, 2010). Their effects on brain function are primarily mediated

by positive or negative allosteric modulation of excitatory (glutamatergic) and inhibitory (GABAergic) neurotransmission (Pack et al, 2011). Endogenous differences in neuroactive steroids and their receptors in males and females are hypothesized to underlie gender differences in patterns of seizure occurrence. As discussed below, estrogen is generally excitatory, and progesterone is generally inhibitory (Beyenburg et al, 2001; Smith & Woolley, 2004; Reddy, 2009; Stevens & Harden, 2011).

1.6.5.1 Estrogen / Estradiol and its Metabolites are Generally Excitatory

Estradiol is the most common type of estrogen in humans, and the two terms are often used interchangeably in the literature. For this thesis, “estradiol” will be used to denote the neurosteroid. Receptors will be referred to as estrogen receptors since they respond to many types of estrogen (King, 2013).

Estradiol plays a complex role in neurosteroid dynamics in the CNS and the periphery (Meitzen & Mermelstein, 2011). Although it is also produced in the brain, estradiol has a high partition coefficient, such that it easily crosses the blood-brain barrier and cell membranes. Thus, estradiol levels in the brain are affected by estradiol levels in the periphery, and vice versa. Estradiol levels in the brain are also affected by endogenous hormonal changes such as menses, pregnancy and puberty (Herzog, 2009; Pack et al, 2011).

Estradiol has many effects in the CNS. In the brain, estradiol has rapid, cell-surface receptor-mediated effects that affect neurotransmission, and longer latency, longer lasting effects on gene transcription and translation. The most well characterized example of the rapid effects of estradiol is the activation of

metabotropic glutamate receptors (Meitzen & Mermelstein, 2011). An example of longer latency effects is that estrogen-induces increases in the densities of dendritic spines and glutamatergic NMDA-containing synapses that occur over days (Smith & Woolley, 2004; Verrotti et al, 2010). Another longer latency effect is the bidirectional effects on CREB phosphorylation in neurons (Meitzen & Mermelstein, 2011). Both of these longer-latency effects are examples of estradiol potentiating neuronal plasticity to affect neuronal excitability (Herzog, 2009). Estrogen also decreases GABA synthesis in some brain regions, decreasing inhibition (Herzog et al, 2009; King, 2013).

Recent evidence suggests that both the membrane and the nuclear effects of estradiol are mediated by the “classical” estrogen receptors α and β originally found in the nucleus (Meitzen & Mermelstein, 2011). Both estrogen receptors α and β , which bind estradiol as well as other estrogen types, can translocate from the nucleus to the cell membrane, where they interact with G-protein coupled receptors, ultimately activating metabotropic glutamate receptors in a region-specific manner (Meitzen & Mermelstein, 2011 throughout).

1.6.5.2 Progesterone and its Metabolites are Generally Inhibitory

Progesterone is an inhibitory neuroactive steroid, primarily via its metabolism to $5\alpha,3\alpha$ -tetrahydropregesterone (THP), also known as allopregnanolone. THP is a powerful GABA_A agonist (**Fig 1.6**). It is produced both in the body and in the brain. Like estradiol, progesterone easily crosses the blood-brain barrier and cell membranes. Levels in the brain, therefore, are influenced

serum levels and vice versa. Progesterone levels are also affected by endogenous hormonal changes such as menses, pregnancy and puberty (Pack et al, 2011).

The main metabolic pathway of progesterone is illustrated in **Fig 1.6**. Progesterone, and its metabolite THP, in particular, have a strong influence on seizure propensity, as is seen in catamenial epilepsy (Beyenburg et al, 2001; Foldvary-Schaefer et al, 2004; Reddy, 2009; Pack et al, 2011). Progesterone and its metabolites will be discussed in detail in the following section.

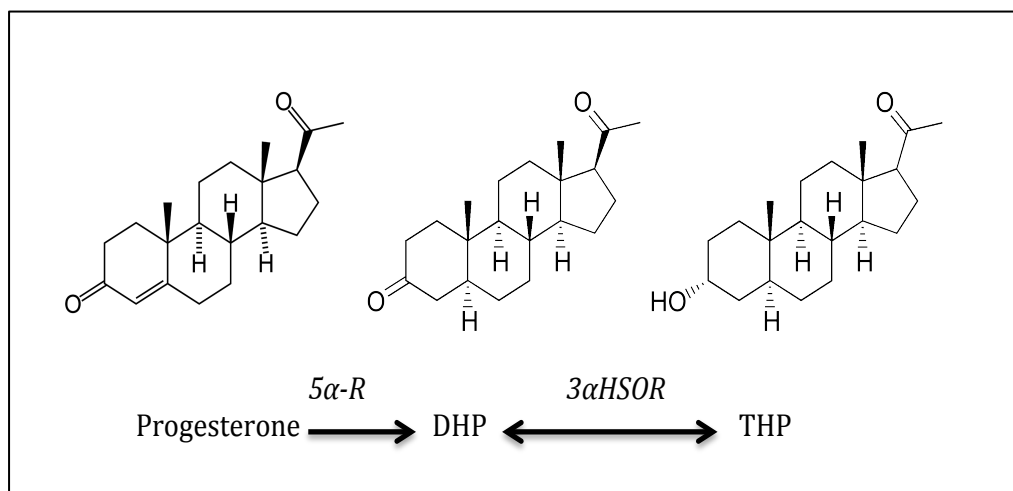


Figure 1.6 Major CNS metabolic pathway of progesterone. Abbreviations: DHP, 5 α -dihydroprogesterone; THP, 5 α ,3 α -tetrahydroprogesterone, also known as allopregnanolone; 5 α -R, 5 α -reductase; 3 α HSOR, 3 α -hydroxysteroid oxidoreductase.

1.7 Progesterone and Its Metabolites

1.7.1 Progesterone

The structures of progesterone and its best-known metabolites are illustrated in **Fig 1.6**. Progesterone is produced from cholesterol in glia and neurons in the CNS, and by Schwann cells in the PNS (Belelli & Lambert, 2005; Schumacher

et al, 2012; King, 2013). Progesterone has diverse effects in the CNS. These include effects on neurogenesis, cognition, affection, neuroprotection, inflammation, synaptic plasticity, receptor levels, and, most relevant for this thesis, neurotransmission (Gyermek et al, 1967; Mellon et al, 2001; Smith & Woolley, 2004; N-Wihlbäck et al, 2006; Koulen et al, 2008; Brinton et al, 2008; Do Rego et al, 2009; Lanxanberger et al, 2011; Bahra et al 2011; King, 2013; Zorumski et al, 2013). Progesterone's effects on neurotransmission are generally inhibitory, which is why it elevates seizure threshold. As noted above, the progesterone metabolite THP enhances GABA_A receptor-mediated inhibition. Progesterone can also change GABA_A receptor gene expression in vitro by an unknown mechanism (Maguire & Mody, 2007, 2009). It is, however, known that progesterone itself has little or no interaction with GABA_A receptors (Bellelli & Lambert, 2005).

Synthetic progestins do not have the complex effects of natural progesterone in the CNS (Do Rego et al, 2009; Brinton et al, 2008; Kaore et al, 2011). Interestingly, the progesterone analog medroxyprogesterone acetate (MPA) can inhibit the enzyme *3 α ,5 α -hydroxysteroid oxidoreductase (3 α -HSOR)*, preventing the re-conversion of 3 α ,5 α -tetrahydroprogesterone (THP) to 5 α -dihydroprogesterone (DHP; **Fig 1.6**). This is hypothesized to be the mechanism of MPA's inhibitory effects on neurotransmission (Bellelli & Lambert, 2005). Unlike progesterone, MPA is not neuroprotective (Nilsen & Brinton, 2002; Ciriza et al, 2006; Bethea, 2011). MPA has, however, been reported to have anticonvulsant effects in women with catamenial seizures, but not in women with non-catamenial seizures (Bellelli & Lambert, 2005). In a clinical trial with post-menopausal women with epilepsy, hormone replacement

therapy with MPA actually caused an increase in seizure frequency (Harden et al, 2006).

1.7.2 Progesterone Metabolites

Progesterone can be metabolized via several pathways. In one of them, progesterone is metabolized to the 5 α -pregnanes, 5 α -dihydroprogesterone (DHP) and 3 α ,5 α -tetrahydroprogesterone (THP, also known as allopregnanolone). In the 5 α -pregnane pathway, progesterone is first metabolized to DHP by the unidirectional enzyme *5 α -reductase*. DHP is subsequently metabolized to THP by the bidirectional enzyme *3 α ,5 α -hydroxysteroid oxidoreductase (3 α -HSOR*; Mellon et al, 2001; Do Rego et al, 2009; King, 2013). This metabolic pathway, illustrated in **Fig 1.6**, is the best known and most well studied. In many studies, when finasteride is used to inhibit the metabolism of progesterone to DHP and THP, the anticonvulsant effects of progesterone are lost (Belelli & Lambert, 2005). Progesterone can also be metabolized to 5 β -pregnanes, 4-pregnenes, corticosteroids, androgens, and estrogens (Wiebe, 2006). Enzyme levels determine which metabolic pathway progesterone will follow in a region-dependent manner (Agís-Balboa et al, 2006). In the CNS, however, progesterone is primarily metabolized to DHP and THP (Reddy, 2009). DHP and THP are themselves neuroactive steroids (Ciriza et al, 2004), and are particularly relevant to this thesis. Both DHP and THP affect adult neurogenesis (Giachino et al, 2003) and can suppress neuropathic pain in vitro (Meyer et al, 2010). In addition, both DHP and THP can increase myelination in a model of peripheral neuropathy in diabetes

(Pesaresi et al, 2010). As noted below, both DHP and THP have been reported to have anticonvulsant effects.

1.7.3 5 α -dihydroprogesterone (DHP)

Progesterone's primary metabolite, 5 α -dihydroprogesterone (DHP), has been reported to have a number of physiological actions. It has been found to inhibit cortical interictal spikes in a penicillin cat model (Landgren et al, 1987), and to protect against hippocampal neuronal injury in a rat kainate model (Ciriza et al, 2006). DHP is also carcinogenic-like in cell-line models of breast cancer, and is produced at higher levels in tumorous than non-tumorous breast cancer cell lines (Weiler & Wiebe, 2000; Wiebe et al, 2005; Wiebe, 2006; Wiebe et al, 2010).

There is currently not a known binding site for DHP in the CNS, although Weiler & Wiebe (2000) have discovered a "membrane" binding site in cultured breast cancer cells. Of particular importance to this thesis, DHP suppressed focal and generalized seizures in previous studies in amygdala-kindled rats. This seizure suppression by DHP in both male and female rats was not associated with behavioral sedation or ataxia (Lonsdale & Burnham, 2003; Lonsdale et al, 2006). The mechanism of DHP's anticonvulsant effects is unknown.

1.7.4 5 α ,3 α -tetrahydroprogesterone (THP)

The anticonvulsant effects of progesterone are generally attributed to its 3 α ,5 α -reduction to THP (Frye et al, 2002; Reddy et al, 2004; Saalman et al, 2007; Herzog, 2008a; Finocchi & Ferrari, 2011). THP has anticonvulsant actions because it binds to the GABA_A receptor. THP binds to both synaptic and extrasynaptic GABA_A

receptors on the post-synaptic membrane. GABA itself has high efficacy at synaptic receptors, but low efficacy at extrasynaptic receptors. THP, however, has a very strong effect on extrasynaptic GABA_A receptors. THP increases channel open time (<1 μ M) or opens the channel directly (>1 μ M). THP can reduce neuronal excitability 20-fold more than benzodiazepines, and 200-fold more than barbiturates at similar doses (King, 2013). As such, THP is sedative at higher anticonvulsant doses. (Belelli & Lambert, 2005; Belelli et al, 2009, throughout). The GABA_A receptor binding site for THP is not yet known, but it is known that THP's binding site is distinct from the benzodiazepine and barbiturate binding sites (Hosie et al, 2006, 2007; Lambert et al, 2009; Belelli et al, 2009). Since THP can increase GABA_A channel open time or open the channel directly, there are hypothesized to be at least 2 binding sites for neurosteroids in GABA_A receptors to mediate these two different effects (Walker & Kullman, 2012). THP is hypothesized enhance GABA binding to α and β subunits of the GABA_A receptor (King, 2013). The GABA-modulatory effect of THP promotes the open state of GABA-gated ion channels to allow more chloride into a neuron, increasing inhibition (Belelli et al, 2009).

As noted above, when GABA binds at synaptic GABA_A receptors, THP promotes the open state of channel. This enhances a transient or phasic inhibitory postsynaptic event, transiently increasing inhibition. GABA_A receptors that mediate phasic inhibition usually contain $\alpha\beta\gamma$ subunits (Belelli et al, 2009). When THP binds extrasynaptic GABA_A receptors, it allosterically modulates them such that they increase tonic inhibitory conductance (Walker & Kullman, 2012). The extrasynaptic GABA_A receptor is activated by GABA "spillover" from the synapse. Extrasynaptic

GABA_A receptors have high affinity, but low efficacy, for GABA. The extrasynaptic GABA_A receptors contain the relatively rare subunits: $\alpha 4$ and/or $\alpha 6$, and δ (Belelli et al, 2009). The δ subunit is very sensitive to low ambient levels of GABA, and is also particularly sensitive to THP-mediated inhibition. Of note, dysfunction of GABA_A receptors with $\alpha\beta\delta$ subunits in particular is associated with idiopathic generalized epilepsy (Belelli et al, 2009). It is hypothesized that THP also potentiates the efficacy of GABA at these extrasynaptic receptors. The anticonvulsant effects of THP are generally attributed to its enhancement of persistent tonic inhibition in the CNS (Belelli & Lambert, 2005; King, 2013).

THP is generally devoid of progesterone's hormonal effects, but it does have many effects on neural function (King, 2013). THP can modulate the release of specific neurotransmitters, by unknown mechanisms, in a region-specific manner. For example, in the pre-optic area of the hypothalamus, THP affects NMDA receptors to increase luteinizing hormone releasing hormone (LHRH) release by an unknown mechanism (Giuliani et al, 2011). In the medial frontal cortex, THP can inhibit the L-type calcium channel, resulting in decreased presynaptic glutamate release in vitro via an unknown mechanism (Hu et al, 2006). Hence, THP can decrease excitation by blunting glutamatergic excitation, as well as by enhancing GABAergic inhibition (Puia et al, 2012). Pathological alterations in THP levels, therefore, could have profound effects on excitatory and inhibitory signaling in the CNS and PNS. It has been suggested that altered THP levels are associated with mental health disorders, including depression and anxiety, the two most common comorbidities of epilepsy (Schüle et al, 2011; 2014).

In healthy women, changes in progesterone levels during the menstrual cycle result in subsequent changes in THP levels. Catamenial seizure exacerbation is due to pathophysiological decreases of progesterone, and hence, THP (Reddy, 2009). This decrease in THP significantly diminishes both the phasic and tonic inhibition in the brain that is mediated by GABA_A receptors.

1.7.5 The Progesterone Metabolizing Enzymes *5 α -Reductase (5 α -R)* and *3 α -hydroxysteroid oxidoreductase (3 α -HSOR)*

Both *5 α -reductase* and *3 α ,5 α -hydroxysteroid oxidoreductase (3 α -HSOR)* are widely distributed in human and rodent brains. They occur in both neurons and glia, in a regionally dependent manner (Mellon, 2001; Do Rego et al, 2009; Pelletier, 2010; King, 2013). They are considered promising targets for the treatment of steroid-related diseases. Interestingly, both *5 α -reductase* and *3 α ,5 α -HSOR* enzymes are differently distributed in humans with drug-refractory temporal lobe epilepsy (Stoffel-Wagner, 2003). For the purposes of this thesis, the hippocampal and cortical distributions of these enzymes will be discussed in detail.

The levels of *5 α -reductase* vary in different regions of the mouse brain (Roselli & Snipes, 1984; Do Rego et al, 2009). Areas high in *5 α -reductase* mRNA include the hippocampal cornu ammonis (CA) areas CA3, CA1, and in the dentate gyrus, as well as in the cerebral cortex (Do Rego et al, 2009). Interestingly, in women with polycystic ovarian syndrome, a common comorbidity of catamenial epilepsy, there are significant genetic variants of *5 α -reductase* type I, the most common isoform in the brain (Graupp et al, 2011).

The 3α -*HSOR* enzymes are also known as the “AKR1C” enzymes. Different isoforms have unique distributions across tissues (Rižner & Penning, 2014). In the hippocampus, 3α -*HSOR* enzymes are particularly highly expressed (Steckelbroeck et al, 2010). Cytosolic isoforms function as dehydrogenases at the 3α position, preferentially metabolizing THP to DHP. Microsomal isoforms preferentially hydrogenate DHP at the 3α position to form THP (Chetyrkin et al, 2001; Belelli & Lambert, 2005). Since 3α -*HSOR* enzymes are highly expressed in the hippocampus, reduction of DHP to THP, or oxidation of THP to DHP, is likely to occur. Medroxyprogesterone acetate (MPA) and dexamethasone inhibit 3α -*HSOR* enzymes (Lee et al, 1999).

1.7.6 Blockade of 5α -reductase: Finasteride

Finasteride is a 4-azasteroid (17 β -(N-t-butyl)carbamoyl-4-aza- 5α -androst-1-en-3-one). **Fig 1.7** shows the molecular structure of finasteride. Finasteride is a competitive 5α -reductase inhibitor. It is widely used experimentally and in clinical settings.

In clinical applications, finasteride prevents the 5α -reduction of testosterone. Finasteride is used clinically for benign prostate hyperplasia as a chemoprotective agent, and for male pattern baldness (Rittmaster, 1994; Chaudhary & Turner, 2010; Ferraldeschi et al, 2013). Finasteride will prevent the formation of all steroids' 5α and $3\alpha,5\alpha$ -reduced metabolites (Finn et al, 2006). Hence finasteride also blocks the metabolism of progesterone to DHP, and, therefore, to THP (Finn et al, 2006; Brunton et al, 2008; Mukai et al, 2008;

Chaudhary & Turner, 2010). Finasteride, therefore, is frequently used in animal studies to distinguish the effects of progesterone from the effects of DHP and THP (Frye et al, 1998; Kokate et al, 1999; Ciriza et al, 2006; Reddy & Mohan, 2011; Reddy et al, 2012; Reddy & Ramanathan, 2012; Puia et al, 2012). In studies of premenstrual and postpartum dysphoric disorder, catamenial epilepsy, depression, and alcohol finasteride is often used to prevent the formation of 5 α - and 3 α ,5 α -reduced steroid metabolites that are antidepressant and anticonvulsant (Finn et al, 2006).

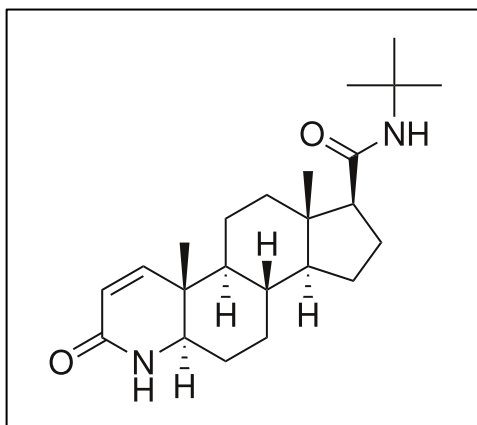


Figure 1.7 Molecular structure of finasteride. Wikimedia commons, <http://en.wikipedia.org/wiki/File:Finasteride.svg> Used with permission.

There are two *5 α -reductase* isoforms in rodents and humans, type I and type II. In the rodent CNS, type I is the dominant isoform. (Finn et al, 2006). In humans *5 α -reductase* type I is the most common isoform in brain, liver, muscle and skin. The type II *5 α -reductase* isoform is distributed in human prostate, seminal vesicle, epididymis, hair follicles, and liver. Finasteride is a classical competitive and reversible inhibitor of the type I *5 α -reductase* enzyme, the most common

isoform in the rodent and human CNS. Finasteride also binds irreversibly to type II *5 α -reductase*. Finasteride, therefore, binds to and irreversibly modifies the *5 α -reductase* isoform most common in the rodent and human periphery (Finn et al, 2006).

In rodents, finasteride binds with equal affinity to both type I and type II *5 α -reductase* isoforms. Unlike rodents, in humans, finasteride has 100 times higher affinity for type II *5 α -reductase* than it does for type I *5 α -reductase*. Finasteride's specificity for type II *5 α -reductase* in humans is hypothesized to be the reason, when used to treat benign prostate hyperplasia or male pattern baldness, that finasteride has few adverse effects in off-target organs, including the brain (Finn et al, 2006, throughout).

In an oft-cited case study, Herzog and Frye (2003) report a woman with drug-refractory catamenial epilepsy who achieved improved seizure control with supplemental progesterone therapy (in the "inadequate" luteal phase of her cycle). Her seizure control was lost when a dermatologist prescribed finasteride for rapidly progressing male pattern baldness, but was regained when finasteride treatment ceased (Herzog & Frye, 2003).

Of interest to this thesis, in the maximal electroshock (MES) model of generalized seizures, very high doses of progesterone were able to prevent the seizure measure (tonic hind-limb extension) despite finasteride pre-treatment. This might indicate an anticonvulsant effect of progesterone independent of its metabolism to THP (Kokate et al, 1999; Reddy et al, 2004).

1.8 Progesterone Receptors

Progesterone has both membrane and nuclear receptors, with the membrane receptors mediating rapid responses, and the nuclear receptors mediating slower responses based on changes in gene transcription. Membrane receptors also may have effects on nuclear gene transcription, and vice versa (Rupprecht et al, 1996; Carbajal et al, 2009). Both membrane and nuclear progesterone receptors are found in many different types of cells (Rupprecht et al, 1993; Brinton et al, 2008; Mani, 2008; Dressing et al, 2011), including neural cells, at varying receptor densities (King, 2013). Both nuclear and membrane progesterone receptors have been studied as possible contributors to the anticonvulsant effects of progesterone (Brinton et al, 2008).

1.8.1 Nuclear Progesterone Receptors

The classical nuclear progesterone receptor mediates downstream signaling cascades as well as gene transcription. There are at least three receptor isoforms, A, B, and C, which associate as dimers. When progesterone binds to dimerized nuclear progesterone receptors, gene transcription can occur (Lu et al, 2006; Rekawieki et al, 2011).

Classical nuclear progesterone receptors are not necessary for progesterone's acute anticonvulsant effects. Reddy et al (2004) found that nuclear progesterone receptor knock out mice were, in fact, more protected by high doses of progesterone (100-200 mg/kg) than wild-type mice. Reddy & Mohan (2004) also found, however, that the nuclear progesterone receptor may play a role in

epileptogenesis. The development of epileptogenesis in kindling model was impaired in mice lacking progesterone receptors. They hypothesized that this retarded kindling process was due to a significant role of nuclear progesterone receptors in hippocampal signaling, although exact molecular mechanisms remained unclear (Reddy & Mohan, 2004).

Another neurosteroid receptor has also been discovered in the nuclei of cells, and is under investigation. This receptor is not the “classical” nuclear progesterone receptor (above). It is known as the pregnane X receptor (PXR), or as the steroid and xenobiotic receptor (SXR). It binds promiscuously to steroids and their metabolites (Brinton et al, 2008).

1.8.2 Non-Nuclear Progesterone Receptors

In recent years, it has been discovered that progesterone can also bind to non-nuclear receptors. Multitudinous effects are mediated by these receptors (Lösle et al, 2008; Thomas, 2008; Brinton et al, 2008; King, 2013), and they are widely distributed in mammals (Dressing et al, 2011). Nomenclature for these receptors changed significantly as discoveries were made. Progesterone receptor membrane component 1 (PGRMC1) is a receptor that is now known to be the metabotropic sigma-2 receptor (σ -2 receptor; Ahmed, 2012). In the past, it had also been known as 25-Dx and IZAg (Krebs et al, 2000; Guennoun et al, 2008; Brinton et al, 2008; King, 2013).

The σ receptors are widely distributed in the CNS (Waarde et al, 2011). The σ receptors regulate ion channel signaling, steroid signaling, drug-metabolizing

enzymes, and tumor survival in cancerous cells. Progesterone is an antagonist at σ -2 receptors (Rohe et al, 2009). When progesterone binds to σ -2 receptors, it prevents ligand activation of both σ -1 and σ -2 receptors (King, 2013). When bound by progesterone, the excitatory effects of σ receptor-mediated modulation of voltage-gated ion channels is prevented (Johannessen et al, 2010).

Another progesterone receptor group is localized to cell membranes. They are known as “the G_i protein-coupled membrane-associated progesterone receptor (MAPR) family,” and are highly conserved in all eukaryotes (Cahill, 2007; Do Rego et al, 2009; King, 2013). This receptor is also referred to as: 1] membrane progesterone receptor (mPR) α , β , or γ ; 2] transmembrane progesterone receptors α , β , or γ (7TMPR α , β , or γ); or 3] progestin and adipoQ receptors (PAQR; Thomas, 2008). When bound to a progestin, these G-protein coupled receptors (GPCRs) block adenylyl cyclase activity to reduce excitation in the CNS (Thomas, 2008; Brinton et al, 2008; Do Rego et al, 2009; King, 2013).

In the brain, progesterone is thought to inhibit excitatory neurotransmission and increase inhibitory neurotransmission by its interaction with many neurotransmitter-binding sites, by unknown mechanisms. These neurotransmitter-binding sites include the transient receptor potential superfamily, sigma, glycine, muscarine, nicotine, serotonin, dopamine, oxytocin, and GABA_A receptors (Zheng, 2009). The many different functions of progesterone and its metabolites on receptor-mediated neurotransmission are areas of active investigation (King, 2013).

1.9 The GABA_A System

As noted above, one target for progesterone's metabolites is clearly known. In both normal and pathological brains THP is a powerful, positive allosteric modulator of the GABA_A receptor. Subunits of the GABA_A receptor determine its function and structure. As mentioned above, the δ -subunit-containing GABA_A receptor is a peri- or extrasynaptic receptor (Belelli & Lambert, 2005). GABA_A receptors with δ -subunits are insensitive to benzodiazepines (Uusi-Oukari & Korpi, 2010). Neurosteroids, however, are particularly strong activators of GABA_A receptors that contain δ -subunits (Spigelman et al, 2003; Belelli et al, 2006). There is reduced sensitivity to neuroactive steroids in mice that do not express the δ -subunit of GABA_A receptor (Mihalek et al, 1999). GABA_A receptor-modulating neurosteroids are under investigation for novel anticonvulsant drugs (Murashima & Yoshii, 2010).

Progesterone and THP can dynamically alter expression of the δ -subunit with shorter- and longer-term exposures (Follesa et al, 2004; Peng et al, 2004; Shen et al, 2005; Mostallino et al, 2006; Smith et al, 2007). GABA_A receptors, therefore, can powerfully increase tonic inhibition when exposed to neurosteroids (Stell et al, 2003; Lagrange, 2005; Glykys & Mody, 2007; Glykys et al, 2008; Belelli et al, 2009). Thus, progesterone fluctuations affect neuronal excitability, at least in part, by their effects on GABA_A receptor plasticity (Majewska et al, 1986; Harrison et al, 1987; Richerson, 2004; Belelli & Lambert, 2005; Belelli et al, 2006; Maguire & Mody, 2009; Gangisetty & Reddy, 2010).

GABA_A receptors' structure and function change in response to progesterone fluctuations, changing excitability in the brain in a region- and even neuron-specific manner (Paul & Purdy, 1992; Smith & Woolley, 2004; Belelli & Lambert, 2005; Lagrange, 2005; Akk et al, 2007; Herd et al, 2007; 2008; Uusi-Oukari & Korpi, 2010). In chronic models of epilepsy, GABA_A receptors' subunits are altered (Zhang et al, 2007). GABA_A subunit composition thus varies with both physiological and pathological conditions, such as menses, puberty, stress, pregnancy, depression, anxiety, and epilepsy (Purdy et al, 1991; Pirker et al, 2003; Sanna et al, 2004; Belelli & Lambert, 2005; Sethi & Harden, 2008; Maguire & Mody, 2007; 2009; Lambert et al, 2009; Smith, 2012; Schüle et al, 2011; 2014).

The endogenous “neurosteroid withdrawal” of THP occurs in certain points of the menstrual cycle when progesterone levels fall (**Fig 1.2**). This withdrawal lowers seizure threshold in a manner similar to alcohol or benzodiazepine withdrawal (Smith & Woolley, 2004). Women with catamenial epilepsy are particularly vulnerable to seizures at these times, at least in part due to reduced extrasynaptic GABAergic inhibition (Belelli & Lambert, 2005; Reddy, 2009). THP and the GABAergic inhibition it induces have been foci of catamenial epilepsy research (Pack et al, 2011b; Reddy, 2010; Lambert et al, 2009). Anticonvulsant effects of progesterone independent of its metabolism to DHP and THP, however, have not been rigorously assessed.

1.10 Other Potential Targets of Progesterone in the CNS

There are multiple targets of progesterone in the CNS, and a number of these are ion channels (King, 2013). Progesterone has been shown to inhibit voltage-dependent calcium, sodium and potassium channels with high specificity in vitro (Luoma et al 2011; Kelly & Mermelstein, 2011). As mentioned earlier, progesterone antagonizes σ receptors, blocking the downstream activation of excitatory ion channels (Johannessen et al, 2010).

Thus channels, as well as non-nuclear progesterone receptors, are potential targets for progesterone and related anticonvulsants. While it is certain that progesterone exerts anticonvulsant effects via its metabolite THP, other more subtle inhibitory effects of progesterone itself are also under study (King, 2013).

1.11 Previous Work on Progesterone, its Metabolites, and Analogs as Anticonvulsant Compounds

A large body of research has confirmed progesterone's anticonvulsant effects (Bayenburg et al, 2001; Lonsdale & Burnham, 2003; Smith & Woolley, 2004; Rhodes et al, 2004; Biagini et al, 2010; Reddy, 2010; King, 2013). Some of the most convincing data come from experiments involving animal models.

1.11.1 Animal Studies

The anticonvulsant activity of progesterone and its metabolites is well established in animal models of seizures and epilepsy (Reddy, 2010), including the pilocarpine-induced seizure model, the NMDA-induced status epilepticus model

(Kokate et al, 1996), the pentylenetetrazol model (Kokate et al, 1999; Reddy et al, 2004; Rhodes & Frye, 2004), the maximal electric shock model (Kokate et al, 1999; Reddy et al, 2004), and the amygdala-kindling model (Lonsdale & Burnham, 2003; Reddy et al, 2004; Lonsdale et al, 2006; Lonsdale & Burnham, 2007). The present thesis focuses on the effects of progesterone in kindled seizures.

1.11.1.1 Progesterone and Kindled Seizures

In female rats, previous kindling studies in our laboratory have found that, at a pharmacological dose of 120 mg/kg, progesterone suppressed ~50% of focal, and 100% of generalized seizures approximately 60 minutes post progesterone injection (Lonsdale & Burnham, 2003). About 20% of female animals had significant sedation at 60 minutes post-injection, and more sedation was seen at later time points. Since these sedative effects mostly occurred later than the anticonvulsant effects, it was hypothesized that the primary progesterone metabolite DHP might be anticonvulsant without sedation, whereas THP might be inherently sedative at anticonvulsant doses (Lonsdale & Burnham, 2003).

Subsequently, kindling studies in our laboratory were conducted in male rats. Lonsdale et al (2006) found that at a pharmacological dose of 160 mg/kg, progesterone suppressed ~35% of focal seizures and 100% of generalized seizures at about 30 minutes post injection. Also, as early as at 30 minutes post progesterone injection, ~40-50% of male subjects showed ataxia (Lonsdale et al, 2006).

1.11.1.2 DHP and Kindled Seizures

DHP has not been as well characterized in the literature as progesterone or THP. Our laboratory has previously found DHP to be anticonvulsant in male and female rats in the amygdala-kindling model. Seizure suppression occurred without obvious adverse sedative side effects (Lonsdale & Burnham 2003; Lonsdale et al, 2006). In male rats, 7.5 mg/kg of DHP suppressed over 50% of focal and generalized seizures without ataxia (Lonsdale et al, 2006). In female animals, focal and generalized seizure-suppressing doses differed; 60% of focal seizures were suppressed by 5 mg/kg of DHP (projected focal ED₅₀=4.3 mg/kg), and 100% of generalized seizures were suppressed by 5 mg/kg of DHP (generalized ED₅₀=2.9 mg/kg). DHP did not produce sedation or ataxia in female or male rats at anticonvulsant doses (Lonsdale & Burnham, 2003; Lonsdale et al, 2006).

1.11.1.3 THP and Kindled Seizures

Numerous studies have demonstrated the potency of THP as an anticonvulsant (Reddy, 2010). Previous studies in our laboratory with amygdala-kindled rats found no suppression of focal seizures by THP, but 100% of generalized seizures were suppressed at 30 mg/kg in females (Lonsdale & Burnham, 2007) and males (Lonsdale et al, 2006). At 30 mg/kg, THP produced ataxia in 75% of the subjects tested (Lonsdale et al, 2006).

1.11.1.4 Ganaxolone Studies

An analog of THP, ganaxolone, is showing some therapeutic promise in animal models, but like THP, is sedative (Nohria & Giller, 2007). Investigators have

found ganaxolone to be anticonvulsant in many seizure models in vivo that respond to GABAergic drugs (Gasior et al, 2000; Reddy & Rogawski, 2010), including pentylenetetrazol kindling, bicuculline, and amygdala kindling.

In amygdala kindled animals, ganaxolone was as effective as clonazepam. Neither drug, however, suppressed partial seizures (Reddy & Rogawski, 2010). In the pentylenetetrazol model, Gasior et al (2000) found that ganaxolone had anticonvulsant-like effects, and was not as sedative as valproate and diazepam. Reddy & Rogawski (2010) do not report sedation of ganaxolone relative to seizure suppression, although Nohria & Giller (2007) reported that ganaxolone was sedative in animal models. The advantage of ganaxolone compared to benzodiazepines in animal studies is that tolerance does not seem to develop (Reddy & Rogawski, 2010). Like other drugs that enhance GABA_A receptor function, ganaxolone exacerbates absence seizures (Snead, 1998).

1.11.2 Human Studies

1.11.2.1 Progesterone

Progesterone-based hormonal therapies for epilepsy are currently under investigation (Harden et al, 2006; Herzog, 2009; Reddy & Rogawski, 2009; Reddy, 2009; Reddy 2010; Stevens & Harden, 2011; Herzog et al, 2012; Najafi et al, 2013). Pharmacological treatments with natural progesterone or the synthetic progestins, including ganaxolone, have been investigated, albeit in very small sample sizes (Belelli & Lambert, 2005; Nohria & Giller, 2007; Reddy, 2009; Reddy & Rogawski,

2009; Reddy & Rogawski, 2010; Pack, 2010; Stevens & Harden, 2011; Zorumski et al, 2012). Some smaller studies of progesterone supplementation have found it to be effective (Motta et al, 2013; Najafi et al, 2013). The first large, NIH-sponsored phase 3 clinical trial recently investigated the treatment of catamenial epilepsy with natural progesterone. It found that progesterone supplementation treatment was ineffective in women with intractable focal epilepsy. A subset of women with perimenstrual (C2) seizure exacerbation, however, was somewhat responsive to treatment (Herzog et al, 2012).

Neurosteroid replacement therapies for catamenial epilepsy, however, can have undesirable hormonal effects. Endogenous variations in the inter-individual metabolism of progesterone to DHP and THP may influence treatment outcomes (Harden, 2008; Reddy & Rogawski, 2009; Steven & Harding, 2011).

1.11.2.2 DHP, THP and Ganaxolone

No large-scale clinical studies of the anticonvulsant efficacy of DHP and THP have ever been conducted. The THP analog ganaxolone, however, has shown some promise as an anticonvulsant drug in ongoing human trials (Nohria & Giller, 2007; Biagini et al, 2010; King, 2013; Mula, 2013), particularly for women with catamenial epilepsy (Reddy & Rogawski, 2010). The most commonly reported side effects in humans are somnolence and diarrhea (Nohria & Giller, 2007).

1.12 Objectives

1.12.1 General Objectives

Our objectives in the present studies were three-fold: 1] to investigate the potential psychotoxicity of anticonvulsant doses of DHP and THP; 2] to investigate the effects of progesterone and its metabolites in kindled mice; and 3] to investigate the effects of progesterone and its metabolites in vitro in the entorhinal cortex.

This thesis, therefore, has three main components: 1] Behavioral studies in naïve (unkindled) male and female rats (chapter 2); 2] electrophysiological studies in hippocampal-kindled male mice in vivo (chapter 3); and 3] electrophysiological studies of entorhinal brain slices of naïve male mice in vitro (chapter 4). The specific objectives of each component are detailed below.

1.12.2 Objectives of the Behavioral Studies

A major focus of our laboratory is the investigation of treatments for drug-refractory focal seizures. Previous studies found DHP and THP to be effective anticonvulsants in male and female rats in the amygdala-kindling model of drug-refractory seizures of partial onset (Lonsdale & Burnham, 2003; Lonsdale et al, 2006). This suggested that these metabolites might be developed as anticonvulsants. Novel anticonvulsants, however, should have minimal side effects. Thus we investigated potential toxicities of anticonvulsant doses of DHP and THP in models of learning and memory, depression, anxiety, and locomotion in male and female rats.

1.12.3 Objectives of the Studies in Hippocampal-Kindled Subjects

To further characterize the anticonvulsant effects of progesterone and its metabolites, electrophysiological studies were conducted in hippocampal kindled male mice. Mice were used so that future molecular/transgenic studies based on these experiments could be conducted to determine possible mechanisms of action.

Experiments were conducted in the presence and absence of finasteride, such that the effects of progesterone could be separated from the effects of DHP and THP. Unexpectedly, anticonvulsant effects of high doses of progesterone were discovered in the presence of finasteride in our experiments. Therefore, *in vivo*, we sought to characterize these effects in a mouse model of drug-refractory, partial seizures (Albright & Burnham, 1980).

1.12.4 Objectives of Studies in Entorhinal Slices

To further characterize the non-GABAergic effects of progesterone, electrophysiological studies were conducted in naïve male mouse brain slices *in vitro*, using standard epileptiform preparations (Heinemann et al, 2006), in the presence of the GABA_A receptor antagonist picrotoxin.

Chapter 2: Behavioral Studies of Progesterone

Metabolites

This experimental series was conducted in the laboratory of Dr. Mac Burnham, my supervisor. In the present chapter, I recorded data, conducted experiments, performed statistical analyses while “blinded” to treatment groups, and subsequently built figures.

With Dr. Deborah Lonsdale, I prepared the experimental apparatuses and video recording equipment. Dr. Lonsdale and I conducted the behavioral experiments and animal handling together with project students.

Special thanks to project students Deepali Bhatta, Sneha Patel, Frank Wang, and Yen-an Xu.

Papers submitted for peer-reviewed publication are in **Appendix 1** and **Appendix 2**.

2.1 Introduction

As mentioned previously, studies in our laboratory found the progesterone metabolites DHP and THP to be anticonvulsant in male and female rats in the amygdala kindling model of epilepsy. The progesterone metabolites DHP and THP induced no obvious sedation or ataxia at anticonvulsant doses (Lonsdale & Burnham, 2003; Lonsdale et al, 2006; Lonsdale & Burnham, 2007). It seemed possible that advanced behavioral tests could reveal potential psychotoxicities missed by the simple ataxia/sedation rating scales.

The purpose of the behavioral studies in this thesis was to determine whether anticonvulsant doses of DHP and THP had psychotoxic effects. We conducted studies of anticonvulsant doses of DHP and THP on the following: 1] depression and locomotion, using the forced swim test (FST) and open field test (OFT), respectively, 2] learning and memory in the Morris water maze (MWM), and 3] anxiety in the elevated plus maze (EPM), in male and female rats.

2.2 General Methods

2.2.1 Subjects

In the behavioral studies we used naïve rats that were not implanted, kindled or altered, genetically or otherwise. They were normal rats that did not model epilepsy or seizures in any way. In this respect, our behavioral studies measure psychotoxicity per se, rather than toxicity in animal epilepsy models.

The Animal Care Committee (Faculty of Medicine) of the University of Toronto approved all protocols. Adult male and female Wistar rats (Charles River, Quebec, Canada) were housed individually in 24 x 24 x 45 cm transparent, plastic cages. Subjects were allowed free access to food and water. The vivarium was kept at a constant temperature of 21°C and maintained on a 12-hour light/dark cycle (lights on at 7:00 a.m.). Rats were 60 days old on the date of delivery to the vivarium and approximately 90 days old at the time of behavioral testing. Different subjects were used for each experiment.

The experimental room had low light levels from indirect sources, and was kept quiet. Animals were allowed to acclimatize to the experimental room in their home cages for at least thirty minutes on each testing day before experimental procedures began. All tests were conducted between 9 am and 4 pm.

2.2.2 Drugs

DHP, THP, and the vehicle β -cyclodextrin were obtained from Sigma-Aldrich Canada Ltd (Oakville, Ontario). All drugs were dissolved in a 45% solution of β -cyclodextrin in physiological (0.9%) saline. Fresh solutions of vehicle and drugs were made for each day of drug testing. As determined by previous experiments, the interval between injection and testing was 15 minutes for DHP, THP and vehicle in male and female animals (Lonsdale & Burnham, 2003; Lonsdale et al, 2006; Lonsdale & Burnham, 2007). All injections were intraperitoneal (i.p.).

In the MWM, each animal was given the same drug treatment on day 1 and day 4. In the FST, OFT, and EPM, each animal was given only one drug treatment. In the FST, OFT, and MWM 5 mg/kg was the only dose of DHP and THP given. The 5 mg/kg dose is within the anticonvulsant dose range, but not the ataxic range, for males and females in our previous kindling experiments (Lonsdale & Burnham, 2003; Lonsdale et al, 2006; Lonsdale & Burnham, 2007). In the EPM, both DHP and THP were tested not only at 5 mg/kg, but also at 7.5 mg/kg and 10 mg/kg (higher doses of DHP could not be tested due to solubility problems). All vehicle control treatments were matched in volume to the largest drug treatments. One investigator (DL) prepared and coded all drugs; a second investigator, blinded to treatment (MJ), conducted statistical analyses. The drug codes were only broken when analyses were complete.

2.2.3 Handling Procedure

Each animal was gently handled for 5 minutes each weekday for four weeks before the experiments began. The purpose of this handling paradigm was to match the amount of human contact that the animals had received in previous kindling experiments.

2.2.4 Statistics

SPSS Statistics versions 20 or 21 were used for all data analyses. Parametric data were analyzed using one-way ANOVAs (ie, comparing 3 drug treatments) or two-way ANOVAs (ie, comparing doses and drugs in the EPM) by an experimenter blinded to the treatment groups. When appropriate, pairwise comparisons were made using Tukey's honest significant difference (HSD) *post hoc* test. Non-parametric data were analyzed for the MWM only. These data were analyzed using the independent samples Kruskal-Wallis test. All tests were 2-tailed, with $p < 0.05$ considered significant. Figures show mean $\pm$ SE.

2.2.5 Forced Swim Test (FST) and Open Field Test (OFT)

The FST, also known as the "Porsolt test," is a drug-validated animal model of acute, depression-like behavior in rodents (Porsolt et al, 1978). The FST is used to screen novel antidepressant compounds (Cryan et al, 2002). This model responds to a variety of depressant and antidepressant compounds (Borsini & Meli, 1988; Cryan et al, 2005). The FST apparatus consisted of a clear plastic cylindrical apparatus 45 cm high and 20 cm wide (Geneq Inc., Quebec, Canada). The apparatus

was filled with water (25° C) to a level of 27 cm, such that animals could not either touch the bottom or escape.

The FST was used in the present experiments because THP has been reported to have both antidepressant-like and depressant-like effects (Walf & Frye, 2006; Reddy, 2010; Schüle et al, 2011; 2014). The purpose of this experiment was to assess whether anticonvulsant doses of DHP and THP have effects with respect to mood and/or affect. Mood was assessed by comparing the amount of time the animal struggles to escape from the forced swim apparatus to the amount of time it floats in an “immobile” position (Porsolt et al, 1978; Bogdanova et al, 2013). To ensure that any results are not contaminated by a drug’s effect on movement capability, locomotion is tested in an open field apparatus (Walsh & Cummins, 1976; Cryan et al, 1999; Bogdanova et al, 2013). We therefore tested anticonvulsant doses of DHP and THP in the forced swim and open field apparatuses.

The FST protocol occurred over two days. On the first day, in the absence of drug, the animal was placed in the swimming apparatus for ten minutes without any treatment (training day). On the following day (test day), the animal was injected i.p. with 5 mg/kg of DHP, THP or with volume-matched vehicle (the injection-test interval was 15 minutes for both male and female animals). Fifteen minutes after injection, the animal was placed in the swimming apparatus for 5 minutes. Active (swimming) and passive (immobile or floating) behaviors were measured using a stopwatch by an experimenter who was blinded to the treatment groups. Following each exposure to the FST apparatus, animals were gently dried and placed under a heat lamp before being returned to their home cages.

The OFT apparatus was constructed of ¼ inch thick plywood. The apparatus had a 100 x 100 cm base and 60 cm high walls. It was illuminated by a 60-Watt light bulb, positioned 1.5 meters above the apparatus floor. The interior of the apparatus was painted white with 10 x 10 cm squares outlined in black on the floor, and was varnished for cleaning purposes. The apparatus was cleaned between subjects with dilute Virox.

Fifteen minutes after an i.p. injection of vehicle, DHP or THP, the animals were placed in the northwest corner of the OFT apparatus. Using Labview video and motion-tracking software, the following data were collected: horizontal speed, distance, and velocity, vertical activity (rearing). Labview recordings of the OFT were subsequently scored by an experimenter blinded to treatment (M)].

2.2.6 Morris Water Maze (MWM)

The MWM “matching to place” paradigm is a behavioral test of learning and memory. It is used to determine the acute effects of a drug on both new and well-learned tasks. The task involves learning to find a submerged platform, which is hidden under the surface of the water. Various forms of learning and memory can be assessed in the presence and absence of drug (Schwabe et al, 2007). The present study was designed to assess whether anticonvulsant doses of DHP or THP affected learning or memory. Drug and vehicle treatments were given on day 1 and day 4 (the injection-test interval was 15 minutes for both male and female animals).

The MWM apparatus is a cylindrical white polypropylene pool (158 cm diameter, 40 cm depth) with no seams or graphical cues. It was filled with water to

a depth of 28 cm. The animals must find a hidden circular platform to escape the water (26.5 cm from the bottom of the pool, 1.5 cm below the surface of the pool, 14 cm in diameter). Non-toxic tempura paint is mixed in the water to ensure opacity, such that the submerged platform is not visible. Four different, visible cues are placed on the walls of the experimental room outside of the pool, designated N, S, E, or W. Each quadrant of the pool where the platform was placed is designated as NW, NE, SE, or SW. The platform location was moved to different quadrants of the pool between days 1 and 2, 2 and 3, and 3 and 4, but on day 5 stayed in the same position as on day 4. Also on day 5, the first 4 trials were to the submerged platform as in all previous trials, but the platform was raised 2 cm above the water level for the last 4 trials. All trials were recorded with Labview video and motion-tracking software.

In the present study, on each of the 5 days of testing, each animal had to locate the submerged platform over eight trials. A trial was 60 seconds long. In each trial, the animals had to swim from different start positions to a constant platform position. For example, when the platform is located in the “NE” corner of the pool, the start positions for the eight trials were N, S, E, W, N, S, E, W. When the animal found the platform, it was left to stand on the platform for 5 seconds before it was removed from the pool. If the animal did not find the platform in 60 seconds, it was gently guided to it, and was left standing on the platform for 5 seconds before being removed from the apparatus.

The distance and velocity an animal travelled in each trial were averaged for analysis on days 1-4. On day 5, subgroupings of trials were averaged (trials 1-2;

3-4; 1-4; 5-8) for analysis. A trial was considered over when an animal reached the platform or the 60 second trial ended.

Using a modification of the MWM protocol of Schwabe et al (2007), we assessed the following measures: 1] the initial acquisition of memory (average latency day 1 all trials with drug, average latency day 5 trials 5-8 when the platform was raised, no drug); 2] working and reference memory (the effect of drug treatment on average latency in a well-learned task, day 4); 3] spatial memory, the average latency to the spatially known platform on day 5, trials 1-4; 4] comparing the average velocity of the animals in all trials in the presence (day 1, trials 1-8) and the absence (day 5, trial 1-4) of drug treatment. There are several measures of learning and memory that can be taken in the Morris Water maze (MWM).

Discussion of the different measures is presented below.

We used two measures to assess the animals' ability to learn a new task in the presence of drug or vehicle on day 1. The first was the average latency to platform over all trials in day 1 (**Fig 2.7**, males; **Fig 2.8**, females). The second measure of ability to learn a new task in the presence of drug was the thigmotaxic response on day 1 (**Fig 2.9** males; **Fig 2.10** females). This measure takes advantage of the animals' natural tendency circle to perimeter of the pool (thigmotaxis) until it learns to cross the pool to find the platform. Working memory data analyzed included latency to platform on day 4 (**Fig 2.13**, males; **Fig 2.14**, females). Another measure of working memory was whether or not the animal found the platform within 60 seconds in each trial after the first trial in which they found the platform

on their own (**Fig 2.15**, males; **Fig 2.16**, females), ie, without an experimenter guiding them to the correct platform location when the 60 second trial had ended.

Two measures of reference memory were taken on day 4; whether or not the animals swam directly to the platform position from the previous day (**Fig 2.15** males; **Fig 2.16**, females), and the number of times the animal swam over day 3's platform position in the first trial of day 4 (**Fig 2.17**, males; **Fig 2.18**, females). On day 5 another measure of reference memory was taken. Since the platform did not move between days 4 and 5, we assessed whether or not the animals swam directly, in trials 1-4 on day 5, to the platform position (data not shown). This is a measure of the effect of drug on both on the acquisition and recollection of the platform position learned the previous day. Velocity in the presence of drug is measured on day 1 (**Fig 2.19**, males; **Fig 2.20** females) and day 4 (**Fig 2.21**, males; **2.22**, females). Velocity is also measured in the absence of drug on day 2 (**Fig 2.23**, males; **Fig 2.24** females), day 3 (**Fig 2.25**, males; **Fig 2.26**, females), and day 5, trials 1-4 (**Fig 2.27**, males; **Fig 2.28**, females). Another measure of velocity was made on day 5, in the absence of drug; velocities of animals in trials 3-4 (submerged platform) and 5-8 (raised platform) were compared (data not shown).

There are several measures in the Morris water maze that can be used to assess how drug affects the acquisition and recall of spatial memory (Schwabe et al, 2007). For the recall of spatial memory, latency measures were made on day 5 (24 hours after drug treatment) when the platform was submerged and its position was unchanged from day 4. We measured latency to platform using the mean of all trials 1-4 on day 5 as a rough measure of spatial memory recall in males (**Fig 2.31**) and

females (**Fig 2.32**) in the absence of drug treatment. This spatial memory measure was designed to assess the effects of drug on recalling the submerged platform position from the previous day. The effects of drug on spatial memory can also be assessed using latency measures from trial groups 1-2 and 3-4 on day 5. Latency to platform in trials 1-2 models spatial memory from day 4 to day 5, since the platform on day 5 is in the same position as it was in day 4. Latency to a spatially known location is also measured on day 5 in trials 3-4.

2.2.7 Elevated Plus Maze (EPM)

Potential anxiolytic/anxiogenic effects were evaluated in the EPM, a sensitive behavioral test of anxiety in rodents (Lister, 1987; Reddy & Kulkarni, 1996; Hogg, 1996). The EPM apparatus consists of an elevated, plus symbol-shaped, black Plexiglas runway elevated 80 cm from the floor. Two of the opposing arms are open, with slightly raised edges (0.5 cm). The other two opposing arms have 40 cm high black Plexiglas walls. All arms are 50 cm long and 10 cm wide.

The EPM test presents the animals with a choice between two conflicting, inherent rodent behaviors: aversion to open spaces, and exploration of novel areas. Anxious animals avoid the open spaces (Hogg, 1996). Anxiolytic drugs tend to increase the proportion of time rodents spend in the open arms and/or increase the proportion of open arm entries. The purpose of the EPM in the present study was to determine whether anticonvulsant doses of DHP and THP changed the number of open arm entries and/or the time spent in the open arms (the injection-test interval was 15 minutes for both male and female animals).

The percentage of time spent in the open arms and the percentage of open arm entries (open arm entries/total arm entries) were determined for the 5-minute test period, and recorded by Labview video and motion-tracking software to provide estimates of overall locomotor activity. An entry was scored when all four of the animals' paws entered an arm.

2.3 Results

2.3.1 The Forced Swim and Open Field Tests

FST data are presented in **Fig 2.1** for male animals and **Fig 2.2** for female animals. Active and passive behaviors in the FST were very similar for vehicle-, DHP-, and THP-treated male and female animals. No significant differences were found (ANOVA $p>0.05$)

For the OFT, behavior was similar in all treatment groups and no significant differences were found (ANOVA $p>0.05$). Horizontal (distance travelled) and vertical activity (rearing) in the OFT are represented in **Fig 2.3** and **2.5** for male animals, and **Fig 2.4** and **2.6** for female animals, respectively. A statistical summary for the FST and OFT experiments is presented in **Table 1**.

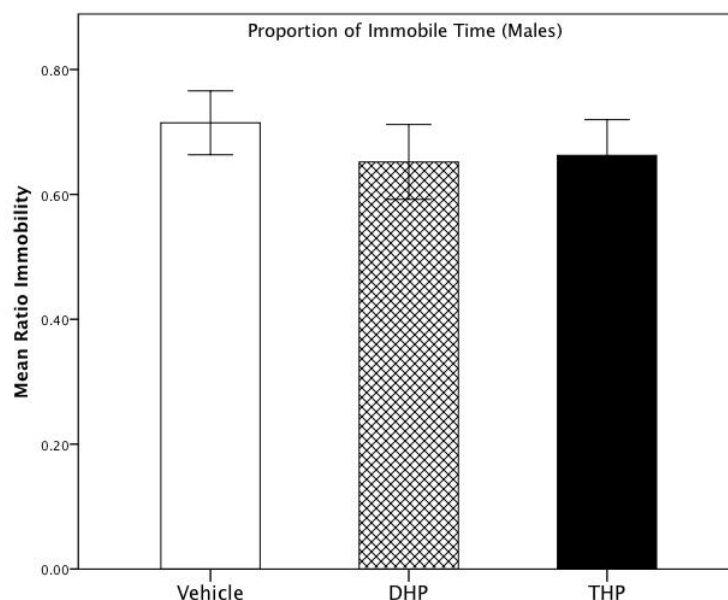


Figure 2.1 Male immobility ratio in the forced swim test.

The y-axis measures the ratio of immobility time to swimming time over the 5-minute test period. There were no significant differences related to the type of drug treatment (ANOVA, $p > 0.05$, $n = 12$ per group). White bars, vehicle; hatched bars, DHP; black bars, THP. Mean $\pm$ SE.

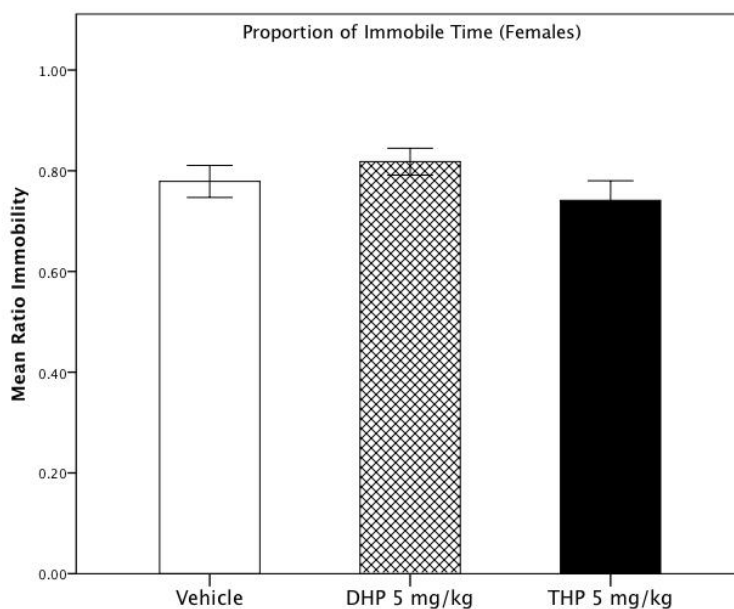


Figure 2.2 Female immobility ratio in the forced swim test.

The y-axis measures the ratio of immobility time to swimming time over the 5-minute test period. There were no significant differences related to the type of drug treatment (ANOVA, $p > 0.05$, $n = 12$ per group). White bars, vehicle; hatched bars, DHP; black bars, THP. Mean $\pm$ SE.

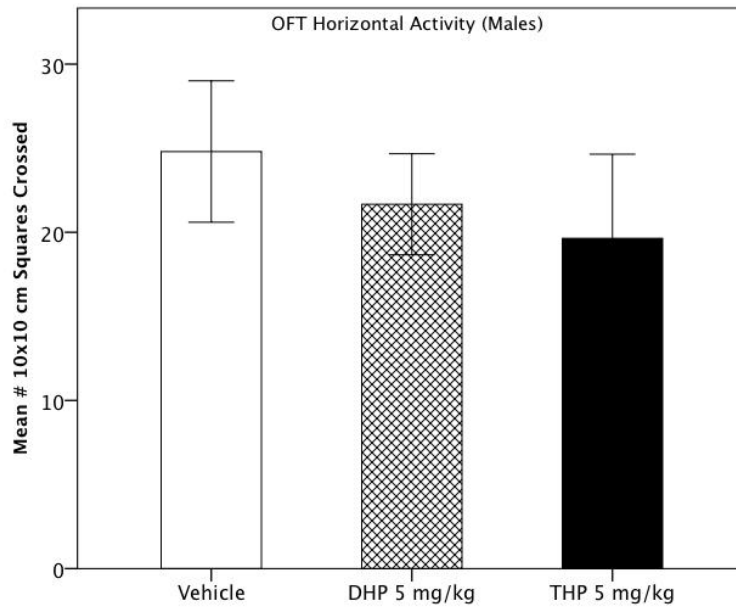


Figure 2.3 Horizontal activity (distance travelled) by males in the open field test. The y-axis represents the number of 10x10cm squares crossed in the apparatus over the 5-minute test period. There were no significant differences related to the type of drug treatment (ANOVA, $p>0.05$, $n=12$ per group). White bars, vehicle; hatched bars, DHP; black bars, THP. Mean $\pm$ SE.

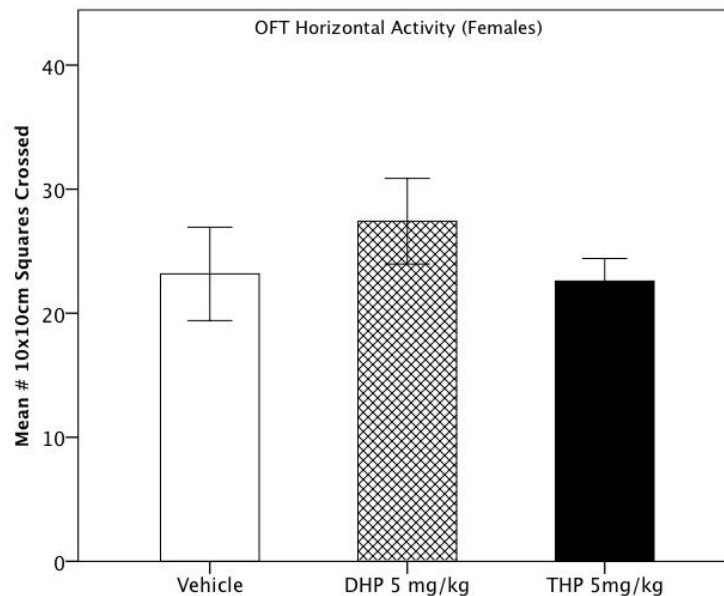


Figure 2.4 Horizontal activity (distance travelled) by females in the open field test. The y-axis represents rearing incidents over the 5-minute test period. There were no significant differences related to the type of drug treatment (ANOVA, $p>0.05$, $n=12$ per group). White bars, vehicle; hatched bars, DHP; black bars, THP. Mean $\pm$ SE.

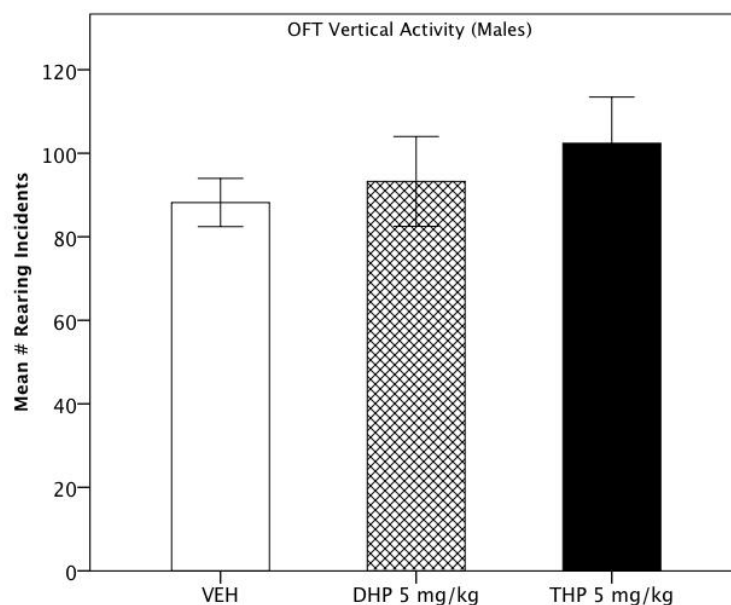


Figure 2.5 Vertical activity (rearing) by males in the open field test. The y-axis represents the number of times the animal reared on hindlimbs over the 5-minute test period. There were no significant differences related to the type of drug treatment (ANOVA, $p > 0.05$, $n = 12$ per group). White bars, vehicle; hatched bars, DHP; black bars, THP. Mean $\pm$ SE.

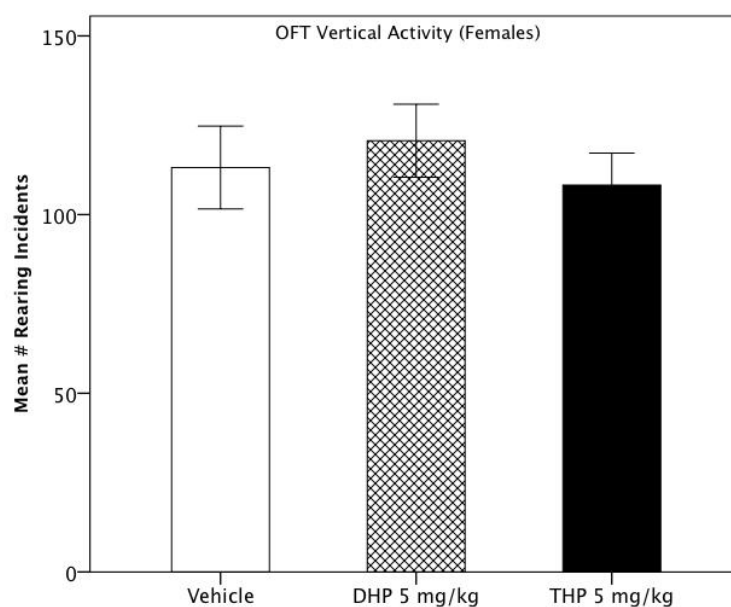


Figure 2.6 Vertical activity (rearing) by females in the open field test. The y-axis represents number of times the animal reared on hindlimbs over the 5-minute test period. There were no significant differences related to the type of

drug treatment (ANOVA, $p>0.05$, $n=12$ per group). White bars, vehicle; hatched bars, DHP; black bars, THP. Mean $\pm$ SE.

<i>FST/OFT Measure</i> (n=12 per treatment group)	<i>Male Statistic</i> ANOVA, p-value Vehicle: Mean $\pm$ SE DHP: Mean $\pm$ SE THP: Mean $\pm$ SE Figure	<i>Female Statistic</i> ANOVA, p-value Vehicle: Mean $\pm$ SE DHP: Mean $\pm$ SE THP: Mean $\pm$ SE Figure
FST Proportion of time immobile: Anti-depressant-like effect	ANOVA $p=0.702$ Vehicle: 0.715 ± 0.051 DHP: 0.652 ± 0.060 THP: 0.662 ± 0.057 Fig 2.1	ANOVA $p=0.260$ Vehicle: 0.715 ± 0.051 DHP: 0.652 ± 0.060 THP: 0.662 ± 0.057 Fig 2.2
OFT Horizontal activity (overall locomotion)	ANOVA $p=0.269$ Vehicle: 24.800 ± 4.298 DHP: 21.667 ± 3.924 THP: 19.636 ± 4.098 Fig 2.3	ANOVA $p=0.499$ Vehicle: 23.170 ± 3.762 DHP: 27.420 ± 3.448 THP: 22.580 ± 1.823 Fig 2.4
OFT Vertical activity / rearing (overall activity)	ANOVA $p=0.602$ Vehicle: 88.200 ± 10.294 DHP: 93.250 ± 9.397 THP: 102.364 ± 9.815 Fig 2.5	ANOVA $p=0.695$ Vehicle: 113.17 ± 11.597 DHP: 120.67 ± 10.201 THP: 108.250 ± 8.949 Fig 2.6

Table 1. Summary of results in the FST and OFT.

2.3.2 The Morris Water Maze

A summary of the entire Morris water maze experimental series is presented in table 2, at the end of section 2.3.2.

2.3.2.1 Ability to learn a new task

There were no significant differences related to the type of drug treatment in either the average latency to platform over all trials in day 1 (**Fig 2.7**, males; **Fig 2.8**, females). There were also no significant differences in average thigmotaxic response related to the type of drug treatment on day 1 (**Fig 2.9** males; **Fig 2.10** females). This may mean that anticonvulsant doses of DHP and THP do not affect the rats' ability to learn a new task.

2.3.2.2 Working and Reference Memory

Working and reference memory were measured on days 4 (working memory, average latency to platform, **Fig 2.11-2.14**) and 5 (reference memory, average latency to platform **Fig 2.15-2.18**). In male and female animals, drug treatment did not affect any measures of working or reference memory. In general, these results suggest that anticonvulsant doses of DHP and THP do not affect working and reference memory.

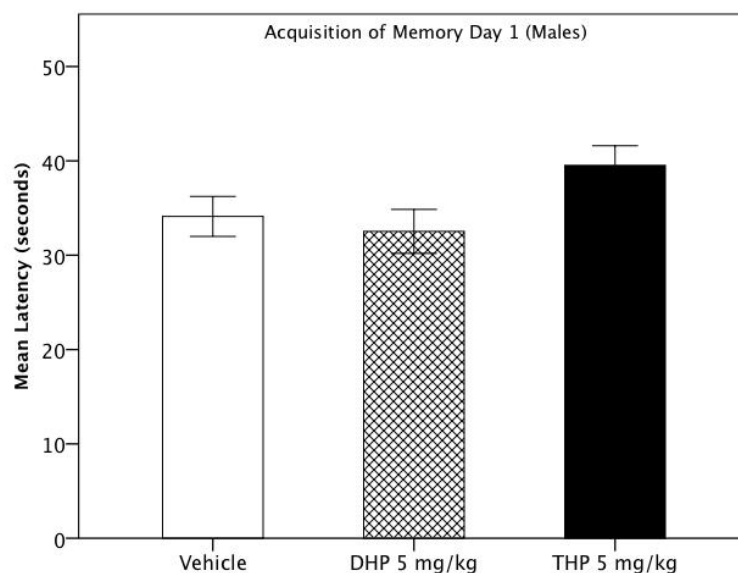


Figure 2.7 Acquisition of memory: average male latency to the platform across all trials on day 1. The y-axis represents the average amount of time, in seconds, taken to reach the platform across all 8 trials of day 1. There were no significant differences related to the type of drug treatment (ANOVA, $p > 0.05$, $n = 12$ per group). White bars, vehicle; hatched bars, DHP; black bars, THP. Mean $\pm$ SE.

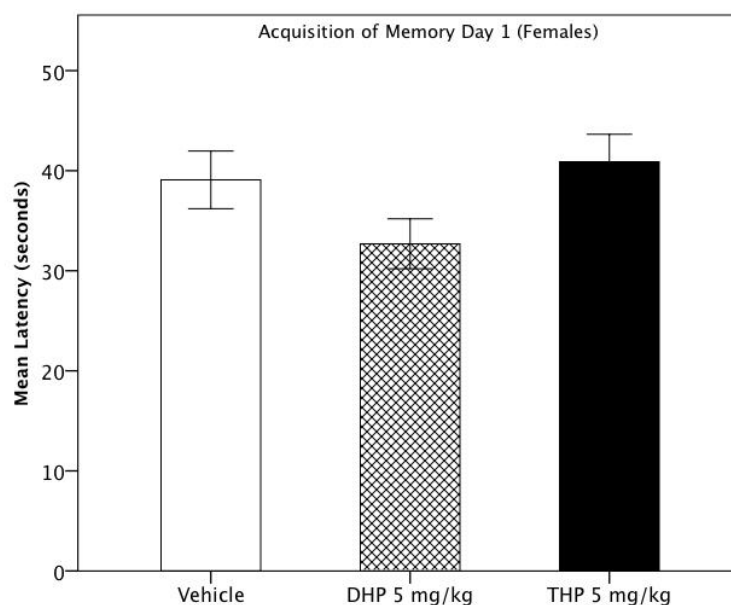


Figure 2.8 Acquisition of memory: average female latency to the platform across all trials on day 1. The y-axis represents the average amount of time, in seconds, taken to reach the platform across all 8 trials of day 1. There were no significant differences related to the type of drug treatment (ANOVA, $p > 0.05$, $n = 12$ per group). White bars, vehicle; hatched bars, DHP; black bars, THP. Mean $\pm$ SE.

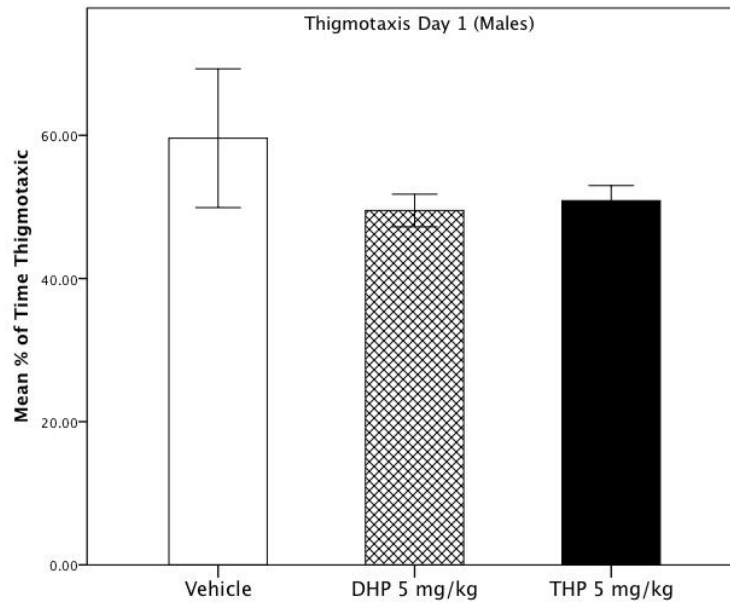


Figure 2.9 Learning a new task: thigmotaxic response of male animals on day 1. The y-axis represents the average percentage of time the animal spends circling the perimeter of the pool during all trials on day 1. There were no significant differences related to the type of drug treatment (ANOVA, $p > 0.05$, $n = 12$ per group). White bars, vehicle; hatched bars, DHP; black bars, THP. Mean $\pm$ SE.

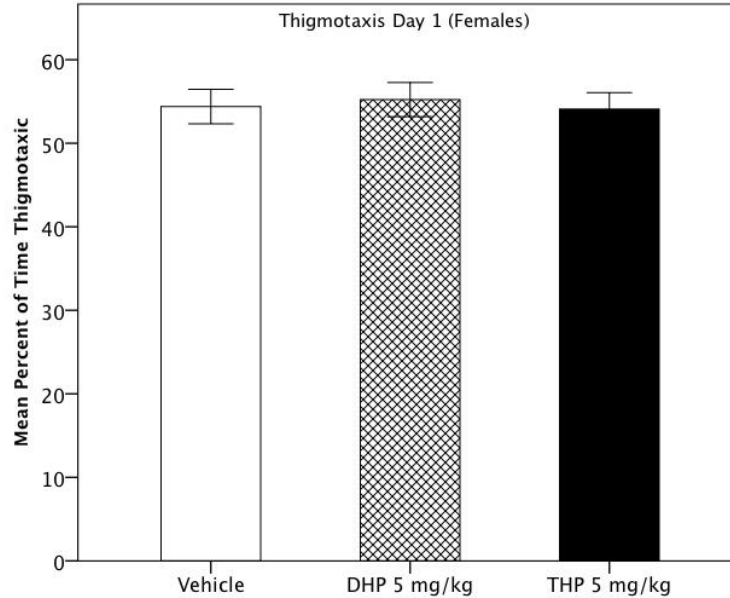


Figure 2.10 Learning a new task: thigmotaxic response of female animals on day 1. The y-axis represents the average percentage of time the animal spends circling the perimeter of the pool during all trials on day 1. There were no significant differences related to the type of drug treatment (ANOVA, $p > 0.05$, $n = 12$ per group). White bars, vehicle; hatched bars, DHP; black bars, THP. Mean $\pm$ SE.

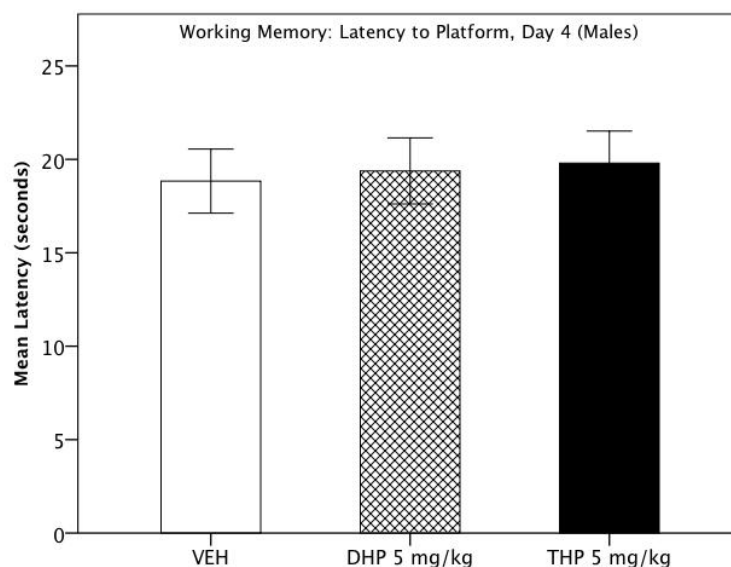


Figure 2.11 Working memory: Latency of male animals to platform on day 4: the ability to recall a well-learned task in the presence of drug. The y-axis represents the average amount of time, in seconds, taken to reach the platform across all 8 trials of day 4. There were no significant differences related to the type of drug treatment (ANOVA, $p > 0.05$, $n = 12$ per group). White bars, vehicle; hatched bars, DHP; black bars, THP. Mean $\pm$ SE.

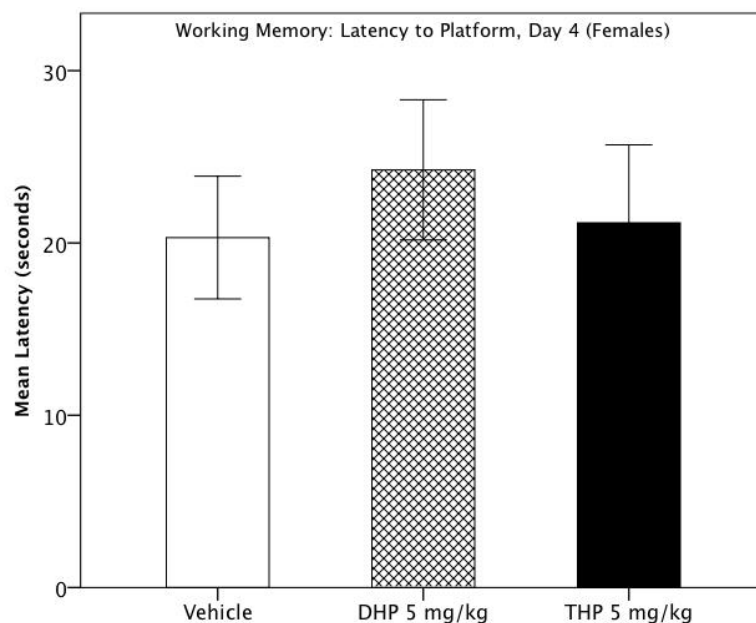


Figure 2.12 Working memory: Latency of female animals to platform on day 4: the ability to recall a well-learned task in the presence of drug. The y-axis represents the average amount of time, in seconds, taken to reach the platform across all 8 trials of day 1. There were no significant differences related to the type of drug treatment (ANOVA, $p > 0.05$, $n = 12$ per group). White bars, vehicle; hatched bars, DHP; black bars, THP. Mean $\pm$ SE.

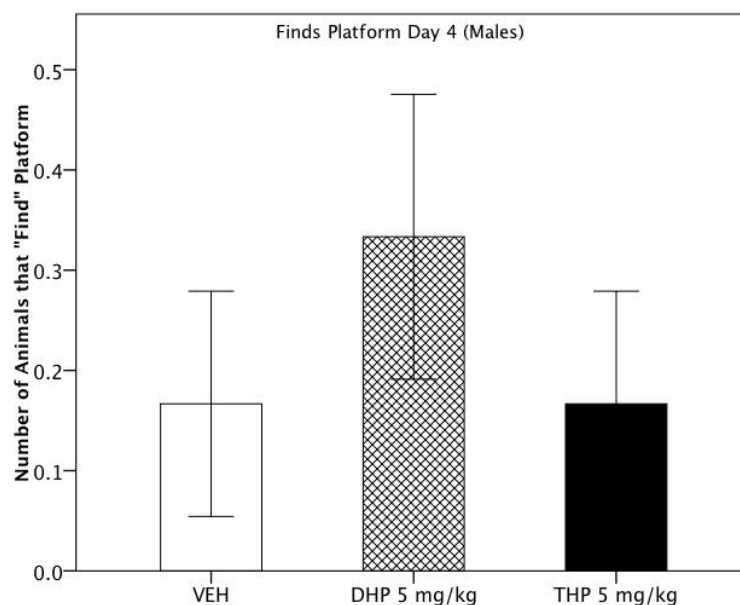


Figure 2.13 Working memory: Ability of male animals to find the platform without assistance after being guided to it on day 4 (with drug). The y-axis represents the average proportion of animals that were able to find the platform after being guided to it. There were no significant differences related to the type of drug treatment (Independent samples Kruskal-Wallis test, $p > 0.05$, $n = 12$ per group). White bars, vehicle; hatched bars, DHP; black bars, THP. Mean $\pm$ SE.

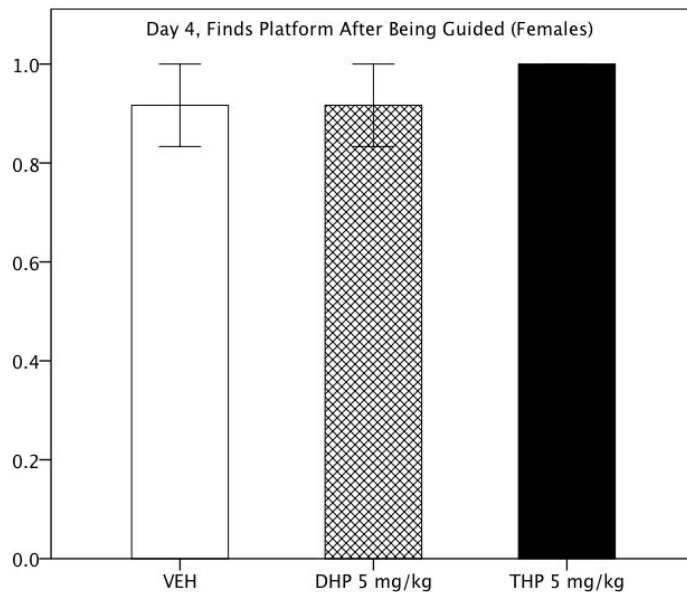


Figure 2.14 Working memory: Ability of female animals to find the platform without assistance after being guided to it on day 4 (with drug). The y-axis represents the proportion of animals that were able to find the platform after being guided to it. There were no significant differences related to the type of drug treatment (Independent samples Kruskal-Wallis test, $p > 0.05$, $n = 12$ per group). White bars, vehicle; hatched bars, DHP; black bars, THP. Mean $\pm$ SE.

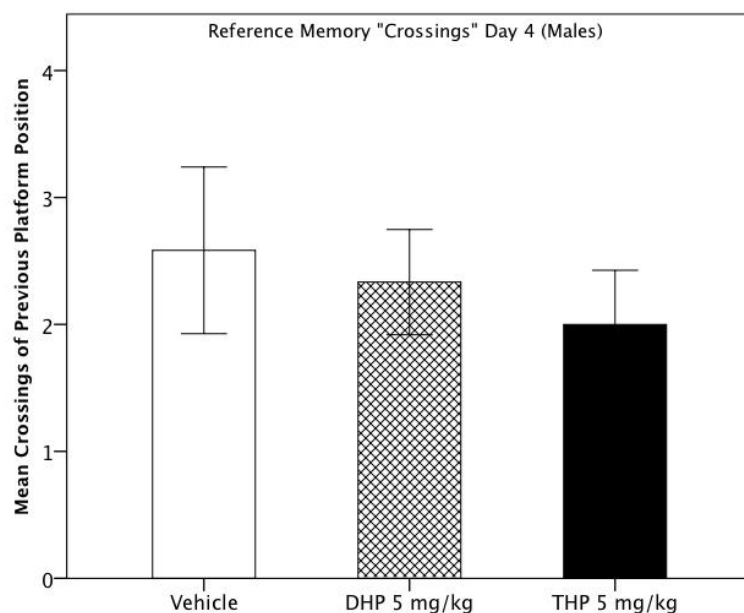


Figure 2.15 Reference memory: Crossings. Number of times on day 4 that male animals swim across the previous (day 3) platform position. There were no significant differences related to the type of drug treatment (ANOVA $p > 0.05$, $n = 12$ per group). White bars, vehicle; hatched bars, DHP; black bars, THP. Mean $\pm$ SE.

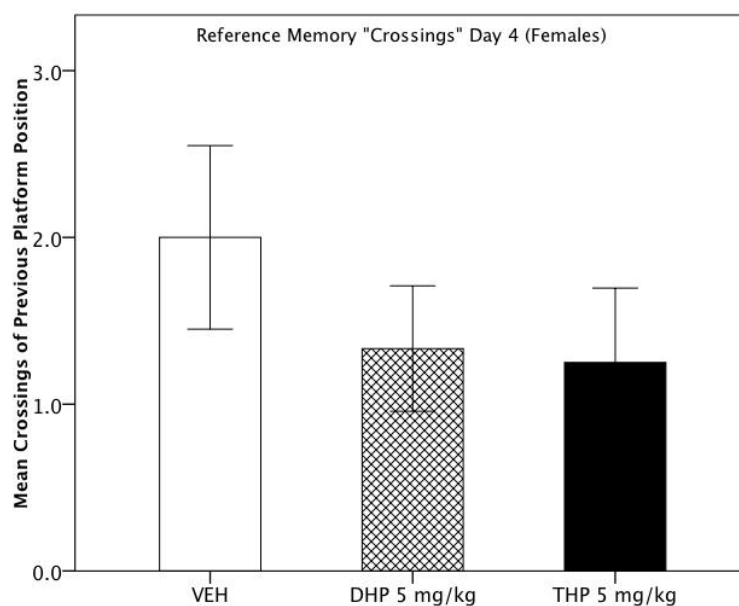


Figure 2.16 Reference memory: Crossings. Number of times on day 4 that female animals swim across the previous (day 3) platform position. There were no significant differences related to the type of drug treatment (ANOVA $p > 0.05$, $n = 12$ per group). White bars, vehicle; hatched bars, DHP; black bars, THP. Mean $\pm$ SE.

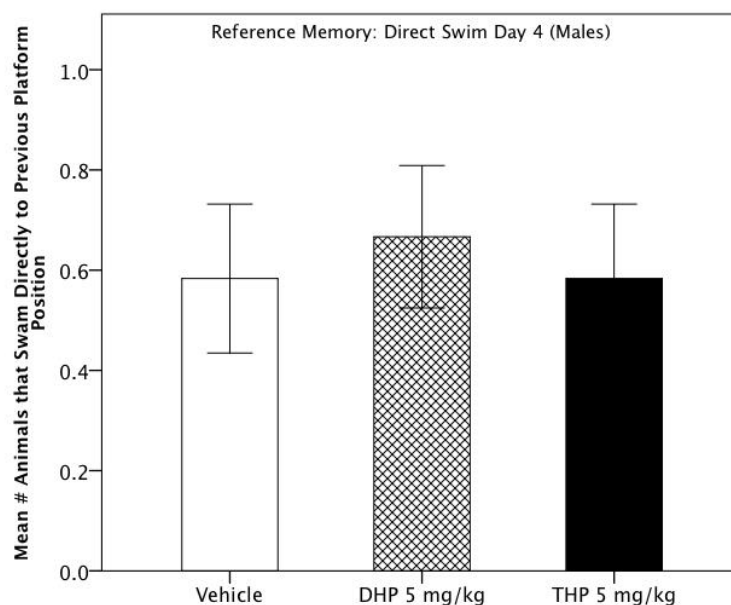


Figure 2.17 Reference memory: Direct swim, day 4. Male animals that swim directly to the previously learned day 3 platform position. The y-axis represents the proportion of males in each drug treatment group that swam directly to the day 3's platform position in the 1st trial of day 4. There were no significant differences related to the type of drug treatment (Independent samples Kruskal-Wallis test, $p > 0.05$, $n = 12$ per group). White bars, vehicle; hatched bars, DHP; black bars, THP. Mean $\pm$ SE.

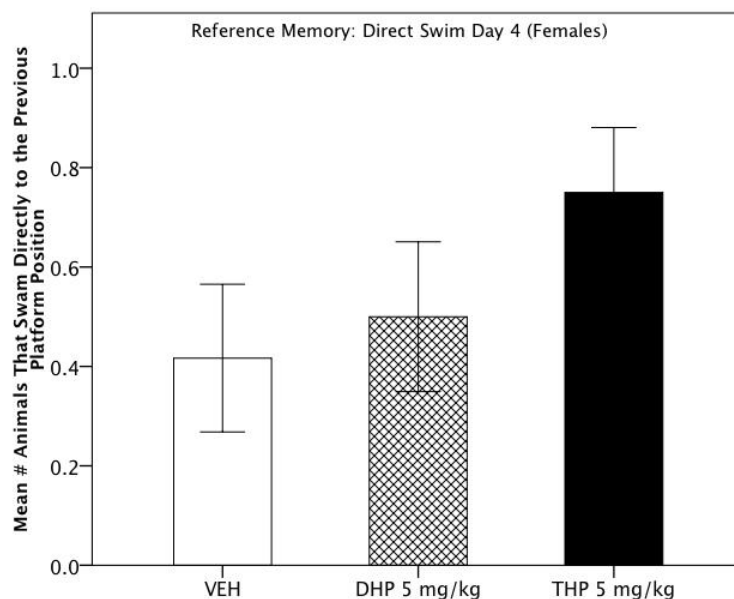


Figure 2.18 Reference memory: Direct swim day 4. Female animals that swim directly to the previously learned day 3 platform position. The y-axis represents the proportion of males in each drug treatment group that swam directly to the day 3's platform position in the 1st trial of day 4. There were no significant differences related to the type of drug treatment (Independent samples Kruskal-Wallis test, $p > 0.05$, $n = 12$ per group). White bars, vehicle; hatched bars, DHP; black bars, THP. Mean $\pm$ SE.

2.3.2.3 Velocity

In the presence of drug on day 1, there were significant differences in velocity in male animals (ANOVA $p=0.000$; **Fig 2.19**), but not in female animals (ANOVA $p>0.05$; **Fig 2.20**). Male animals' velocity on day 1 was slower in THP-treated animals compared to vehicle (Tukey's HSD *post hoc* $p=0.000$), but not compared the DHP (Tukey's HSD *post hoc* $p>0.05$). In the presence of drug on day 4, male and female results also differed. In male animals only, there was a significant difference in velocities (ANOVA $p=0.002$; **Fig 2.21**); DHP-treated male animals' velocities were decreased compared to control (Tukey's HSD *post hoc* $p=0.002$), but not compared to THP (Tukey's HSD *post hoc* $p>0.05$). In female animals, there were no significant velocity differences related to drug treatment (ANOVA $p>0.05$; **Fig 2.22**).

In the absence of drug on day 2, day 3, and day 5, trials 1-4, results were mixed. There were no significant effects of the type of drug treatment on day 2 in male or female animals (**Fig 2.23, 2.24**). On day 3, however, male animals' velocities were affected by previous drug treatment (ANOVA $p=0.013$; **Fig 2.25**). Male animals that had been treated with THP on day 1 were slower than DHP-treated animals (Tukey's HSD *post hoc* $p=0.009$) on day 3, but not significantly different from vehicle control (Tukey's HSD *post hoc* $p>0.05$). There were, however, no significant differences in velocity on day 3 in female animals (ANOVA $p>0.05$; **Fig 2.26**). On day 5 (trials 1-4), there were no significant differences in velocity in male animals related to the type of drug treatment (ANOVA $p>0.05$; **Fig 2.27**). There were, however, velocity differences in female animals on day 5 (trials 1-4) related to previous drug treatment (ANOVA $p=0.005$; **Fig 2.28**). The female animals

treated with THP on day 4 were significantly slower on day 5 than female animals that had been treated with either DHP (Tukey's HSD *post hoc* $p=0.020$) or vehicle (Tukey's HSD *post hoc* $p=0.010$) on day 4. Given the results above, the effects of drug treatment on velocity, therefore, were mixed.

In males there were significant differences in swimming velocity in day 1 (ANOVA $p=0.000$) with THP treatment (Tukey's HSD *post hoc* $p=0.000$, **Fig 2.19**). Thus THP treatment slowed male swim velocity learning a new task. On day 4, however, drug treatment had a different significant effect (ANOVA $p=0.002$, **Fig 2.21**): the velocity of DHP-treated animals was significantly less than the velocity of vehicle control (Tukey's HSD *post hoc* $p=0.002$), and slightly less than THP in males (Tukey's HSD *post hoc* $p=0.096$; **Fig 2.21**). Therefore, DHP decreased velocity in a well-learned task, but not in a new task. Also in males, drug treatment on day 1 did not have significant effects on velocity on day 2 (**Fig 2.23**). Drug treatment on day 4 did not affect velocity in the first four trials of day 5 (**Fig 2.27**). On day 3, 48 hours after treatment, velocity of different treatments differed significantly (ANOVA $p=0.013$). Male animals treated with THP on day 1 were slower than DHP-treated animals on day 3 (Tukey's HSD *post hoc* $p=0.009$), but were not significantly slower than vehicle controls (Tukey's HSD *post hoc* $p=0.226$; **Fig 2.25**). Therefore, THP decreased velocity relative to DHP in a well-learned task 48 hours after treatment in male animals. In a comparison of velocities of trials 3-4 and 5-8 of day 5 in males, there were no significant differences in the interaction term trial group/drug, nor were there significant differences between trial groups or between drugs (ANOVA $p>0.05$; data not shown). Drug treatment on day 4, therefore, did not affect velocity

in a well-learned task (trials 3-4, submerged platform) as compared to a new task (trials 5-8, raised platform) in male rats.

In females, there were no significant differences associated with the type of drug treatment, in the presence of drug, on swimming velocity on day 1 (**Fig 2.20**) or day 4 (**Fig 2.22**). On days 2 and 3 as well, one and two days after drug treatment, respectively, the velocity of female animals did not differ by drug treatment (**Fig 2.24, Fig 2.26**). On day 5, however, one day after drug treatment, female velocities in trials 1-4 differed by drug treatment (ANOVA $p=0.005$; **Fig 2.28**). Female animals that had been treated with THP on day 4 were significantly slower on day 5, trials 1-4, than those that were treated with DHP (Tukey's HSD *post hoc* $p=0.020$) or vehicle (Tukey's HSD *post hoc* $p=0.010$). Therefore, THP may have slowed female velocity to a spatially known location 24 hours after treatment, but did not affect velocity when learning a new task or recalling a task on the same day as drug treatment. Also in females, in a comparison of velocities of trials 3-4 and 5-8 of day 5, there were no significant differences related to the drug treatment and/or trial group (ANOVA $p=0.308$).

Overall, drug treatment with THP or DHP affected some measures of swim velocity in male and female rats. There was not a consistent pattern with respect to drug-induced effects on velocity in male or female animals.

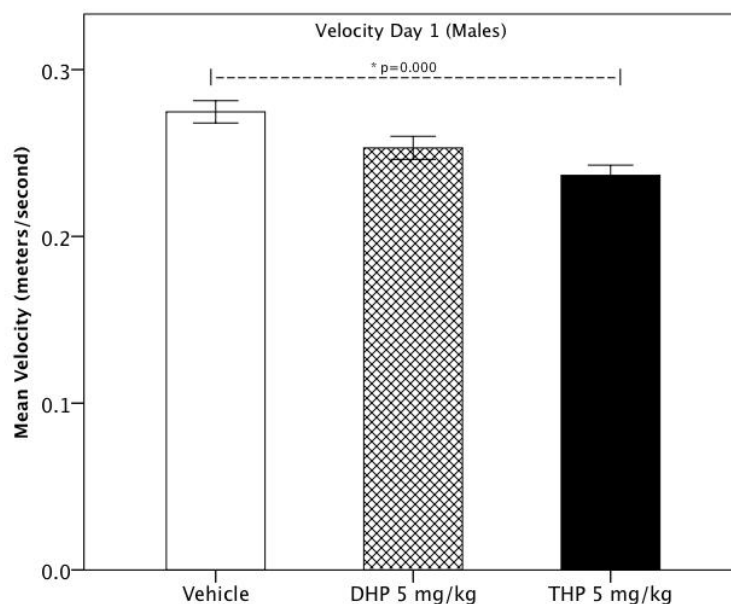


Figure 2.19 Drug effects on male velocity, day 1. The y-axis represents velocity (meters/second). THP was significantly slower than vehicle. There were not other significant differences by drug treatment (ANOVA, $p>0.05$, $n=12$ per group). White bars, vehicle; hatched bars, DHP; black bars, THP. Mean $\pm$ SE.

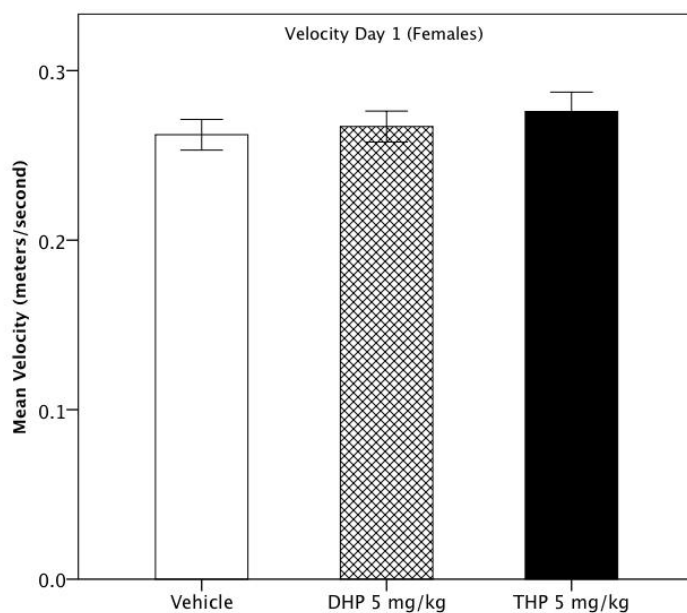


Figure 2.20 Drug effects on female velocity, day 1. The y-axis represents velocity (meters/second). There were no significant differences related to the type of drug treatment (ANOVA, $p>0.05$, $n=12$ per group). White bars, vehicle; hatched bars, DHP; black bars, THP. Mean $\pm$ SE.

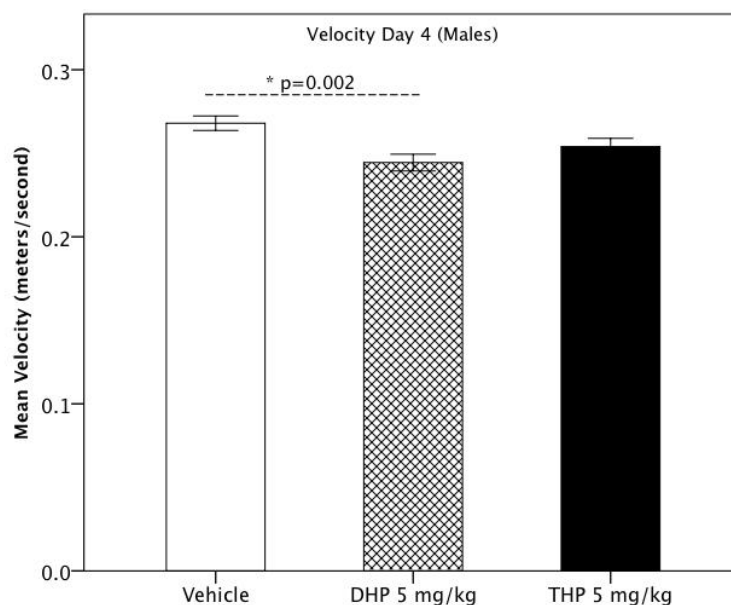


Figure 2.21 Drug effects on male velocity, day 4. The y-axis represents velocity (meters/second). THP treatment slowed velocity relative to vehicle control (ANOVA, $F=6.160$, $p=0.002$, THP<VEH, $p=0.002$ Tukey's HSD *post hoc*, $n=12$ per group). White bars, vehicle; hatched bars, DHP; black bars, THP. One-way ANOVA, Tukey's HSD *post hoc*. Mean $\pm$ SE. *, $p<0.05$ compared to vehicle. Mean $\pm$ SE.

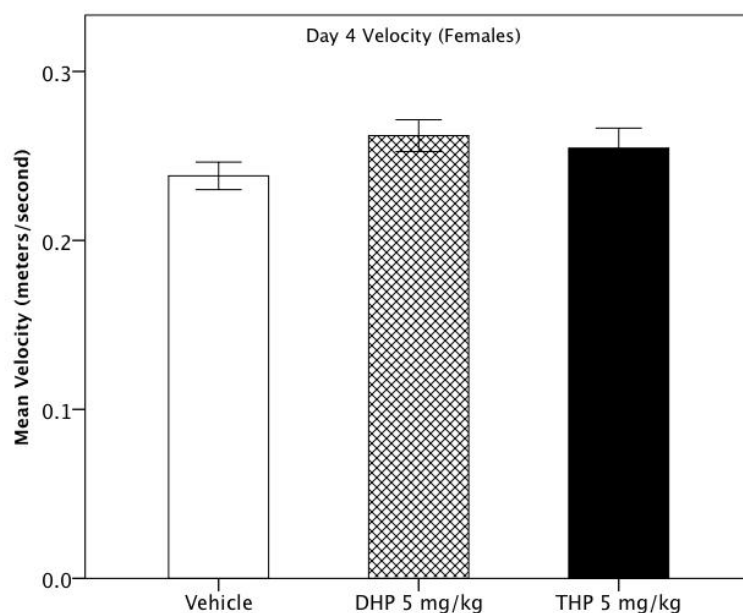


Figure 2.22 Drug effects on female velocity, day 4. The y-axis represents velocity (meters / second). There were no significant differences related to the type of drug treatment (ANOVA, $p>0.05$, $n=12$ per group). White bars, vehicle; hatched bars, DHP; black bars, THP. Mean $\pm$ SE.

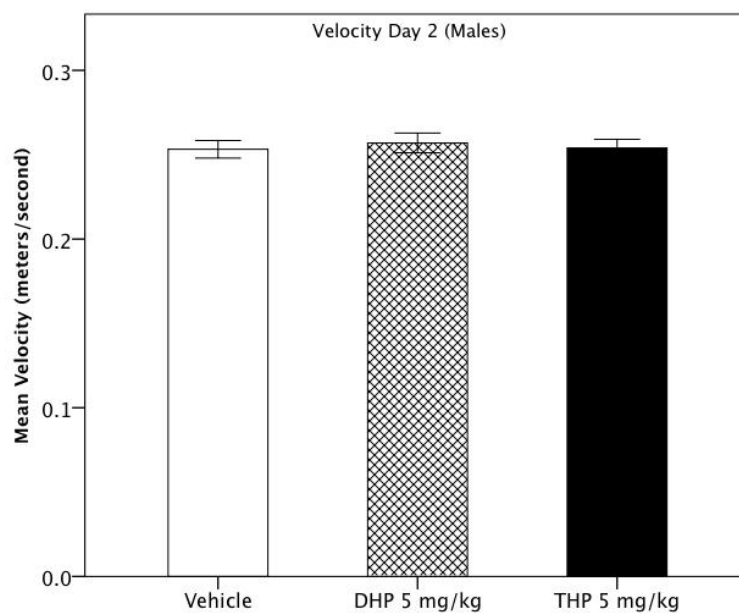


Fig 2.23 Male velocity 24 hours after treatment, day 2. The y-axis represents velocity (meters/second). There were no significant differences related to the type of drug treatment (ANOVA, $p>0.05$, $n=12$ per group). White bars, vehicle; hatched bars, DHP; black bars, THP. Mean $\pm$ SE.

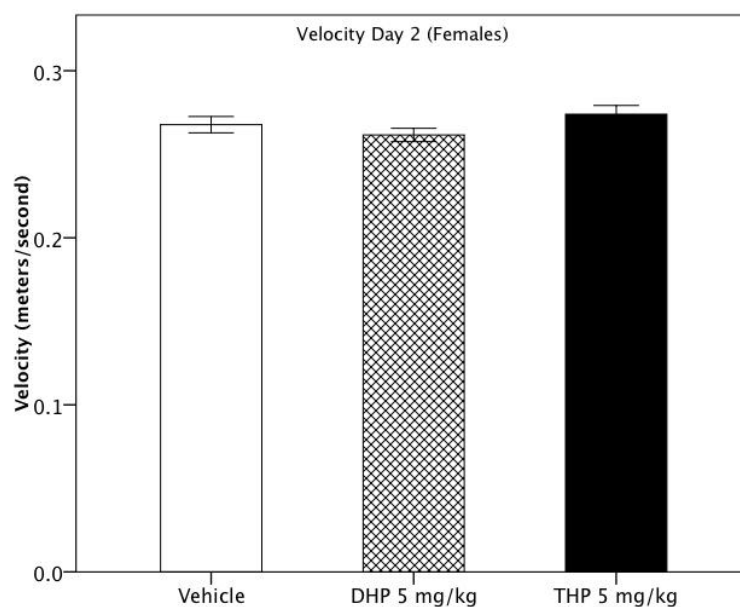


Fig 2.24 Female velocity 24 hours after treatment, day 2. The y-axis represents velocity (meters/second). There were no significant differences related to the type of drug treatment (ANOVA, $p>0.05$, $n=12$ per group). White bars, vehicle; hatched bars, DHP; black bars, THP. Mean $\pm$ SE.

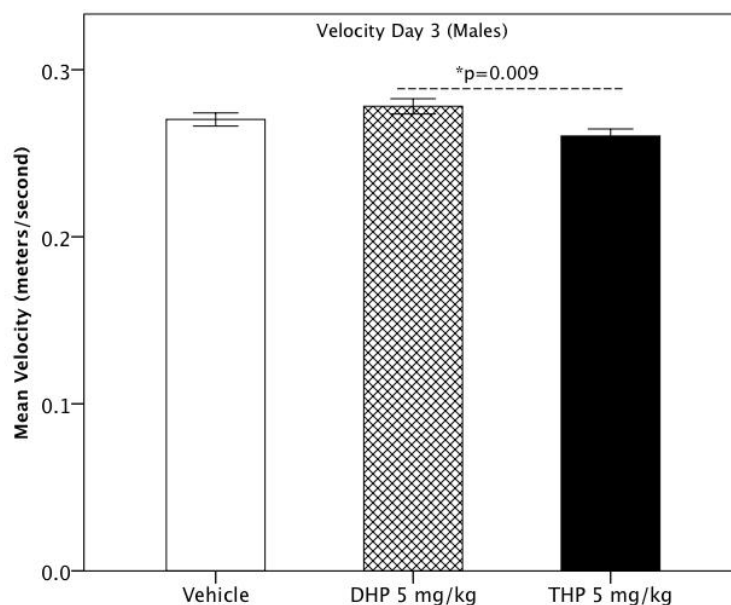


Figure 2.25 Male velocity 48 hours after treatment, day 3. The y-axis represents velocity (meters/second). The type of drug treatment caused significant differences (ANOVA $F=4.405$, $p=0.013$). DHP and THP were significantly different from each other (Tukey's HSD *post hoc* $p=0.009$). White bars, vehicle; hatched bars, DHP; black bars, THP. Mean $\pm$ SE. * $p<0.05$.

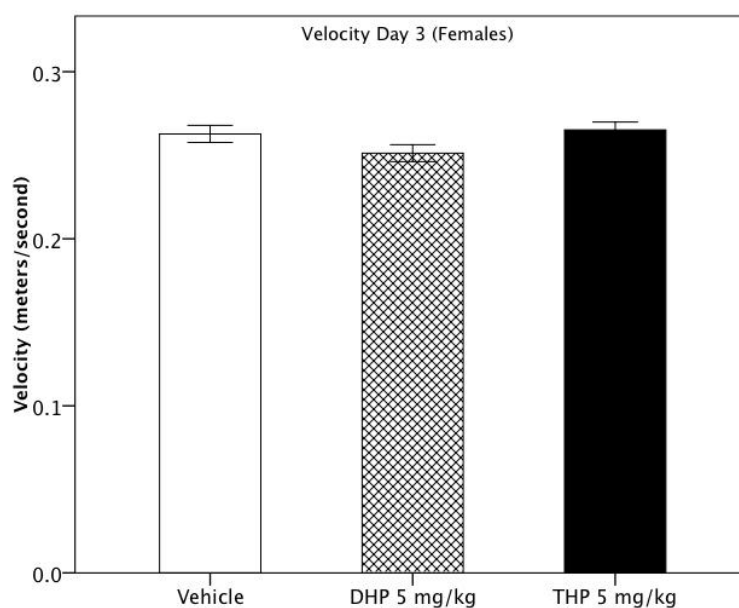


Figure 2.26 Female velocity 24 hours after treatment, day 3. The y-axis represents velocity (meters/second). There were no significant differences related to the type of drug treatment (ANOVA, $p>0.05$, $n=12$ per group). White bars, vehicle; hatched bars, DHP; black bars, THP. Mean $\pm$ SE.

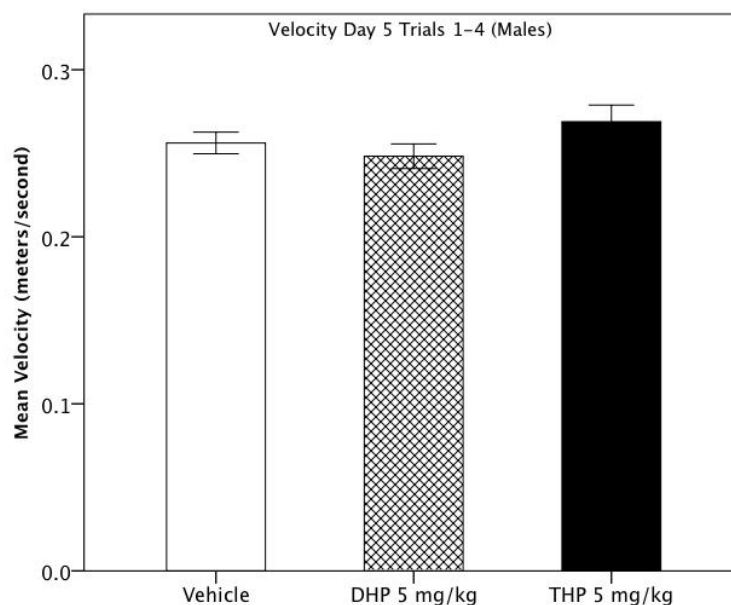


Figure 2.27 Drug effects on male velocity one day after treatment, on day 5 trials 1-4 (submerged platform). The y-axis represents velocity (meters/second). There were no significant differences related to the type of drug treatment (ANOVA, $p > 0.05$, $n = 12$ per group). White bars, vehicle; hatched bars, DHP; black bars, THP. Mean $\pm$ SE.

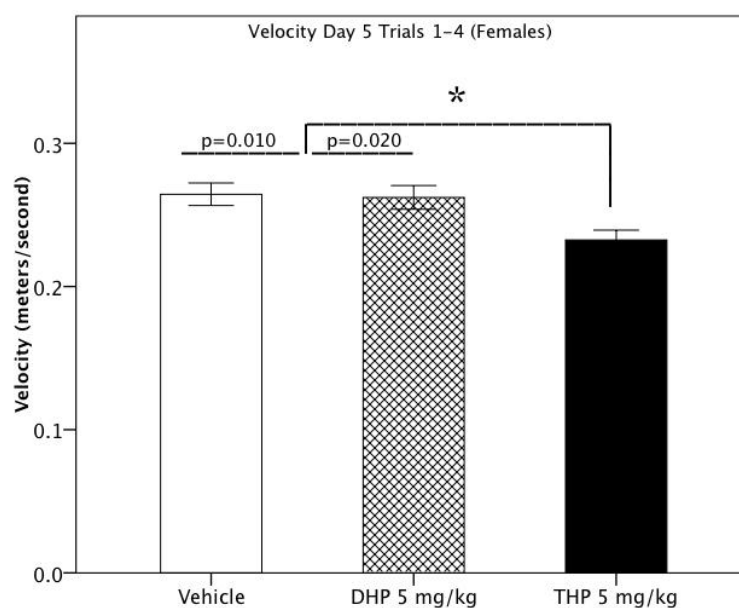


Figure 2.28 Drug effects on female velocity one day after treatment, on day 5 trials 1-4 (submerged platform). The y-axis represents velocity (meters/second). The type of drug treatment on day 4 caused significant differences (ANOVA $F = 5.434$, $p = 0.005$). DHP and THP are significantly different from each other (Tukey's HSD *post hoc* $p = 0.020$), as are THP and vehicle (Tukey's HSD *post hoc* $p = 0.010$). Vehicle and DHP were not significantly different from each other (Tukey's HSD *post hoc* $p > 0.05$). White bars, vehicle; hatched bars, DHP; black bars, THP. Mean $\pm$ SE. * $p < 0.05$.

2.3.2.4 Spatial Memory

In males, there were no significant differences in latency to platform related to drug treatment on day 5 during trials 1-4. This might mean that spatial memory in male animals is not affected by anticonvulsant doses of DHP and THP (**Fig 2.29**). In female animals, however, there were significant differences related to drug treatment (ANOVA $p=0.028$; **Fig 2.30**): THP treatment on day 4 was associated with decreased latency to platform on day 5 relative to both DHP (Tukey's HSD *post hoc* $p=0.010$) and vehicle (Tukey's HSD *post hoc* $p=0.010$). It is possible that THP treatment in females on day 4 improved the retention of the spatial memory used to recall the platform position on day 5. In male and female animals, no measures of latency/spatial memory on day 5 during trials 1-2 or 3-4 (**Fig 2.31-2.34**) were significantly related to drug treatment. Anticonvulsant doses of DHP and THP, therefore, do not impair spatial memory in male or female animals as compared to vehicle. In fact, in female animals, an anticonvulsant dose of THP may actually improve the retention of a spatial memory.

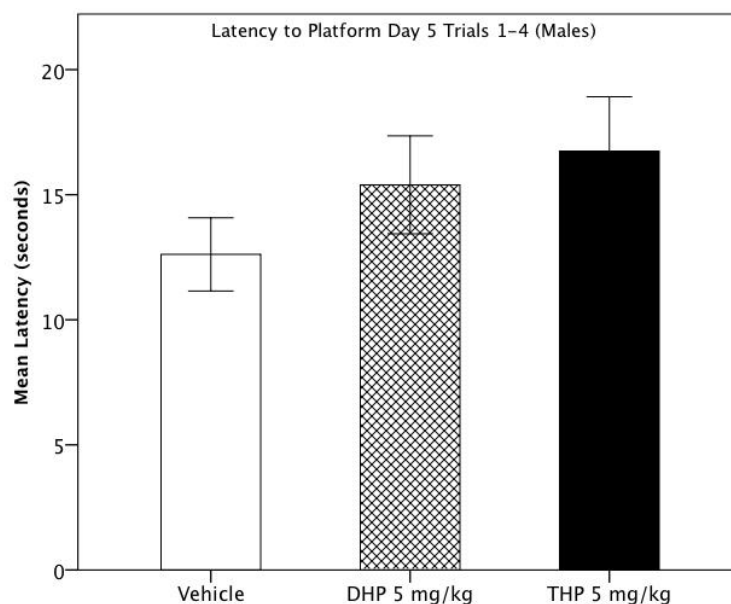


Figure 2.29. Spatial memory measures in male animals on day 5. The y-axis represents the latency to platform in seconds. There were no significant differences related to the type of drug treatment. White bars, vehicle; hatched bars, DHP; black bars, THP. One-way ANOVA. Mean $\pm$ SE.

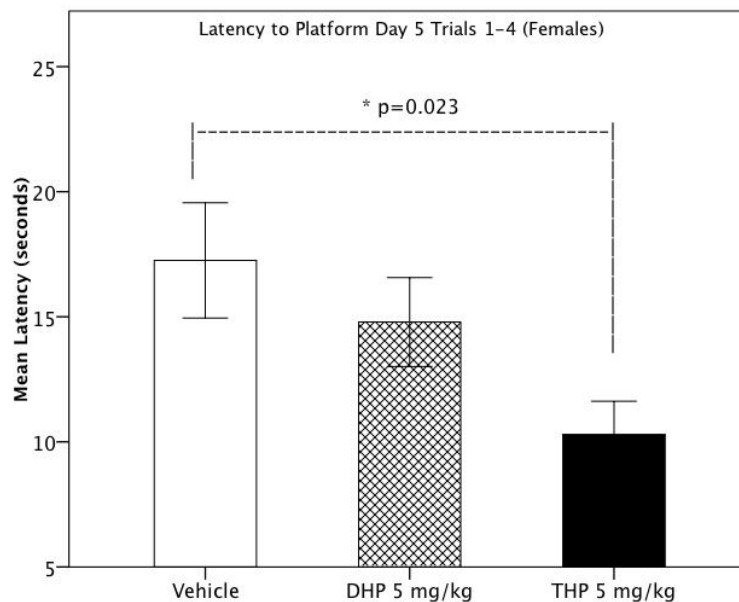


Figure 2.30 Spatial memory measures in female animals on day 5. The y-axis represents the latency to platform in seconds. Female latencies were affected by the type of drug treatment in trials. THP treatment on day 4 decreased latency to platform on day 5: ANOVA $F=3.656$, $p=0.028$, post hoc THP < vehicle, ($p=0.023$). One-way ANOVA, Tukey's HSD *post hoc*. Mean $\pm$ SE. *, $p<0.05$ compared to vehicle.

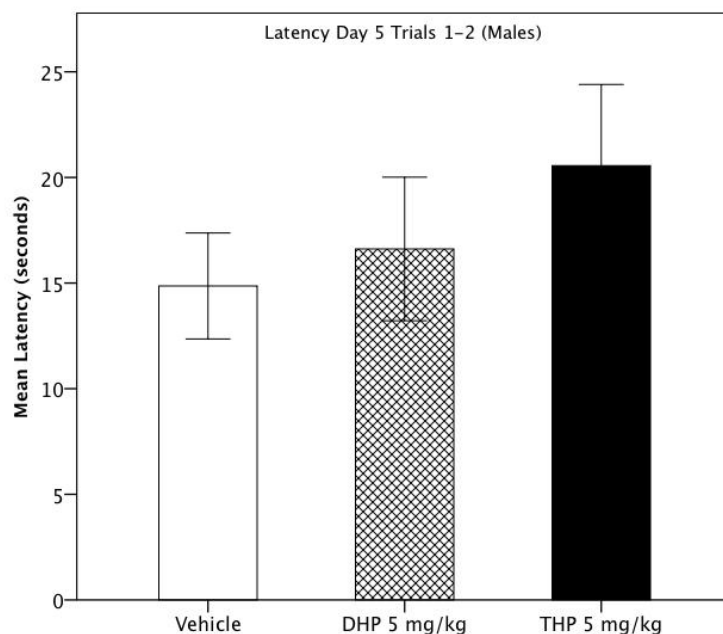


Figure 2.31 Spatial memory measures in male animals on day 5. The y-axis represents the latency to platform in seconds. There were no significant differences related to the type of drug treatment. White bars, vehicle; hatched bars, DHP; black bars, THP. One-way ANOVA. Mean $\pm$ SE.

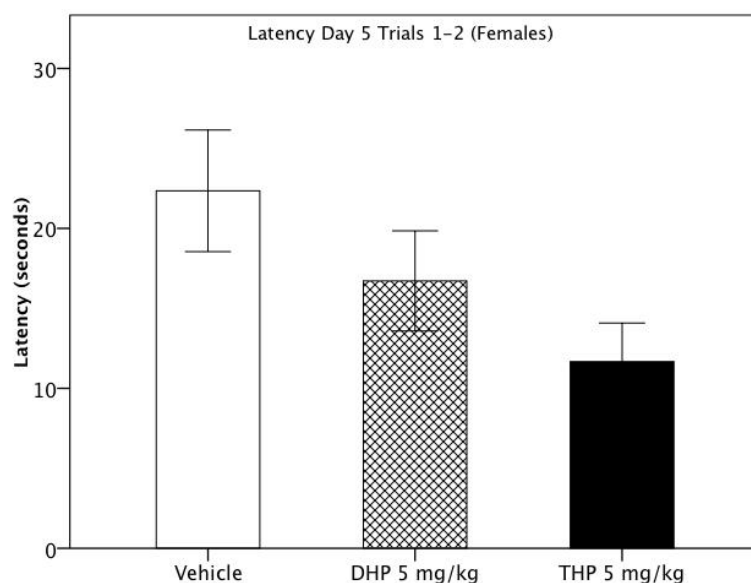


Figure 2.32 Spatial memory measures in female animals on day 5. The y-axis represents the latency to platform in seconds. There were no significant differences related to the type of drug treatment, although significance is approached ($F=2.841$, $p=0.065$). White bars, vehicle; hatched bars, DHP; black bars, THP. One-way ANOVA. Mean $\pm$ SE.

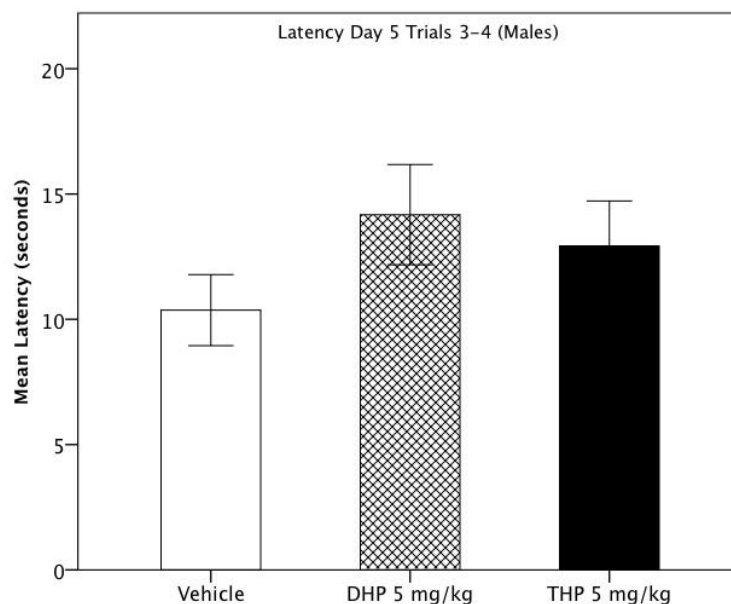


Figure 2.33 Spatial memory measures in male animals on day 5. The y-axis represents the latency to the platform in seconds. There were no significant differences related to the type of drug treatment (ANOVA, $p > 0.05$). White bars, vehicle; hatched bars, DHP; black bars, THP. Mean $\pm$ SE.

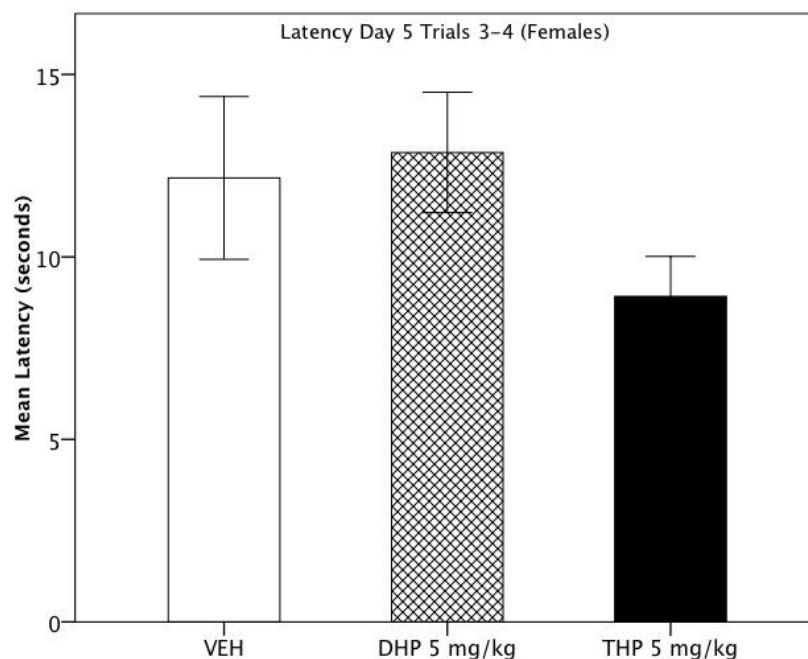


Figure 2.34 Spatial memory measures in female animals on day 5. The y-axis represents the latency to platform in seconds. There were no significant differences related to the type of drug treatment (ANOVA, $p > 0.05$). White bars, vehicle; hatched bars, DHP; black bars, THP. One-way ANOVA. Mean $\pm$ SE.

Morris Watermaze <i>Learning/memory measure (n=12 per treatment group)</i>	Male statistic <i>Statistical test, p-value post hoc test as appropriate Vehicle: Mean±SE DHP: Mean±SE THP: Mean±SE Figure</i>	Female statistic <i>Statistical test, p-value post hoc test as appropriate Vehicle: Mean±SE DHP: Mean±SE THP: Mean±SE Figure</i>
Acquisition of memory in presence of drug: Day 1 latency to platform (s)	ANOVA p=0.060 Vehicle: 34.108±2.122 DHP: 35.529±2.330 THP: 39.518±2.086 Fig 2.7	ANOVA p=0.919 Vehicle: 39.082±2.715 DHP: 32.690±2.715 THP: 40.887±2.715 Fig 2.8
Learning a new task in the presence of drug: Day 1 time thigmotaxic (%)	ANOVA p=0.434 Vehicle: 59.591±9.690 DHP: 49.501±2.284 THP: 50.875±2.098 Fig 2.9	ANOVA p=0.919 Vehicle: 4.397±2.062 DHP: 5.221±2.050 THP: 4.082±1.972 Fig 2.10
Working memory: Ability to recall a well-learned task in presence of drug, day 4 latency to platform (s)	ANOVA p=0.925 Vehicle: 18.836±1.714 DHP: 19.381±1.771 THP: 19.801±1.711 Fig 2.11	ANOVA p=0.775 Vehicle: 20.313±4.073 DHP: 24.239±4.073 THP: 21.168±4.073 Fig 2.12
Working memory: Animal finds platform on own once guided to it, day 4	Independent samples Kruskal-Wallis test, p=0.893 (M±SE n/a) Fig 2.13	Independent samples Kruskal-Wallis test, p=0.598 (M±SE n/a) Fig 2.14
Reference memory: Crossings Number of times on day 4 that animal crosses day 3 platform position	ANOVA p=0.363 Vehicle: 2.583±0.657 DHP: 2.333±0.414 THP: 2.000±0.426 Fig 2.15	ANOVA p=0.463 Vehicle: 2.000±0.463 DHP: 1.333±0.463 THP: 1.250±0.463 Fig 2.16
Reference memory: Direct swim. On day 5 animal swims directly to the unchanged platform position	Independent samples Kruskal-Wallis test, p=0.897 (M±SE n/a) data not shown	Independent samples Kruskal-Wallis test, p=0.128 (M±SE n/a) data not shown

Velocity with drug: Day 1 (m/s)	ANOVA p=0.431 Vehicle: 0.275±0.007 DHP: 0.253±0.007 THP: 0.237±0.004 Fig 2.19	ANOVA p=0.304 Vehicle: 0.262±0.010 DHP: 0.267±0.010 THP: 0.276±0.010 Fig 2.20
Velocity with drug: day 4 (m/s)	ANOVA *p=0.002 Tukey's HSD <i>post hoc</i> : <u>DHP<vehicle, p=0.002</u> Vehicle: 0.268±0.004 DHP: 0.244±0.005 THP: 0.254±0.005 Fig 2.21	ANOVA p=0.237 Vehicle: 0.238±0.010 DHP: 0.262±0.010 THP: 0.255±0.010 Fig 2.22
Velocity without drug: day 2 (m/s)	ANOVA p=0.872 Vehicle: 0.253±0.005 DHP: 0.257±0.006 THP: 0.254±0.005 Fig 2.23	ANOVA p=0.184 Vehicle: 0.268±0.005 DHP: 0.262±0.004 THP: 0.274±0.047 Fig 2.24
Velocity without drug: day 3 (m/s)	ANOVA *p=0.013 M±SE=0.270±0.002 Tukey's HSD <i>post hoc</i> : <u>THP<DHP, p=0.009</u> Vehicle: 0.270±0.004 DHP: 0.278±0.005 THP: 0.260±0.002 Fig 2.25	ANOVA F= p=0.107 Vehicle: 0.263±0.005 DHP: 0.251±0.050 THP: 0.265±0.046 Fig 2.26
Velocity without drug: day 5 trials 1-4 (m/s)	ANOVA p=0.194 Vehicle: 0.256±0.006 DHP: 0.248±0.007 THP: 0.269±0.010 Fig 2.27	ANOVA *p=0.005 Tukey's HSD: <u>THP<vehicle, p=0.010</u> <u>THP<DHP, p=0.020</u> Vehicle: 0.264±0.054 DHP: 0.260±0.056 THP: 0.232±0.048 Fig 2.28
Day 5 velocities in trials 3-4 (submerged platform) are compared to velocities in trials 5-8 (raised platform) (m/s)	2-way ANOVA Drug*Trial set p=0.789 Drug p=0.941 Trial set p=0.063 <u>Trials 3-4</u> Vehicle: 0.256±0.010 DHP: 0.251±0.010 THP: 0.252±0.009 <u>Trials 5-8</u> Vehicle: 0.237±0.007 DHP: 0.240±0.053 THP: 0.244±0.041 (data not shown)	2-way ANOVA Drug*Trial set p=0.308 Drug *p=0.033 Trial set p=0.011 <u>Trials 3-4</u> Vehicle: 0.263±0.058 DHP: 0.259±0.063 THP: 0.228±0.048 <u>Trials 5-8</u> Vehicle: 0.229±0.008 DHP: 0.248±0.008 THP: 0.222±0.007 (data not shown)

Spatial memory overall, day 5: Latency day 5, trials 1-4 (s)	ANOVA p=0.291 Vehicle: 12.613±1.462 DHP: 15.393±1.958 THP: 16.745±2.170 Fig 2.29	ANOVA *p=0.028 Tukey's HSD <i>post hoc</i> <u>THP < vehicle, *p=0.023</u> Vehicle: 17.255±2.304 DHP: 14.790±1.775 THP: 10.295±9.200 Fig 2.30
Spatial memory from day 4 to day 5: Latency day 5, trials 1-2 (s)	ANOVA p=0.461 Vehicle: 14.861±2.509 DHP: 16.614±3.397 THP: 20.558±3.844 Fig 2.31	ANOVA p=0.065 Vehicle: 22.347±3.802 DHP: 16.717±3.136 THP: 11.670±2.416 Fig 2.32
Spatial memory, day 5: Latency to spatially known platform trials 3-4 (s)	ANOVA p=0.299 Vehicle: 10.366±1.417 DHP: 14.173±2.000 THP: 12.932±1.790 Fig 2.33	ANOVA p=0.232 Vehicle: 12.163±2.231 DHP: 12.862±1.648 THP: 8.920±1.095 Fig 2.34

Table 2. Summary of results in the MWM experiments.

2.3.3 The Elevated Plus Maze

Behavioral measures in the EPM were similar across all treatment groups. There were no significant differences in the percent of time spent in open and closed arms of the maze, or entries into the open or closed arms (2-way ANOVA $p>0.05$). The effects of drug/dose on the EPM variables open arm time, closed arm time, open arm entries, closed arm entries, and locomotion (total arm entries), respectively, are presented in **Figs 2.35, 2.37, 2.39, 2.41, and 2.43** for male animals, and **2.36, 2.38, 2.40, 2.42, and 2.44** for female animals. Spontaneous locomotor activity, assessed by the sum of closed and open arm entries, was also not affected by treatment condition (2-way ANOVA $p>0.05$; **Fig 2.43-2.44**). This confirms our findings of no effect on locomotion in the open field experiment (**Fig 2.3 and 2.5**). The statistical summary of the EPM is presented in **Table 3**. Overall, these results may suggest that anticonvulsant doses of DHP and THP do not have anxiogenic or anxiolytic effects in male and female animals.

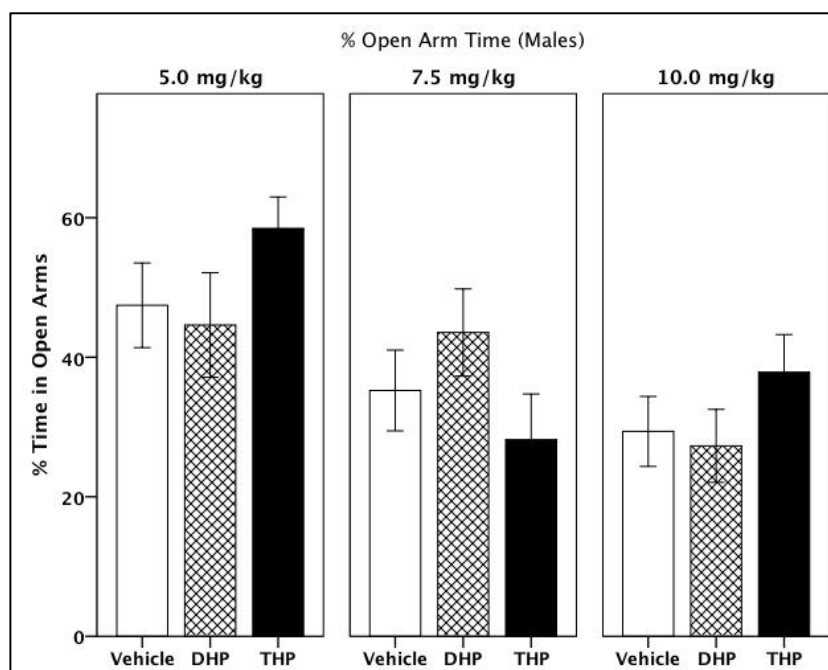


Figure 2.35 Percent of time males spent in the open arms. The y-axis represents the percentage of the 3-minute trial time spent in the open arms. There were no significant differences related to the type of drug treatment or the dose used. White bars, vehicle; hatched bars, DHP; black bars, THP. 2-way ANOVA (drug*dose). Mean $\pm$ SE.

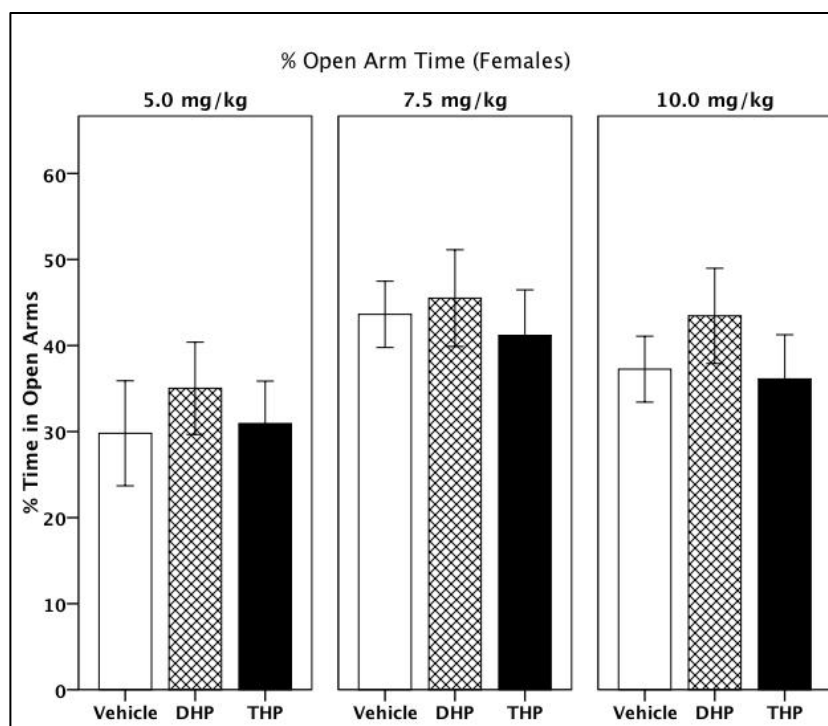


Figure 2.36 Percent of time females spent in open arms. The y-axis represents the percentage of the 3-minute trial time spent in the open arms. There were no significant differences related to the type of drug treatment or the dose used. White bars, vehicle; hatched bars, DHP; black bars, THP. 2-way ANOVA (drug*dose). Mean $\pm$ SE.

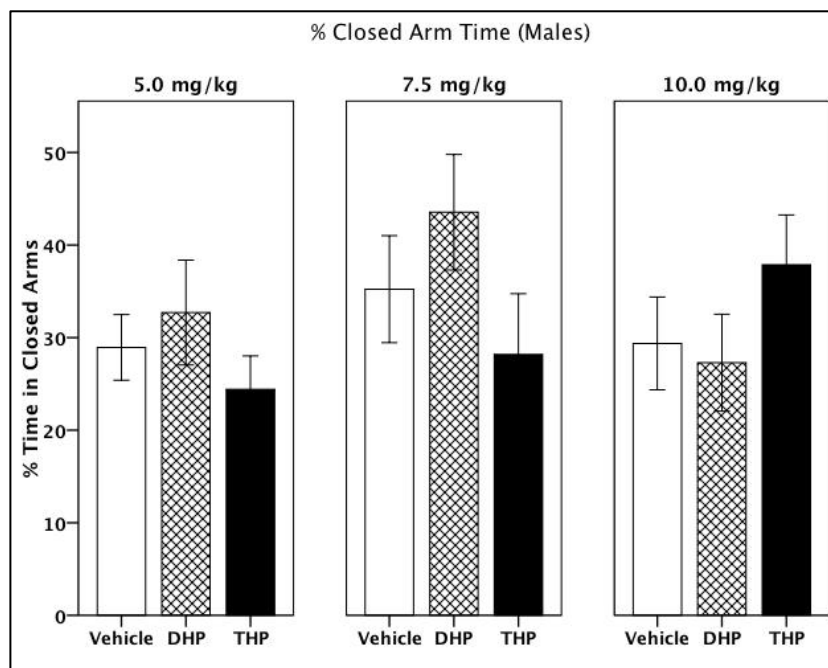


Figure 2.37 Percent of time males spent in closed arms. The y-axis represents the percentage of the 3-minute trial time spent in open arms. There were no significant differences related to the type of drug treatment or the dose used. White bars, vehicle; hatched bars, DHP; black bars, THP. 2-way ANOVA (drug*dose). Mean ± SE.

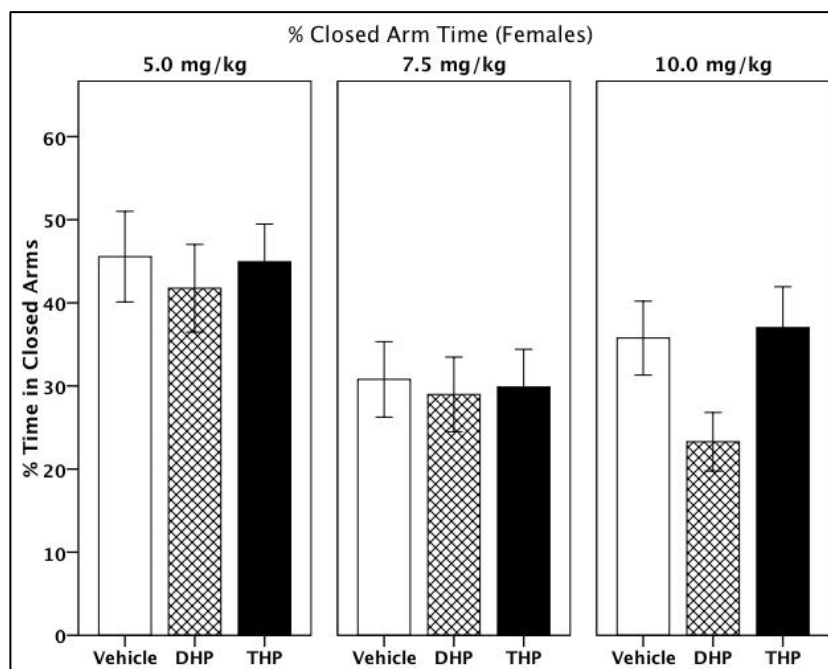


Figure 2.38 Percent of time females spent in closed arms. The y-axis represents the percentage of the 3-minute trial time spent in the closed arms. There were no significant differences related to the type of drug treatment or the dose used. White bars, vehicle; hatched bars, DHP; black bars, THP. 2-way ANOVA (drug*dose). Mean ± SE.

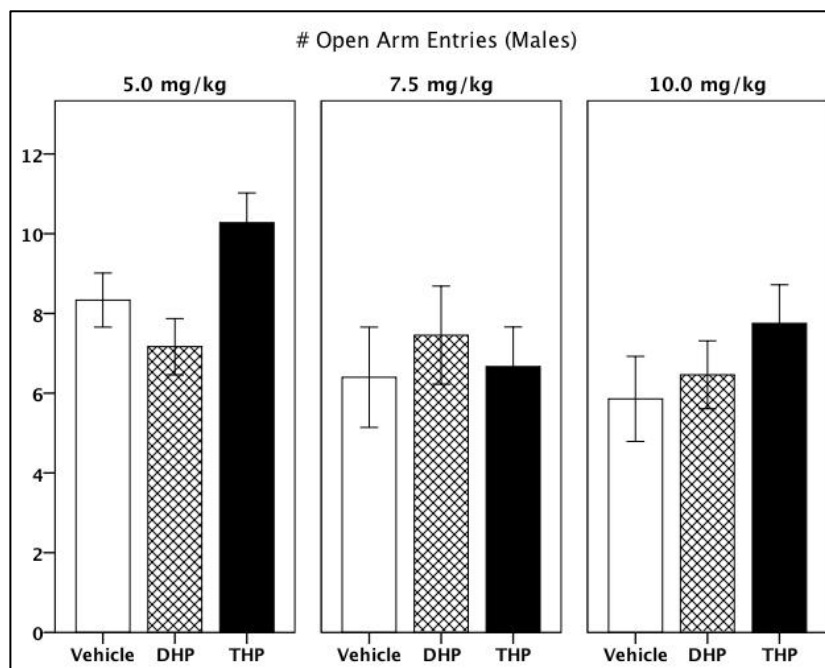


Figure 2.39 Male open arm entries. The y-axis represents the number of open arm entries. There were no significant differences related to the type of drug treatment or the dose used. White bars, vehicle; hatched bars, DHP; black bars, THP. 2-way ANOVA. Mean $\pm$ SE.

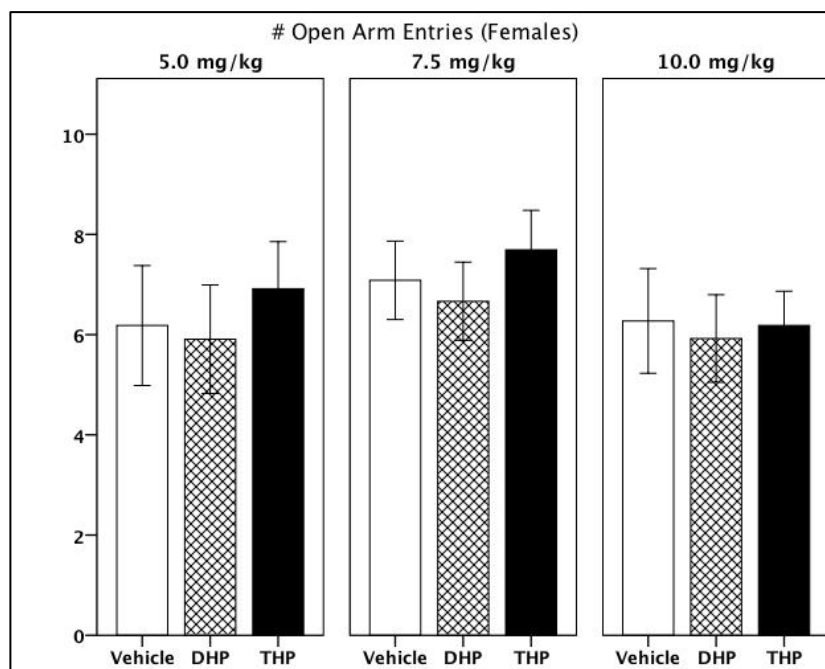


Figure 2.40 Female open arm entries. The y-axis represents the number of open arm entries. There were no significant differences related to the type of drug treatment or dose used. White bars, vehicle; hatched bars, DHP; black bars, THP. 2-way ANOVA. Mean $\pm$ SE.

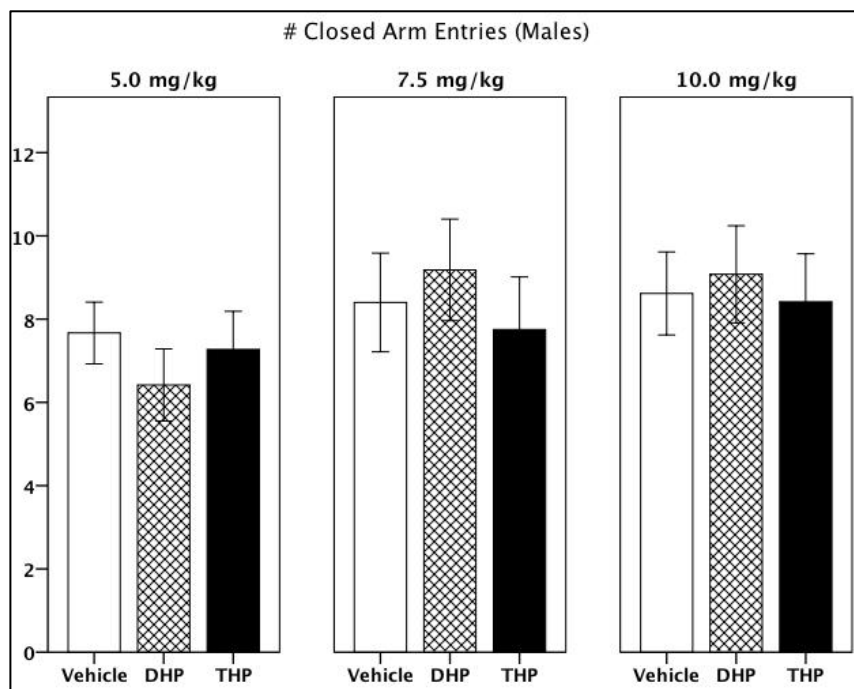


Figure 2.41 Male closed arm entries. The y-axis represents the number of closed arm entries. There were no significant differences related to the type of drug treatment or the dose used. White bars, vehicle; hatched bars, DHP; black bars, THP. 2-way ANOVA (drug*dose). Mean $\pm$ SE.

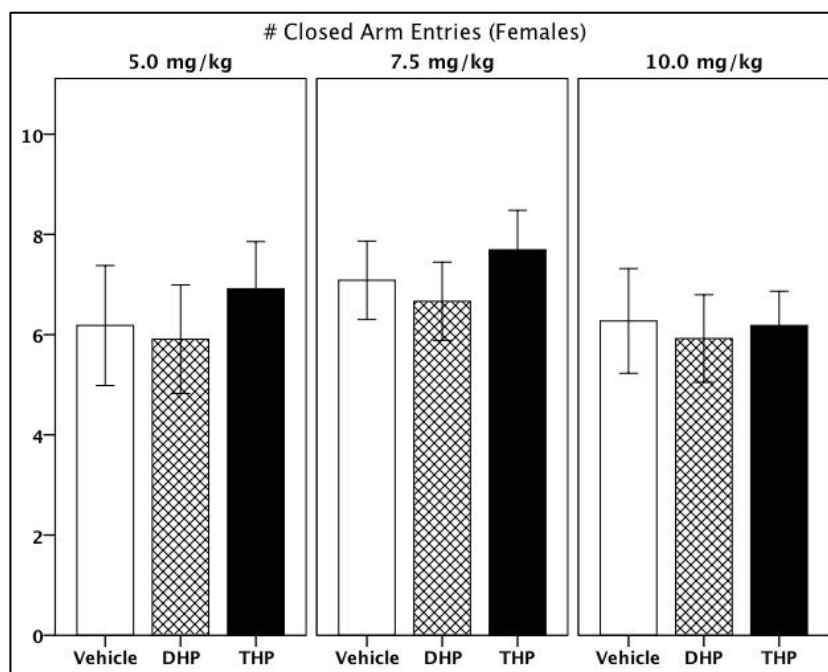


Figure 2.42 Female closed arm entries. The y-axis represents the number of closed arm entries. There were no significant differences related to the type of drug treatment or the dose used. White bars, vehicle; hatched bars, DHP; black bars, THP. 2-way ANOVA (drug*dose). Mean $\pm$ SE.

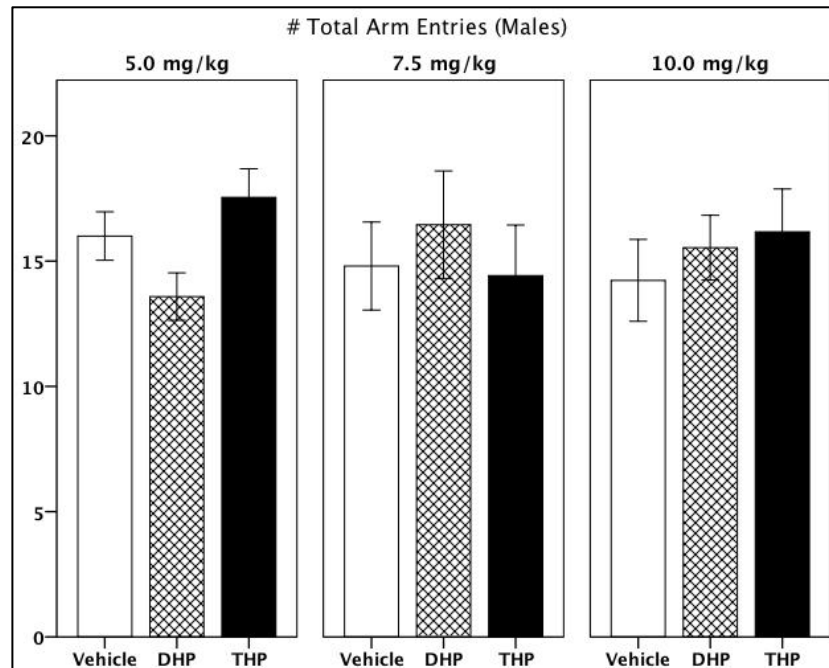


Figure 2.43 Male total arm entries. The y-axis represents the total number of arm entries. There were no significant differences related to the type of drug treatment or the dose used. White bars, vehicle; hatched bars, DHP; black bars, THP. 2-way ANOVA (drug*dose). Mean $\pm$ SE.

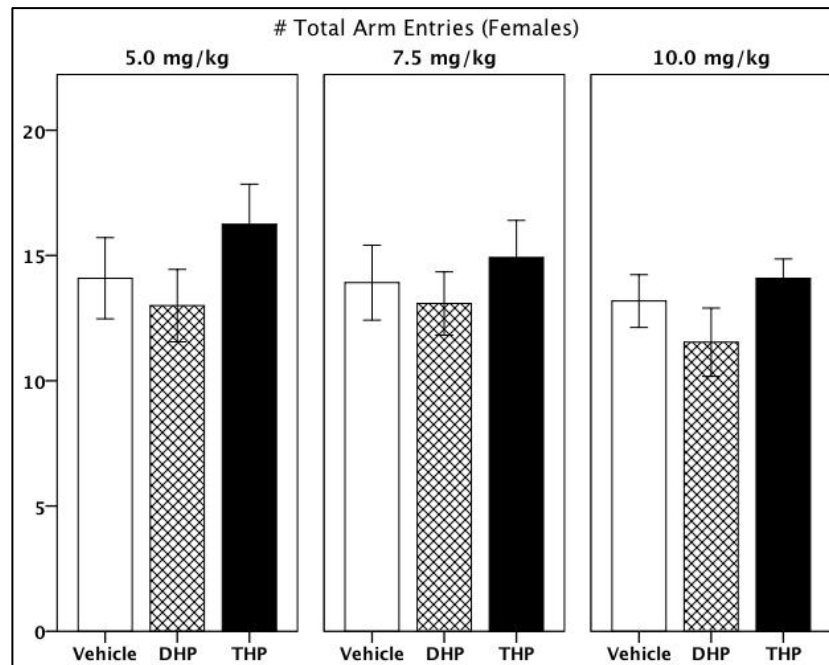


Figure 2.44 Female total arm entries. The y-axis represents the total number of arm entries. There were no significant differences related to the type of drug treatment or the dose used. White bars, vehicle; hatched bars, DHP; black bars, THP. 2-way ANOVA (drug*dose). Mean $\pm$ SE.

<i>EPM Measure</i> (n=10-14 per treatment group)	<i>Male Statistic</i> 2-way ANOVA, p-value Vehicle: Mean±SE DHP: Mean±SE THP: Mean±SE Figure	<i>Female Statistic</i> 2-way ANOVA, p-value Vehicle: Mean±SE DHP: Mean±SE THP: Mean±SE Figure
Open arm time: Increased open arm time indicates decreased anxiety (s)	Drug*Dose: p=0.168 <u>5 mg/kg</u> Vehicle: 142.325±18.193 DHP: 133.875±22.47 THP: 175.409±13.521 <u>7.5 mg/kg</u> Vehicle: 105.690±17.333 DHP: 130.636±22.476 THP: 84.550±19.669 <u>10 mg/kg</u> Vehicle: 88.114±15.046 DHP: 81.877±15.693 THP: 113.600±16.133 Fig 2.35	Drug*Dose: p=0.990 <u>5 mg/kg</u> Vehicle: 89.400±18.288 DHP: 83.472±12.463 THP: 105.100±16.094 <u>7.5 mg/kg</u> Vehicle: 130.858±15.005 DHP: 123.508±14.417 THP: 136.533±15.005 <u>10 mg/kg</u> Vehicle: 111.736±15.673 DHP: 108.364±15.673 THP: 130.338±14.417 Fig 2.36
Closed arm time: Increased closed arm time indicates increased anxiety (s)	Drug*Dose: p=0.175 <u>5 mg/kg</u> Vehicle: 86.825±10.634 DHP: 98.100±16.996 THP: 73.227±10.846 <u>7.5 mg/kg</u> Vehicle: 111.210±12.706 DHP: 107.700±13.594 THP: 134.508±21.566 <u>10 mg/kg</u> Vehicle: 149.749±19.108 DHP: 144.877±19.180 THP: 114.275±18.517 Fig 2.37	Drug*Dose: p=0.629 <u>5 mg/kg</u> Vehicle: 136.664±16.341 DHP: 141.754±12.841 THP: 125.254±15.831 <u>7.5 mg/kg</u> Vehicle: 92.358±13.217 DHP: 89.615±12.698 THP: 86.950±13.217 <u>10 mg/kg</u> Vehicle: 107.282±13.805 DHP: 111.073±13.805 THP: 69.838±12.698 Fig 2.38
Open arm entries: Higher entries indicate decreased anxiety	Drug*Dose: p=0.946 <u>5 mg/kg</u> Vehicle: 8.330±0.678 DHP: 7.170±0.705 THP: 10.270±0.752 <u>7.5 mg/kg</u> Vehicle: 6.400±1.258 DHP: 7.450±1.231 THP: 6.670±0.995 <u>10 mg/kg</u> Vehicle: 5.860±1.068 DHP: 6.460±0.852 THP: 7.750±0.970 Fig 2.39	Drug*Dose: p=0.989 <u>5 mg/kg</u> Vehicle: 6.180±1.197 DHP: 6.730±1.010 THP: 5.910±1.083 <u>7.5 mg/kg</u> Vehicle: 7.083±0.833 DHP: 7.692±0.800 THP: 6.667±0.833 <u>10 mg/kg</u> Vehicle: 6.273±0.870 DHP: 6.182±0.870 THP: 5.923±0.800 Fig 2.40

Closed arm entries: Higher entries indicate increased anxiety	Drug*Dose: p=0.990 <u>5 mg/kg</u> Vehicle: 7.670±0.742 DHP: 6.420±0.866 THP: 7.270±0.915 <u>7.5 mg/kg</u> Vehicle: 8.400±1.185 DHP: 9.180±1.220 THP: 7.750±1.262 <u>10 mg/kg</u> Vehicle: 8.620±0.997 DHP: 6.42±1.168 THP: 8.420±1.151 Fig 2.41	Drug*Dose: p=0.915 <u>5 mg/kg</u> Vehicle: 7.91±0.889 DHP: 9.820±1.007 THP: 7.090±0.595 <u>7.5 mg/kg</u> Vehicle: 6.833±0.939 DHP: 7.231±0.902 THP: 6.417±0.939 <u>10 mg/kg</u> Vehicle: 6.909±0.980 DHP: 7.909±0.980 THP: 5.615±0.902 Fig 2.42
Total arm entries: Spontaneous locomotion	Drug*Dose: p=0.939 <u>5 mg/kg</u> Vehicle: 16.000±0.969 DHP: 13.580±0.949 THP: 17.550±1.139 <u>7.5 mg/kg</u> Vehicle: 14.800±1.756 DHP: 16.450±2.146 THP: 14.420±2.024 <u>10 mg/kg</u> Vehicle: 14.230±1.634 DHP: 15.540±1.294 THP: 16.170±1.709 Fig 2.43	Drug*Dose: p=0.982 <u>5 mg/kg</u> Vehicle: 14.090±1.621 DHP: 16.55±1.718 THP: 13.000±1.446 <u>7.5 mg/kg</u> Vehicle: 13.917±1.292 DHP: 14.923±1.242 THP: 13.083±1.292 <u>10 mg/kg</u> Vehicle: 13.182±1.350 DHP: 14.091±1.350 THP: 11.538±1.242 Fig 2.44

Table 3. Results from the EPM.

2.4 Chapter Discussion

2.4.1 Forced Swim and Open Field Test

In the FST and OFT, no significant drug differences related to the type of drug treatment were found. This is a novel finding for DHP, since there is no literature that we are aware of. Contrary to our findings, other investigators have reported THP to have antidepressant-like effects in rodents in the FST (Khisti & Chopde, 2000; Hirani et al, 2002; Contreras et al, 2011; Shirayama et al, 2011; Evans et al, 2012). These experiments used very different procedures, however, than the ones in the present study. Our findings likely differ due to different species and methods (Bogdanova et al, 2013). For example, mice were used in the studies of Khisti & Chopde (2000) and Harani et al (2002). In one FST study using low doses of 0.5, 1.0, and 2.0 mg/kg of THP, Khisti & Chopde (2000) reported that all three doses of THP, injected i.p., significantly reduced immobility in the FST in mice. Models of depression used also differed. Hirani et al used (2002) an alcohol-withdrawal induced model of depression in the FST. Shirayama et al (2011) used the electric foot-shock/inescapable stress model of depression in rats. Evans et al (2012) found that THP was an effective antidepressant in a social isolation model of depression.

In addition to these species and model differences, routes of injection also differed. Evans et al (2012) used subcutaneously implanted pellets that released 60 mg of THP over 30 days. Hirani et al (2002) and Shirayama et al (2011) injected 0.05-1.00 µg of THP/mouse via intracerebroventricular injections; it is unclear what a similar in vivo dose could be, or how to compare it to the present

studies (acute i.p. injection of 5 mg/kg, 15 minute injection-test latency). In a study of the time-course of THP's antidepressant effect in rats, Contreras et al (2011) used rats in the FST injected with 1 mg/kg of THP. Contreras et al (2011) found that 1 mg/kg of THP did have an antidepressant-like effect in the FST in rats beginning at 30 minutes post-injection. Khisti & Chopde also found antidepressant-like effects of 0.5-2 mg/kg of THP 30 minutes post-injection. Our latency of 15 minutes was chosen based on the time-response curves determined in previous experiments in rats (Lonsdale et al, 2006; Lonsdale & Burnham, 2007). It is possible that no antidepressant-like effect was observed in our FST study due to the difference between optimal time-response of THP's anticonvulsant effects (15 minutes) and its antidepressant-like effects (30 minutes).

The OFT is often done in conjunction with the FST to show that changes in activity in the FST are not due to changes in the animals' general levels of activity (Porsolt et al, 1978; Bogdanova et al, 2013). In the present experiments, changes in motor behavior in the OFT were not observed following administration of 5 mg/kg of THP or DHP to either male or female rats. As mentioned above, we are not aware of any OFT studies of DHP. The results of an OFT experiment that tested the locomotor effects of THP, however, support our results. Frye & Sturgis (1995) assessed ovariectomized female rats in the OFT, and found that neither 3.2 mg/kg nor 6.4 mg/kg of THP affected locomotion as compared to vehicle (Frye & Sturgis, 1995). In our FST/OFT study, we did not find significant effects of anticonvulsant dose of THP (5 mg/kg). In this respect, our OFT results are in good agreement with those of Frye & Sturgis (1995). There were some differences between the drug

administration protocol used by Frye & Sturgis compared to those of the present studies. In the Frye & Sturgis experiment, the injection-test latency THP was 30 minutes, and injections were subcutaneous. Our results may be in agreement despite these differences due to different drug distribution and time course of action of subcutaneous versus i.p. injections.

In general, our results in the FST and OFT are consistent with other FST and OFT experiments using similar doses, test-injection intervals, and protocols. Our results with DHP, however, are novel.

2.4.2 Morris Water Maze

Due to the complexity of the findings, MWM results will be recapitulated here. At the anticonvulsant dose of 5 mg/kg, DHP and THP did not have many significant effects on learning or memory in the MWM. In both males and females, drug treatment did not affect measures of the acquisition of a new memory, learning, working memory or reference memory (**Figs 2.7-2.18**). Velocity and spatial memory were the only measures affected by DHP and THP. These effects differed in males and females (**Figs 2.19-2.34**).

In males, there were significant differences in swimming velocity in day 1 (ANOVA $p=0.000$) with THP treatment relative to vehicle (Tukey's HSD *post hoc* $p=0.000$, **Fig 2.19**). Also in males, velocity in the presence of drug (day 4) was significantly different by drug treatment (ANOVA $p=0.002$; **Fig 2.21**); this time, however, DHP treatment was associated with slower velocity as compared to vehicle (Tukey's HSD *post hoc* $p=0.002$), but THP was not. DHP treatment in males

was associated with decreased velocity in the presence of drug on day 4, but not day 1, and THP treatment was associated with decreased velocity in the presence of drug on day 1, but not on day 4. It is possible that DHP treatment did not affect velocity when learning a new task (day 1), but did affect velocity in a well-learned task (day 4); it is also possible that THP treatment affected velocity in learning a new task (day 1), but not a well-learned task (day 4). Males that were given THP treatment on day 1 also had decreased velocity on day 3 (ANOVA $p=0.013$, **Fig 2.25**) relative to DHP (Tukey's HSD *post hoc* $p=0.009$), but not vehicle. It is not immediately clear how DHP and THP had these effects on velocity 48 hours after treatment in males. It is possible that THP, but not DHP, had some longer-lasting effects that decreased velocity in males 48 hours after drug treatment.

In females, drug treatment on day 4 was not associated with a velocity change on day 4 (**Fig 2.22**). THP treatment on day 4, however, was associated with decreased velocity on day 5, 24 hours after the drug was administered (ANOVA $p=0.005$; **Fig 2.28**), as compared to both vehicle (Tukey's HSD *post hoc* $p=0.010$) and DHP (Tukey's HSD *post hoc* $p=0.020$). It is possible that THP, but not DHP, had some longer-lasting effects that decreased velocity in females 24 hours after drug treatment. In another spatial memory measure, in female animals, unlike in male animals, THP treatment on day 4 resulted in a decreased latency to platform on day 5 in trials 1-4 (ANOVA $p=0.028$; **Fig 2.28**). THP-treated female animals were significantly slower compared to vehicle (Tukey's HSD *post hoc* $p=0.023$), but not compared to DHP (Tukey's HSD *post hoc* $p=0.613$). This suggests that, in female animals, spatial memory recall could be improved by treatment with anticonvulsant

doses of THP the previous day (~24 hours), whereas DHP had no significant effects. It is possible that THP had some longer-lasting effects that improved spatial memory in females.

There are no studies that we are aware of that have tested DHP in the MWM. Our results with DHP, therefore, are novel. There are studies, however, that have tested THP in the MWM. Our finding that THP in particular did not affect many measures in the MWM may seem to conflict with other studies (Frye & Sturgis, 1995; Johansson et al, 2002). These experiments, however, used procedures that were quite different from ours. Future experiments might be able to determine whether these differences in procedures caused different results in the present studies and those of Frye & Sturgis (1995) and Johansson et al (2002).

For example, in an experiment using rats, Frye & Sturgis (1995) reported that 3.2 mg/kg and 6.4 mg/kg of THP significantly decreased latency as compared to vehicle-treated animals. In contrast, we found that 5 mg/kg of THP did not affect affect the latency to platform. We propose that different procedures likely account for these differing results. For example, there were differences in the injection/test interval used for THP. Frye & Sturgis (1995) used a 30 minute interval, whereas we used a 15 minute interval. In addition, we tested animals in the “lights on” period of the animals’ diurnal cycle, whereas Frye & Sturgis (1995) tested animals in the “lights off” phase of the diurnal cycle, when rats are more active. Finally, the MWM protocol that Frye & Sturgis (1995) used also differed from that used in the present experiment, since it involved only two days of testing.

Definitions of spatial and reference memory and experimental protocols also differed. Frye & Sturgis (1995) define spatial memory as the latency to platform in the absence of drug (day 1) compared to latency to platform in the presence of drug (day 2), when the platform was not moved from day 1 (no drug). This definition of spatial memory is unlike our own, which was the latency to platform on day 5 trials 1-4, one day after drug administration, when the platform has not been moved. The measures of spatial memory, therefore, were different between the present experiments and those of Frye & Sturgis (1995). Reference memory was also defined and measured differently as compared to the present experiments. Frye & Sturgis (1995) used one definition for two measures: they used the same definition for “reference/spatial memory,” measured as the latency to platform in the absence of drug (day 1 of their protocol) compared to latency to platform in the presence of drug (day 2 of their protocol), when the platform was not moved (Frye & Sturgis, 1995). The measures of reference memory in the present experiments differed, as per Schwabe et al (2007): one measure of reference memory was whether the animals swam directly to the unchanged platform position from the beginning of trials 1-4 on day 5; the other measure was, on day 4 in the presence of drug treatment, how many crossings animals made of the previously correct platform position (day 3).

The results reported by Johansson et al (2002) also differ from our findings. In their study, Johansson et al (2002) intravenously injected 2 mg/kg of THP, for 11 consecutive days over the course of a 9-day experimental protocol. They found that this “chronic” administration of THP impaired the average latency to

platform over 9 days of testing (Johansson et al, 2002). We might also have found these effects if we had administered THP chronically.

We are aware of one other study of the effects of THP on learning that supports our findings. In Matthews et al's (2002) MWM study in male rats, they found that 17.5 or 20 mg/kg of THP impaired the use of spatial memory in rats, but 12 mg/kg of THP did not, 20 minutes after i.p. injection (Matthews et al, 2002). These data agree fairly well with our data, since we found no effects of 5 mg/kg of THP on spatial memory in male rats. Our dose of THP, 5 mg/kg, is well below the dose that Matthews et al (2002) found no effects on spatial memory in the MWM (12 mg/kg).

2.5.3 Elevated Plus Maze

At the anticonvulsant doses tested (5, 7.5 and 10 mg/kg) DHP and THP were not anxiogenic or anxiolytic as compared to vehicle controls when administered 15 minutes before testing in the EPM. Other investigators, however, have found THP to be a potent anxiolytic at doses between 5-10 mg/kg (Picazo & Fernandez-Gausti, 1995; Rodgers & Johnson, 1998; Reddy et al, 2005; Kita et al, 2008). Differences in species and procedures may explain our differing results.

Reddy et al (2005) report significant anxiolytic effects of THP at doses of 2 and 5 mg/kg, using a 15-minute test-injection interval. Reddy et al (2005), however, used mice and a subcutaneous route of injection. Kita et al (2008) also reported anxiolytic effects of 10 mg/kg of THP, using a 20-minute test-injection interval. Kita et al (2008), however, used mice, a subcutaneous route of injection,

and a social interaction model of anxiety. It is possible that differences in species, route of injection and anxiety model account for differing results in the present study.

Our results are consistent with the previous report of Rodgers & Johnson (1998) that DHP (10-20 mg/kg, i.p. injection-test interval, 10 minutes) was not anxiolytic in the EPM when tested in mice. Our results with THP, however, differ from those of Rodgers & Johnson (1998), who found that 10 mg/kg of THP (injection-test interval, 10 minutes) significantly decreased anxiety behaviors in mice. Rodgers & Johnson (1998) also found that 5 and 10 mg/kg of THP increased open arms entries, and 10 mg/kg of THP increased percent of open arm time in mice. Species differences may account for the differing findings in the present studies as compared to the study of Rodgers & Johnson (1998).

Our results are consistent with those of Picazo & Fernandez-Gausti (1995), who assessed the anxiolytic effects of 1-4 mg/rat of DHP, and 0.12-1.0 mg/rat of THP, in the defensive burying test of anxiety in ovariectomized female rats, and found no anxiolytic effects. Although they used ovariectomized subjects and a different anxiety model, Picazo & Fernandez-Gausti (1995) used the same species and strain of rat and similar doses. Their “per rat” doses translate to ~4-16 mg/kg of DHP 0.48-4 mg/kg of THP (the authors report rat weights of 200-250 g). Hence, our findings of no anxiolytic effects of 5 mg/kg of DHP and THP are consistent with the finding of no effects of 4-16 mg/kg of DHP and 4 mg/kg of THP in the study of Picazo & Fernandez-Gausti (1995). In the study of Picazo &

Fernandez-Gausti's (1995), however, a 4-hour injection-test interval was used. This might have allowed DHP to metabolize to THP.

2.5 Summary

Anticonvulsant doses of DHP and THP in male and female rats did not produce depressant/antidepressant effects in the FST, locomotor effects in the OFT, or anxiogenic/anxiolytic effects in the EPM. Only spatial memory and velocity in the MWM were significantly different relative to the type of drug treatment. No other measures of learning and memory were affected by drug treatment in the MWM. This suggests that DHP and THP might be developed as effective anticonvulsants without attendant psychotoxicity.

2.6 Limitations

There are some limitations to these experiments. The first is the lack of a positive control. In future experiments positive control drugs, such as fluoxetine (antidepressant) in the FST, propranolol (impairs learning) in the MWM, and midazolam (anxiolytic) in the EPM might be used. Another option would be to compare the behavioral effects of clinically relevant doses of anticonvulsant medications in each of these behavioral tests.

Secondly, these experiments were done in naïve subjects rather than kindled subjects. There are neurobiological differences between kindled and non-kindled animals (Goddard et al, 1969; Francis et al, 2002; Morimoto et al, 2004; Gavrilovici et al, 2006). The experiments that showed promising effects of DHP

were done in amygdala-kindled male and female rats. The behavioral experiments, however, were done in animals that had not been kindled. Kindled rats could possibly perform differently in behavioral tests than rats that have not been kindled.

The third limitation to our experiments relates to the extensive handling paradigm used. Subjects were handled for 5 minutes a day, 5 days a week for 4 weeks before testing. Most rats are not handled so extensively before behavioral experiments (Hogg, 1996; Khisti & Chopde, 2000; Rodríguez-Landa et al, 2007). Handling is a form of environmental enrichment for laboratory animals; it reduces stress, affects animal neurobiology, behaviors and drug responses (Wade & Maier, 1986; Boix et al, 1990; Andrews & File, 1993; Hogg, 1996; Schmitt & Hiemke, 1998; van Praag et al, 2000; Moncek et al, 2004; Bayne, 2005; Hutchinson et al, 2005; McNair et al, 2007; VanElzakker et al, 2008; Bortolato et al, 2011; Bogdanova et al, 2013). Results in the EPM in particular are significantly affected by handling protocols in rats (Hogg, 1996). Our handling protocol may have reduced baseline levels of anxiety in the rats, and thereby affected our results (Whimby & Denenberg, 1967; Hogg 1996), such that anxiolytic effects of DHP and/or THP could not be seen in the present experiments. The effects of GABAergic drugs in behavioral tests are also attenuated with extended rodent handling (Boix et al, 1990; Andrews & File, 1993). Since THP acts at GABA_A receptors (Belelli & Lambert, 2005), it is possible that our extended handling protocol attenuated THP's GABAergic effects on mood, memory and anxiety.

Future studies should address the above limitations. Handling should be reduced to a level more typical of laboratory rats in behavioral experiments. Kindled animals or animals in models of seizures and/or epilepsy should be tested. Positive pharmacological controls appropriate for behavioral tests should be used. Future studies should also study the effects of chronic administration of DHP and THP in behavioral models, since in humans anticonvulsants are administered on a chronic basis.

Chapter 3: Electrophysiological Studies In Vivo

This experimental series was conducted in the laboratory of Dr. Liang Zhang, who acted as my co-supervisor.

I designed and conducted this experimental series. Dr. Chiping Wu lent his surgical expertise to develop an appropriate surgery technique. After a suitable surgery method had been established, I conducted hippocampal kindling, made EEG recordings and interpreted them, scored behavioral seizures, analyzed data, and wrote the paper based on the results in this chapter and the results in vitro (Chapter 4). Jonathan Gane and Min Lang assisted with kindling, recording, and drug preparation.

The published paper describing the novel surgical method designed for the purpose of these experiments is in **Appendix 3** of this thesis:

Melanie Jeffrey, Min Lang, Jonathan Gane, Chiping Wu, W. McIntyre Burnham, Liang Zhang. 2013. A reliable method for intracranial electrode implantation and chronic stimulation in the mouse brain. BMC Neuroscience 14:82.
<http://www.biomedcentral.com/1471-2202/14/82>

The published paper of the current experiments' results in vivo and in vitro (chapter 4) is in **Appendix 4** of this thesis:

M. Jeffrey, M. Lang, J. Gane, E. Chow, C. Wu and L. Zhang. 2014. Novel anticonvulsant effects of progesterone in a mouse model of hippocampal electrical kindling. Neuroscience 257: 65-75.
<http://dx.doi.org/10.1016/j.neuroscience.2013.1074>

3.1 Introduction

To further study the anticonvulsant effects of progesterone and its metabolites, we tested them in hippocampal-kindled mice. Mice were used because they are often used in electrophysiological experiments, and they are also the species of choice in experiments involving genetic manipulation. The hippocampus was chosen as a kindling site for three reasons: 1] we had the most success with our novel kindling technique when we stimulated and recorded from the hippocampus, 2] the hippocampal formation is often used in electrophysiological studies in brain slices *in vitro*, and 3] the hippocampus is particularly prone to generating epileptiform activity (McCormack & Contreras, 2001).

As indicated in Chapter 1, progesterone is a known anticonvulsant, with its inhibitory effects generally attributed to its secondary metabolite, THP. THP powerfully enhances the activity of the GABA_A receptors. Accumulating evidence, however, suggests that progesterone may have inhibitory effects that are independent of the GABA_A receptor (Zhu et al, 2008; Hwang et al, 2009; Zheng 2009; Johannessen et al, 2011; Luoma et al, 2011). In particular, Kokate et al (1999) have reported that the protective effects of high doses of progesterone (250-350 mg/kg) in the maximal electrical shock (MES) seizure model are not abolished by pretreatment with finasteride (up to 300 mg/kg). Also in the MES model, Reddy et al (2004) reported that five progesterone receptor knock out mice were protected from seizures by high doses of progesterone (200 mg/kg) in the presence of finasteride (100 mg/kg). These studies might indicate that progesterone has THP-independent anticonvulsant effects.

In the present study, therefore, we examined the effects of progesterone in kindled mice with or without pretreatment with finasteride, a *5 α -reductase* inhibitor

known to block the metabolism of progesterone to DHP and THP (Finn et al, 2006). The actions of the progesterone metabolites DHP and THP were also studied. Adult male mice were electrically kindled via a daily stimulation protocol. EEG discharges were recorded from the contralateral hippocampus or cortex to assess “focal” and “generalized” seizure activity, and motor seizure stages were scored (Albright & Burnham, 1980; McIntyre, 2006; Löscher, 2011). Kindled mice were treated with intraperitoneal (i.p.) injections of progesterone with or without finasteride pretreatment, and with the progesterone metabolites DHP and THP. The anticonvulsant drugs midazolam and carbamazepine served as positive controls. The effects of different treatments on hippocampal and/or cortical AD durations and motor seizure stages were compared.

3.2 Methods

The methods described in this section have been published previously (Jeffrey et al, 2013, **Appendix 3**; Jeffrey et al, 2014; **Appendix 4**).

3.2.1 Animals

Male C57 black mice (Charles River Laboratory, Quebec, Canada), between 6 and 10 months of age, were used in the present study. Male animals were used to avoid complications from the cyclic fluctuation of progesterone and its metabolites in female animals. Each subject was reused for each treatment condition, and, hence, each animal served as its own control. Animals were housed in a vivarium that was maintained at 22 °C with a 12-hour light/dark cycle (lights on at 6:00 am). Food and water were available ad libitum. All experiments were conducted between 10 am and 5 pm. All experimental procedures were reviewed and approved by the Animal Care Committee of the University Health Network, in accordance with the guidelines of the Canadian Council on Animal Care.

3.2.2 Drugs

Progesterone, DHP, THP, carbamazepine, finasteride and β -cyclodextrin were obtained from Sigma–Aldrich (Oakville, Ontario, Canada). Midazolam was obtained in clinically injectable form from Sandoz Canada Inc. (Quebec, Canada). Progesterone, DHP, THP, and finasteride were freshly prepared on the day of treatment and dissolved in physiological saline containing 45% β -cyclodextrin. These solutions were sonicated at ~40°C until the compounds had completely dissolved. Carbamazepine was initially

dissolved in dimethyl sulfoxide (DMSO) and then diluted into the β -cyclodextrin solution. The final amount of DMSO was 630 μ L in individual animals with body weights ranging from 29 to 35 g. All drugs were administered via intra-peritoneal (i.p.) injections, with a total volume 0.2–0.3 mL. Vehicle injections were made with 0.3 mL of the β -cyclodextrin solution. Neurosteroids or anticonvulsant drugs were injected at one dose per test day. The same group of animals was reused for all drug testing, thus treatments were spaced 2–3 days apart to allow complete elimination of the compounds.

Progesterone at 10, 35, 100 or 160mg/kg was injected 30 minutes before hippocampal stimulation. DHP at 5 and 10 mg/kg was injected 15 minutes before stimulation. THP at 1, 3.5, 10 or 30 mg/kg was injected 15 minutes before hippocampal stimulation. These doses and injection protocols were based on previous studies in male rats (Lonsdale et al, 2006) and male mice (Singh et al, 2010). Finasteride at a dose of 50 mg/kg was injected 3 hours before hippocampal stimulation, as this interval has been shown to reduce THP levels in the brain after progesterone injection (Finn et al, 2006; Ciriza et al, 2006; Mukai et al, 2008). Clinically relevant doses of midazolam (2 mg/kg) and carbamazepine (50 mg/kg) were injected 15 minutes before hippocampal stimulation (Higgins et al, 2010; Dihr & Rogawski, 2011).

3.2.3 Electrode Construction

All electrodes were made of polyamide-insulated stainless steel wires (outer diameter 200 μ m, Plastics One, Ranoake, VA). Twisted bipolar wires were used for stimulation and recording, with their intracranial tips $\sim$ 100 μ m apart. The extracranial tips of the twisted wire assembly were soldered to the female ends of two connecting pins,

with one wire bent into an L-shape to separate the connecting pins (**Fig 3.1A**). Care was taken to fully remove the insulation layer before soldering. A liquid solder (Soldering Liquid Flux, Certanium Alloys and Research Company, Cleveland OH, USA) or weak phosphoric acid was used to ensure good contact between the stainless steel wire and the connecting pin. A single wire was similarly soldered to a single pin to serve as a ground electrode. The resistance of each constructed wire electrode was $\leq 1.0 \Omega$. After being soldered to the connecting pins, the bipolar wires were then cut to ~ 3 mm in length to target the desired hippocampal cornu ammonis area 3 (CA3; see below). The single monopolar wire was cut to ≤ 0.5 mm for the epidural position of the ground electrode. These electrodes were cleaned with 75% alcohol and stored in a sterilized glass vial until use.

The connecting pins were detached from standard IC sockets (Samtec series SS socket strips, SS-132-G2, Electrosonic, Toronto, Ontario, Canada). These pins are 7.5 mm long in total, and the male end of the pin is 3.2 mm long. The outer diameter is 1.8 mm and 0.5 mm for the female and male ends of the pin, respectively (**Fig 3.1A**). Measured after being dissected out from implanted animals ($n = 3$), the total weight of the implanted electrode assembly, including electrode wires, plastic base and dental acrylic (**Fig 3.1B**), was 0.50-0.52 grams. As adult mice were used in the present experiments, the weight of the implanted electrode assembly was $\leq 2\%$ of the animals' body weights.

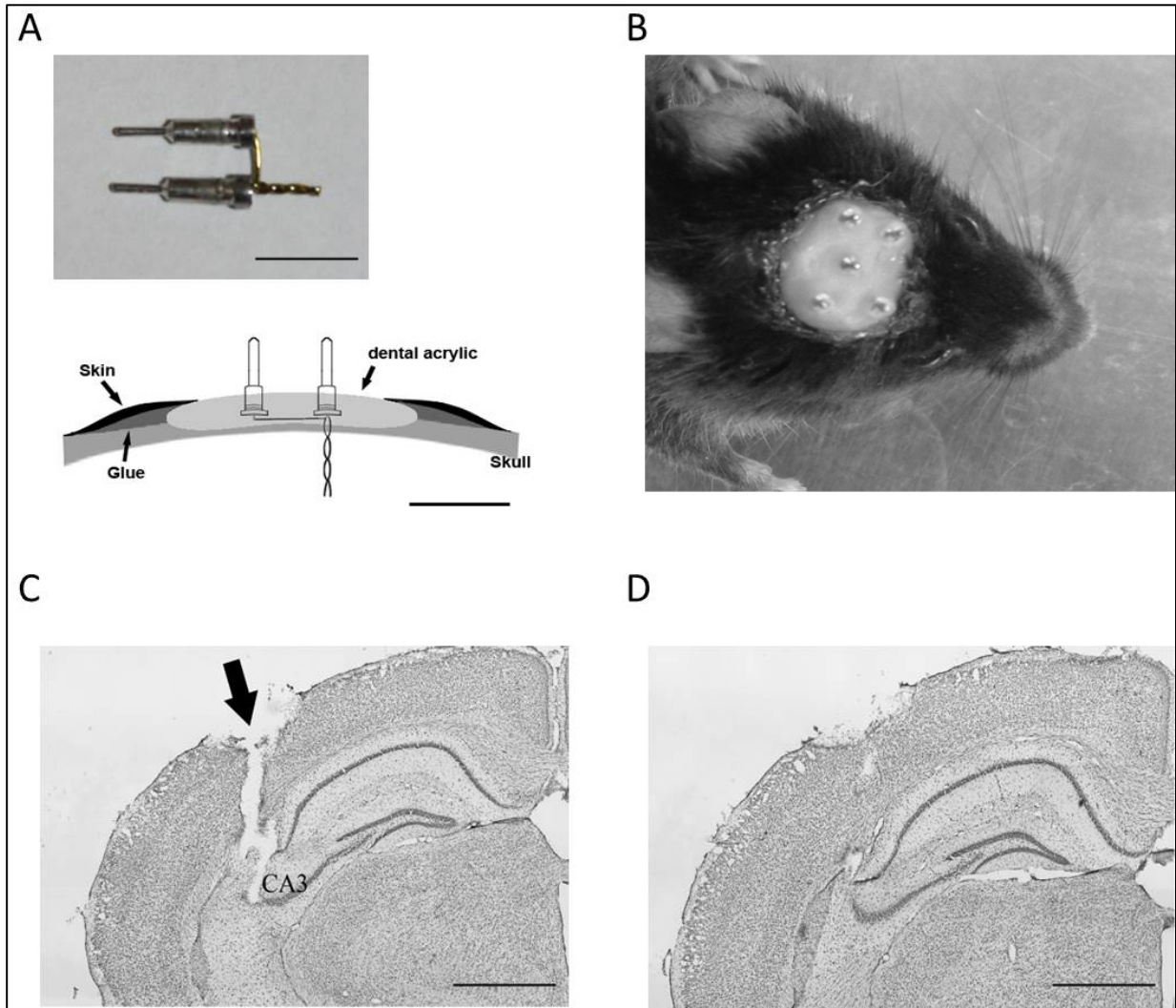


Figure 3.1 Electrode design and histological confirmation of CA3 electrode placement. *A*, a schematic illustration of the electrode mounting and an image of the connecting pins and a bipolar electrode. *B*, a photograph of a subject implanted. *C-D*, images of adjacent brain sections collected from a mouse. The animal was euthanized 3 months after electrode implantation. Note the track of an implanted CA3 electrode (arrowed, *C*) and the densely packed hippocampal neurons in the area near the implanted electrode and in the adjacent section (*D*). Calibrations: 5 mm in *A* and 1 mm in *C-D*.

3.2.4 Surgery for Electrode Implantation

Stimulating and recording electrodes were implanted in the right and left CA3 fields of the hippocampus, and a ground electrode was positioned over the right frontal cortex. Surgical procedures were modified from those previously described (Wu et al, 2008; El-Hayek et al, 2011; Jeffrey et al, 2013). A modified version of a screw-free, glue-based method was used to secure the implanted electrodes. Briefly, the animal was anaesthetized with 2% isoflurane and then placed in a stereotaxic frame, and was held on a horizontal plane by a mouse adaptor (model 926, David Kopf Instruments, Tujunga, CA, USA) that allows for a modified mask for administration of the anaesthetic. After skin incision and exposure of the skull surface, the tip of a mini drill bit (see below) was positioned over bregma via a micromanipulator. After determining the position of bregma, the drill bit was elevated, but its X-Y position was not changed. A thin plastic base then was glued onto the skull surface.

The plastic base was cut from a curved part of plastic weighing boats (polystyrene antistatic weighing dishes, Fisher Scientific, cat #08-732-115). The weighing boats were 140x140 mm in length-width and 25 mm in depth, with a thickness of ~200 μm . The plastic base was soft and could be gently pressed to accommodate the curvature of the skull. Thus, the plastic base was tightly bound to the skull surface after being glued. We used cyanoacrylate glue (Insta-cure+, cure time 5–15 seconds, made in U.S.A., cat# BSI-106C; obtained from Canadian Hobbycraft, Concord, Ontario, Canada).

After the glue had cured, three small holes were drilled through the plastic base and the skull according to the stereotaxic coordinates of the hippocampal CA3 area (bregma -2.5 mm, lateral ± 1.3 mm, and a depth of 3.2 mm from the skull surface). The reference electrode was positioned in the frontal lobe (bregma $+1.0$ mm, lateral 2.0 mm

and a depth of 0.7 mm). The electrode depths were adjusted to accommodate for the thickness (200 μm) of the plastic base.

A motorized drill (model FM3545, Foredom Electric, Bethel, CT, USA) and a mini drill bit (part 115603, Ball Mills Carbide, CircuitMedic, Haverhill, MA, USA) were used to drill small holes (≤ 0.5 mm) through the skull. These holes were large enough for inserting the electrodes, but small enough to prevent dental acrylic leakage into the brain (see below).

In some experiments, a bipolar electrode was implanted in CA3, and two monopolar recording electrodes were implanted contralaterally, one in CA3 (coordinates as above) and the other in the neocortex. The coordinates for cortical implantation, relative to bregma, were: 0.6 mm posterior, 1.5 mm lateral and a depth of 1.0 mm from the skull surface. All electrodes were made of polyamide-insulated stainless steel wires (outer diameter 125 μm ; Plastics One, Ranoake, VA).

Micromanipulators were used to individually insert the electrodes into bilateral hippocampal CA3 areas, or over the anterior cortex. After positioning these electrodes with the micromanipulators, dental acrylic was overlaid onto the plastic base such that the bases of the connecting pins were covered by the dental acrylic. Care was taken to apply acrylic so as not to interfere with electrode positions or to contaminate the connecting pins. We used a dental acrylic with hardening time of 6–9 minutes (Jet Tooth Shade, Reference No. 1404; Lang Dental Mfg. Co., Inc., Wheeling, IL, USA) to carefully cement the implanted electrodes. After the dental acrylic had hardened, the electrodes were released from the micromanipulators. The incised skin was then glued to the dental acrylic (**Fig 3.1B**), which prevents infection in the implanted area. The animals were

released from the stereotaxic frame and allowed to recover for at least one week before further experimentation.

3.2.5 Electrical Stimulation

Unilateral CA3 stimulation was conducted in all present experiments. Animals were stimulated from the right CA3 and recorded from the left CA3 and/or cortex. This protocol was used to avoid the switch artifact that results from stimulating and recording from the same site. Constant current square-wave current pulses (duration of 0.5 ms pulses at 60 Hz, intensities of 10–150 μ A, base to peak) were generated from a Grass stimulator and delivered through a stimulus isolation unit (model S88H, Grass Medical Instruments, Warwick RI, USA) before kindling to establish an input-output plot for evoked CA3 field potentials. A single stimulation was applied every 30 seconds at intensities of 10–150 μ A (10 μ A increments; 5 consecutive responses at a given intensity; **Fig 3.2A-B**).

3.2.6 Hippocampal Kindling

A standard kindling protocol (Albright and Burnham, 1980; Reddy and Rogawski, 2010) was used in the present experiments. An ascending stimulation series was used to determine each animal's individual AD threshold. In the ascending series, stimulation (2 second trains of 0.5 ms 60 Hz pulses) was increased from 10 to 150 μ A with 10- μ A increments, spaced 5 minutes apart, until an AD event of at least 5 seconds in duration was observed. The stimulation intensity that elicited an AD event of at least 5 seconds was considered the AD threshold. The AD threshold stimulation intensities were

between 40-100 μ A for individual animals. The mice were then stimulated daily at 125% of their AD threshold. Animals were considered fully kindled when three consecutive stage 5 motor seizures (see below) were elicited. All animals reached criterion within a month after receiving their first stimulation. Animals were fully kindled after 12.6 ± 2.8 (6-26) ADs.

Subsequently, the animals were stimulated at 125% of their AD threshold on all non-trial days to ensure the stability of ADs (<10% fluctuation in duration) and consistent stage 5 motor seizures during the course of drug testing.

3.2.7 Recordings and Measurements

Recordings were taken from the CA3 or cortical area contralateral to the CA3 stimulation site. We choose this recording procedure because bilateral hippocampi communicate effectively via the ventral and dorsal hippocampal fissures (Witter, 2007), permitting reliable discharge spread from the stimulated site to the contralateral hippocampus. ADs were observed in all animals after 1-2 stimulations.

Local differential recordings were made (**Fig 3.1A**) between the tips of the recording bipolar electrode to reduce artifacts and common-ground EEG signals. Monopolar recordings were made if the local differential recordings were unsuccessful, presumably due to shorts between the tips of the twisted bipolar electrodes. As noted above, in some experiments, two monopolar electrodes were implanted in the contralateral CA3 and neocortical areas for simultaneous recordings of hippocampal and neocortical EEG. AD lengths or standard deviations were normalized as percentiles of kindled untreated responses in individual animals. To measure ADs, corresponding EEG

data were treated with a 0.5 Hz high-pass (Bessel) filter to reduce slow drifts in EEG signals.

Signals were recorded via a 2-channel microelectrode AC amplifier (model 1800, AM Systems, Carlsborg, WA, USA), with the input frequency band set in the range of 0.1-1000 Hz, and the amplification gain at 1000 $\times$. The signals were digitized at 5000 Hz (Digidata 1440A, Axon Instruments/Molecular Devices, Union City, CA, USA). Pclamp software (Version 9 or 10; Axon Instruments/Molecular Devices) was used for data acquisition, storage and analyses.

To detect spontaneous interictal-like spikes, individual animals were recorded for up to 4 hours two weeks after surgery, before kindling was initiated. After animals were fully kindled, interictal spikes were recorded for 4–6 hours, beginning 3-4 hours after the last stimulation (**Fig 3.2C-D**). A spike was only counted as present if the animal had been immobile for at least 10 minutes, the spike amplitude was large (≥ 2 times the amplitude of the background signal) and its waveform was similar to those previously described (Kairiss et al, 1984; Leung, 1988; Leung, 1990; Timofeeva & Peterson, 1996).

Kindling is associated with changes in behavioral states (Leung, 1988; Leung, 1990). To measure hippocampal rhythmic activities associated with “active” and “inactive” behaviors, stable data segments of 5–10 seconds or 30–60 seconds were selected while the animals were moving/exploring or immobile/asleep. Spectral plots were generated from these data segments, and peak frequencies were measured from the spectral plots for individual animals (**Fig 3.2E-F**). To minimize interference of movement-related artifacts, the rates of interictal spikes were measured in the periods (1–2 hours) while the animals were immobile/asleep (**Fig 3.2G-H**).

3.2.8 Behavioral Assessments

Animal behaviors were recorded with a high-definition camera and analyzed by experimenters blinded to treatments. Motor seizures were scored using a modification of the Racine (1972) scale for the mouse (Reddy and Rogawski, 2010): stage 0, no response or behavioral arrest; stage 1, chewing; stage 2, chewing and head nodding; stage 3, single or bilateral forelimb clonus; stage 4, bilateral forelimb clonus and rearing; stage 5, loss of righting reflex (falling).

3.2.9 Confirming Electrode Placement: Brain Histology and Electrophysiological Assessments

To ensure correct electrode position, histological assessments were conducted as previously described (Wu et al, 2008; Jeffrey et al, 2013). Briefly, the animals were anesthetized with pentobarbital (70 mg/kg, i.p.) and trans-cardiacally perfused with saline and then with 10% phosphate-buffered formalin solution before decapitation. Cryostat coronal sections 30 μ m thick were obtained throughout the brain, stained with cresyl violet, and examined under a light microscope to verify electrode placement (representative slice in **Fig 3.1C-D**). Histology was performed on six animals. The tracks of the implanted electrodes were recognizable in brain sections that corresponded to the desired stereotaxic coordinates in the CA3 area of the hippocampus.

Electrophysiological assessment showed significant changes in hippocampal electrophysiology over the kindling process. When fully kindled, individual animals

showed enhanced evoked potentials compared to their own baseline (**Fig 3.2A**), recognizable at all stimulation intensities (**Fig 3.2B**), suggesting that kindling increased overall synaptic strength. The properties of the evoked potential also changed with kindling. The total areas under evoked potential events were 70.33 ± 17.74 mV.ms in mice before kindling, and 174 ± 44.50 mV.ms after kindling in the same subjects ($n=7$, $p=0.049$, paired t-test). Also, interictal spikes were frequently observed in kindled animals (1.77 ± 0.95 events/minute), but were nearly non-detectable in the same animals' baseline recordings ($p=0.013$, paired t-test, $n=5$, **Fig 3.2C-D**).

Since bilateral hippocampi are interconnected by the ventral and dorsal hippocampal commissure, and the CA3 area is critical for the generation of hippocampal EEG rhythms, these data (**Fig 3.2**) provide electrographic evidence for accurately implanted electrodes.

3.2.10 Statistical Analysis

Kindling afterdischarge data was normalized for each subject. This consisted of using the vehicle injection afterdischarge as 100%, and comparing the treatment afterdischarge injection as a percentage of vehicle. The normalized data is used throughout the text and figures. Due to the high standard error of cortical AD length in our small sample size with cortical electrodes ($n=5$), the standard deviation (SD) of the cortical signal was used as a measure of cortical AD amplitudes, and hence, seizure severity.

Statistical tests were performed with SPSS software (Version 20 or 21, SPSS Statistics, IBM). Data are presented as mean and standard error of mean ($M \pm$

SE) throughout the text and figures except where indicated. All ADs reported below were analyzed with one-way repeated measures ANOVAs followed by Tukey's HSD *post hoc* tests or by paired t-tests as appropriate. All motor seizure stages were analyzed using the Wilcoxon signed rank test.

Paired t-tests were used to compare measurements of epileptiform field potentials collected before and following progesterone application. Statistical significance was set at the level of $p < 0.05$.

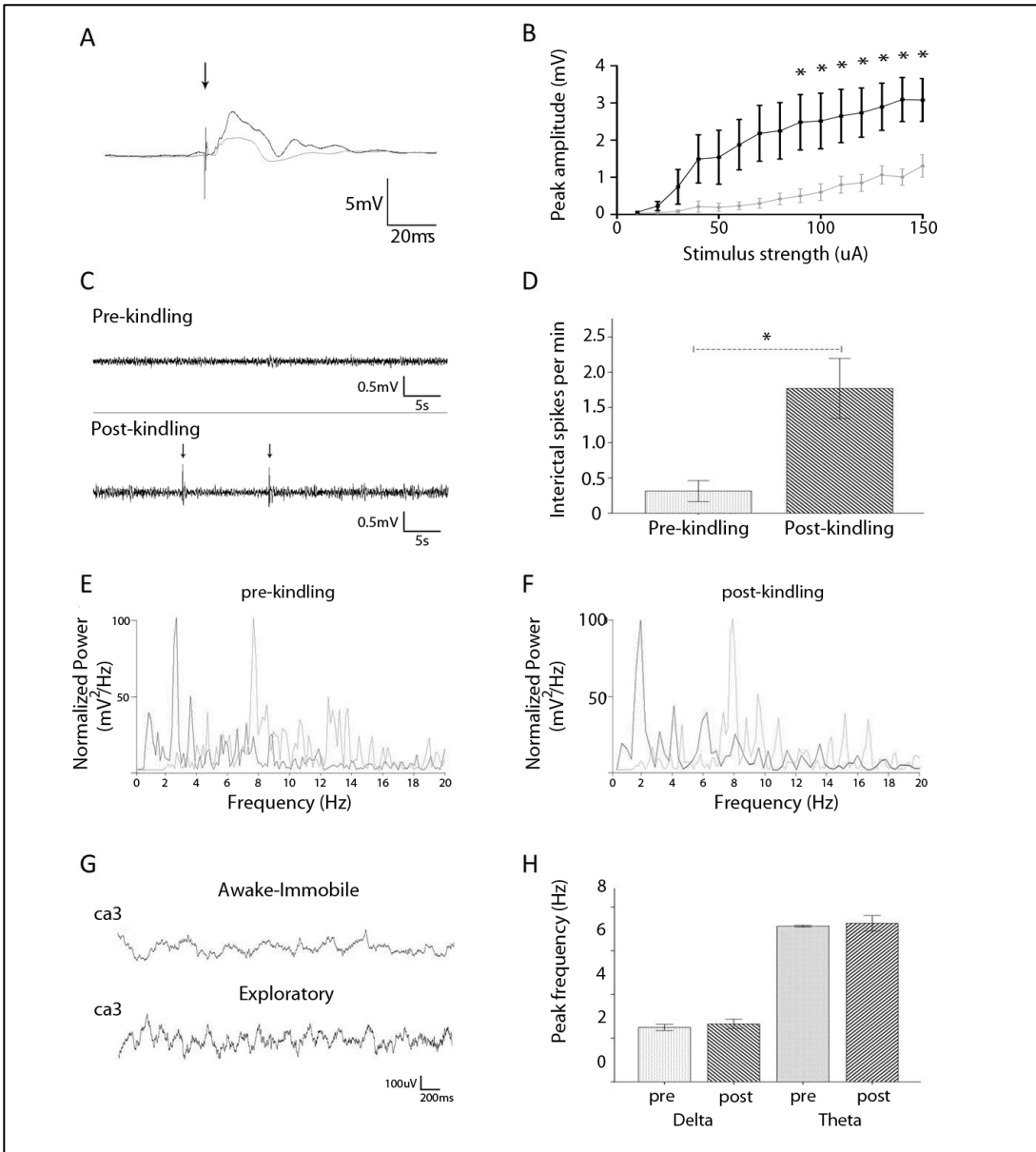


Figure 3.2 Electrophysiological verification of kindling. **A**, left, representative field potentials were collected from a mouse at the beginning the kindling procedure (gray) and after reaching a fully kindled state (black). These field potentials were evoked by unilateral hippocampal CA3 stimulation and recorded from the contralateral CA3 area. Each illustrated trace was averaged from 5 consecutive responses to determine the areas under the curves. **B**, the amplitudes of CA3 field potentials were measured from 5 animals and plotted against stimulation intensities. Note the overall enhancements of field potentials when the animals reached a fully kindled state (pre-kindling gray, kindling black). **C**, interictal spikes (arrowed) were recorded from an animal pre- and post-kindling. Spikes were recorded beginning 3-4 hours after last stimulation in fully kindled animals. **D**, rates of interictal spikes measured pre-kindling and after fully kindled ($p=0.013$, paired t-test, $n=5$). Spikes were recorded beginning 3-4 hours after last stimulation in fully kindled animals. **E-F**, representative spectral plots were generated from EEG signals that were collected from one animal pre- and post-kindling (**G**). Power was normalized to peaks of delta and theta bands. **H**, frequencies of hippocampal theta rhythms and irregular activities were measured from a group of animals. Note that the theta and delta activities were not significantly altered in kindled animal relative to pre-kindling measurements ($p=0.723$ and $p=0.524$, paired t-test).

3.3 Results

3.3.1 Progesterone and THP – But Not DHP – Reduced Hippocampal ADs and Motor Seizures

We first examined the actions of progesterone, DHP and THP in hippocampal-kindled mice, using vehicle injections as a control. Vehicle injections did not produce anticonvulsive effects, as the lengths of hippocampal ADs and motor seizure stages were not significantly different with or without vehicle injections (data not shown).

3.3.1.1 Progesterone

Progesterone was injected 30 minutes before hippocampal electrical stimulation at doses of 10, 35, 100 and 160 mg/kg (n=6-17). The 30-minute test-injection interval was chosen based on existing literature in mice (Reddy & Mohan, 2011; Reddy & Ramanathan, 2012) and previous kindling experiments in our laboratory in rats (Lonsdale et al, 2006). As illustrated in **Fig 3.3**, at 100 (n=12) and 160 mg/kg (n=11), progesterone caused reductions in hippocampal AD lengths (100 mg/kg, $71.03 \pm 7.61\%$ of vehicle control, 12.5-44 seconds long, $p=0.027$; 160 mg/kg, $59.37 \pm 6.64\%$ of vehicle control, 12-21 seconds long, $p=0.024$) and motor seizure stages (100 mg/kg, 4.35 ± 0.28 , $p=0.039$; 160 mg/kg, 2.18 ± 0.50 , $p=0.001$). No complete hippocampal AD suppression was seen, even at the highest dose of progesterone tested.

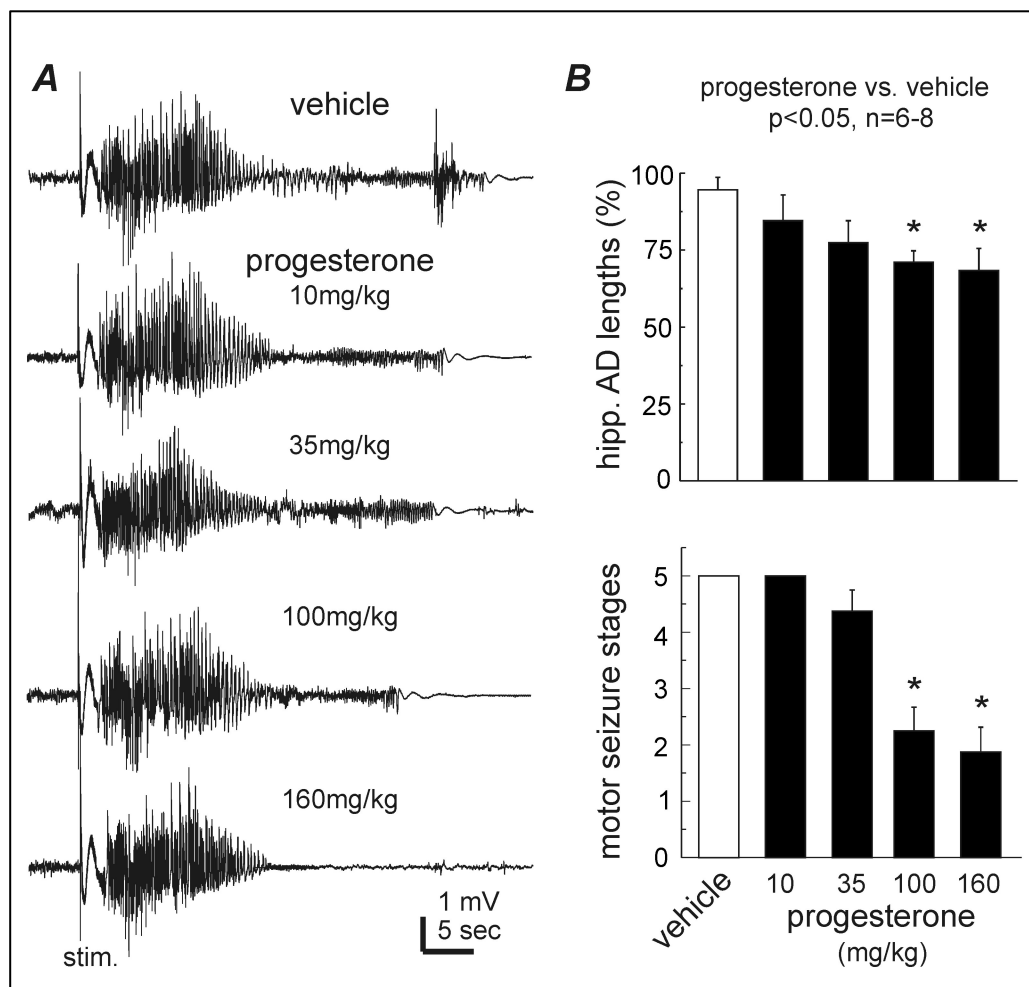


Figure 3.3 Effects of progesterone on hippocampal EEG afterdischarges and motor seizures. *A*, representative hippocampal EEG traces collected from a fully kindled mouse at different doses. *B*, AD lengths and motor seizure stages (*, $p < 0.05$, $n = 6-8$). Intraperitoneal injections of vehicle or progesterone were made 30 minutes before the hippocampal stimulation. AD, ANOVA followed by paired students *t*-test; Seizure stage, Related-samples Wilcoxon signed rank test. *, $p < 0.05$, treatments vs. vehicle. Mean $\pm$ SE.

3.3.1.2 DHP

DHP was injected 15 minutes before hippocampal stimulation and at doses of 5 and 10 mg/kg (n=5 and n=14, respectively). The 15-minute test-injection interval was chosen based on previous kindling studies from our laboratory (Lonsdale & Burnham, 2003; Lonsdale et al, 2006). Higher doses were not tested due to solubility problems. As indicated in **Fig 3.4**, statistical analysis revealed no significant differences among any treatment groups. No anticonvulsant effects of DHP were seen with respect to motor seizure stage at 5 mg/kg (5.00 ± 0.00 , $p=1.000$, $n=5$) or at 10 mg/kg (4.33 ± 0.56 , $p=0.600$, $n=14$; **Fig 3.4**). There were also no significant effects of 5 or 10 mg/kg of DHP on AD (5 mg/kg, $35.05 \pm 15.25\%$ of vehicle control, 17-42 seconds long, $p=0.935$; 10 mg/kg, $104.86 \pm 17.70\%$ of vehicle control, 11-55 seconds long, $p=0.752$).

We noted mild sedation with 10 mg/kg of DHP as compared to vehicle (related-samples Wilcoxon signed rank test, $p=0.038$), suggesting that the drug was in solution and bioavailable (data not shown). Similar levels of sedation were observed for 3.5 mg/kg of THP (related-samples Wilcoxon signed rank test, $p=0.020$, data not shown).

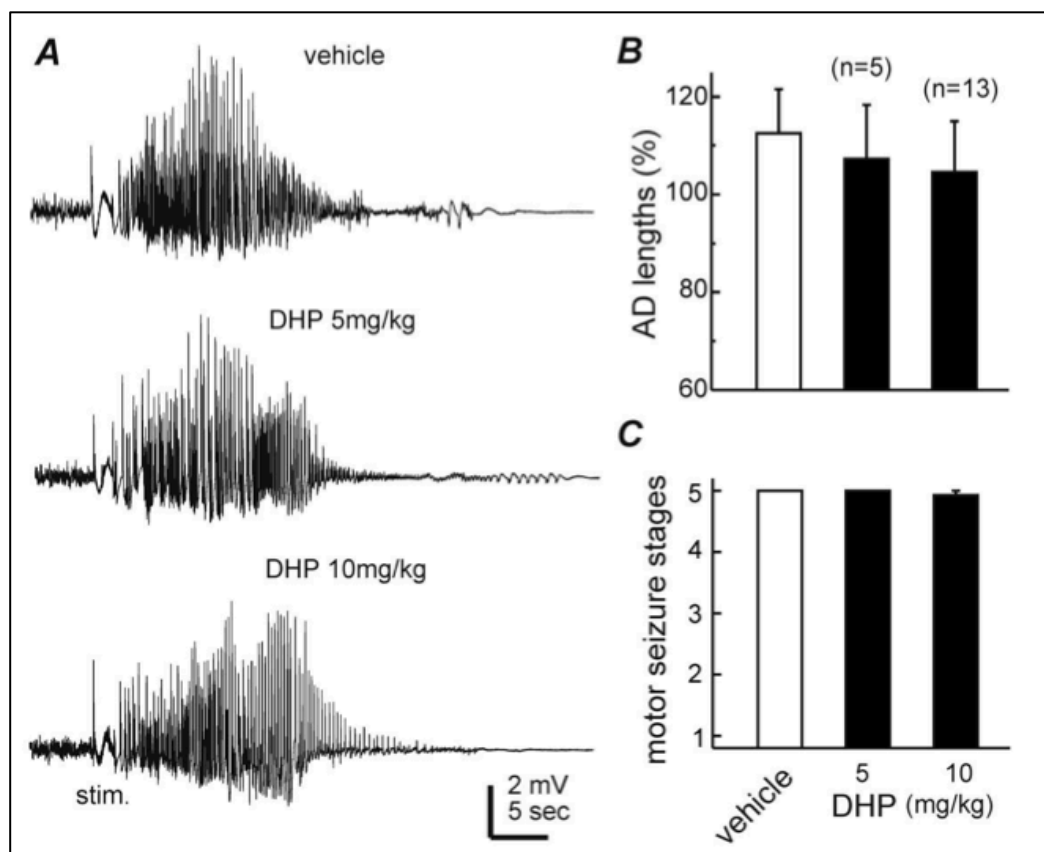


Figure 3.4 Effects of DHP on hippocampal afterdischarges and motor seizures.

A, hippocampal EEG traces collected from a fully kindled mouse. Vehicle or DHP were injected 15 minutes before the hippocampal stimulation. **B**, hippocampal AD. **C**, motor seizure stages. AD, ANOVA followed by paired students t-test; Seizure stage, Related-samples Wilcoxon signed rank test.

3.3.1.3 THP

THP was injected 15 minutes before hippocampal stimulation at doses of 1, 3.5, 10, and 30 mg/kg (n=5-8). As indicated by **Fig 3.5**, THP reduced hippocampal AD lengths and motor seizure stages with significant reductions seen at 10 mg/kg (AD, $18.95 \pm 3.40\%$ of vehicle control, 13-23 seconds long, $p=0.01$; stage, 0.00 ± 0.00 , $p=0.046$) and 30 mg/kg (AD, $12.61 \pm 4.09\%$ of vehicle control, 0-21 seconds long $p=0.001$; stage, 0.00 ± 0.00 , $p=0.005$). Representative EEG traces showing shortened hippocampal ADs by THP are illustrated in **Fig 3.5A**. THP at a dose of 3.5 mg/kg also reduced motor seizure stages (3.67 ± 0.56 , $p=0.034$, $n=6$), but the shortened AD did not reach significance ($65.52 \pm 12.73\%$ of vehicle control, 8.5-34.5 seconds long, $p=0.079$, $n=6$). Although the seizure stages were abolished at 10 and 30 mg/kg, there was no complete suppression of the hippocampal AD even at the highest dose of THP tested (**Fig 3.5B**).

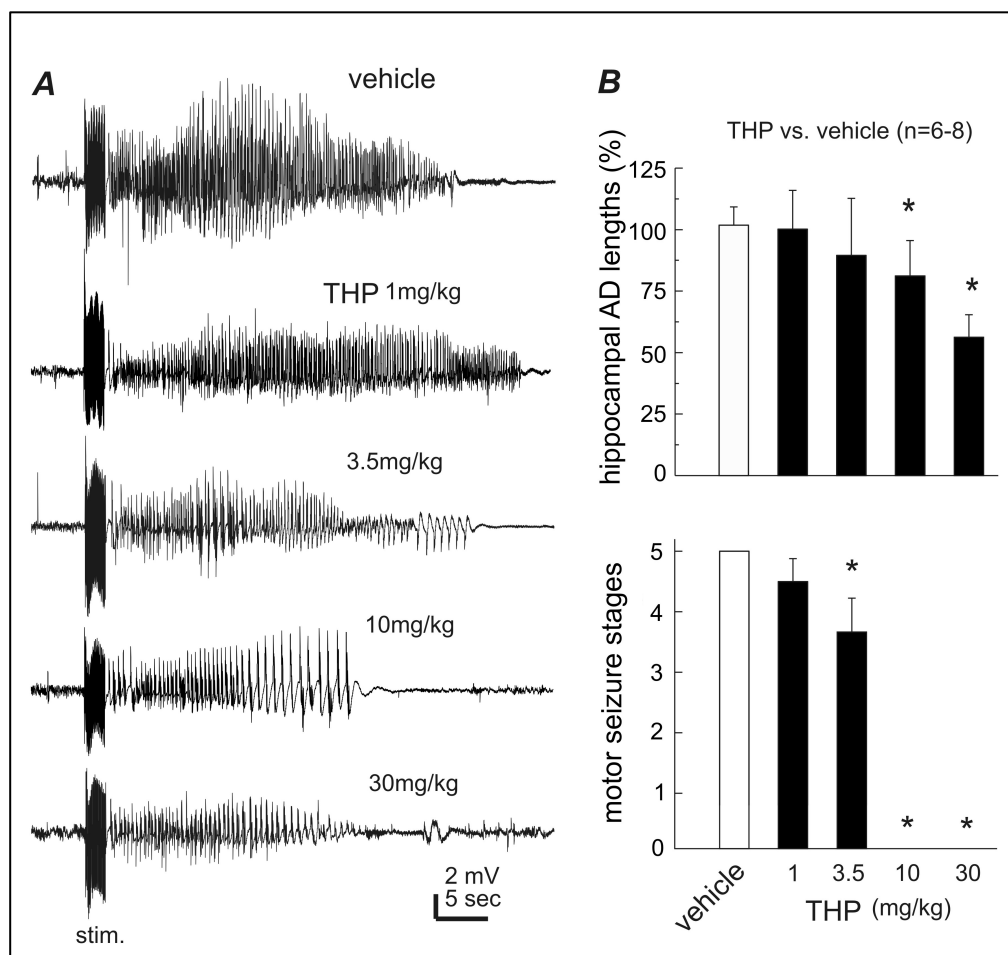


Figure 3.5 Effects of THP on hippocampal EEG afterdischarges and motor seizures.

A, representative hippocampal EEG traces collected from a fully kindled mouse. THP was injected at different doses 15 min before the hippocampal stimulation. **B**, mean hippocampal afterdischarge (AD) lengths and motor seizure stages following vehicle or THP treatments. , $p < 0.05$, THP vs. vehicle. AD, ANOVA followed by paired students t-test; Seizure stage, Related-samples Wilcoxon signed rank test. *, $p < 0.05$, treatments vs. vehicle. Mean $\pm$ SE.

3.3.2 Anticonvulsant Effects of High Dosage Progesterone Were Not Diminished by Finasteride Pretreatment

Finasteride is a classical competitive inhibitor of the predominant *5 α -reductase* isoform in rodent brains (type I). Blockade of *5 α -reductase* prevents the metabolism of progesterone to DHP and THP (Finn et al, 2006). In our experiments, finasteride (50 mg/kg) was injected 2.5 hours before subsequent progesterone injection, or 3 hours (total) before seizure testing. This dose and interval were chosen based on previous studies (Kokate et al, 1999; Frye & Walf, 2002; van Luijtelaaar et al, 2003; Finn et al, 2006; Ciriza et al, 2006). To test whether progesterone exerts its anticonvulsant effects through its metabolism to THP, we treated animals with finasteride prior to progesterone injections and electrical stimulation.

As indicated by **Fig 3.6**, finasteride pretreatment with progesterone abolished progesterone's suppression of hippocampal AD lengths. Hippocampal AD lengths were not significantly different from vehicle controls as compared to 100 mg/kg of progesterone following finasteride pre-treatment (n=14, 20-43 seconds long, p=0.937). Finasteride alone did not affect AD or seizure stage (ANOVA p>0.05, n=5)

Interestingly, finasteride pretreatment did not abolish progesterone's suppression of motor seizures. In mice that were not pretreated with finasteride, progesterone at 100 and 160 mg/kg reduced motor seizure stages as compared to vehicle controls (100 mg/kg progesterone alone, 4.35 ± 0.28 , p=0.039; 160 mg/kg progesterone alone, 2.18 ± 0.50 , p=0.001). In finasteride-pretreated mice, progesterone at 100 and 160 mg/kg also significantly reduced motor seizure stages relative to vehicle controls (100 mg/kg progesterone with finasteride, 0.93 ± 0.25 , p=0.039; 160 mg/kg progesterone with finasteride, 1.0 ± 0.26 , p=0.016, respectively; **Fig 3.6B**).

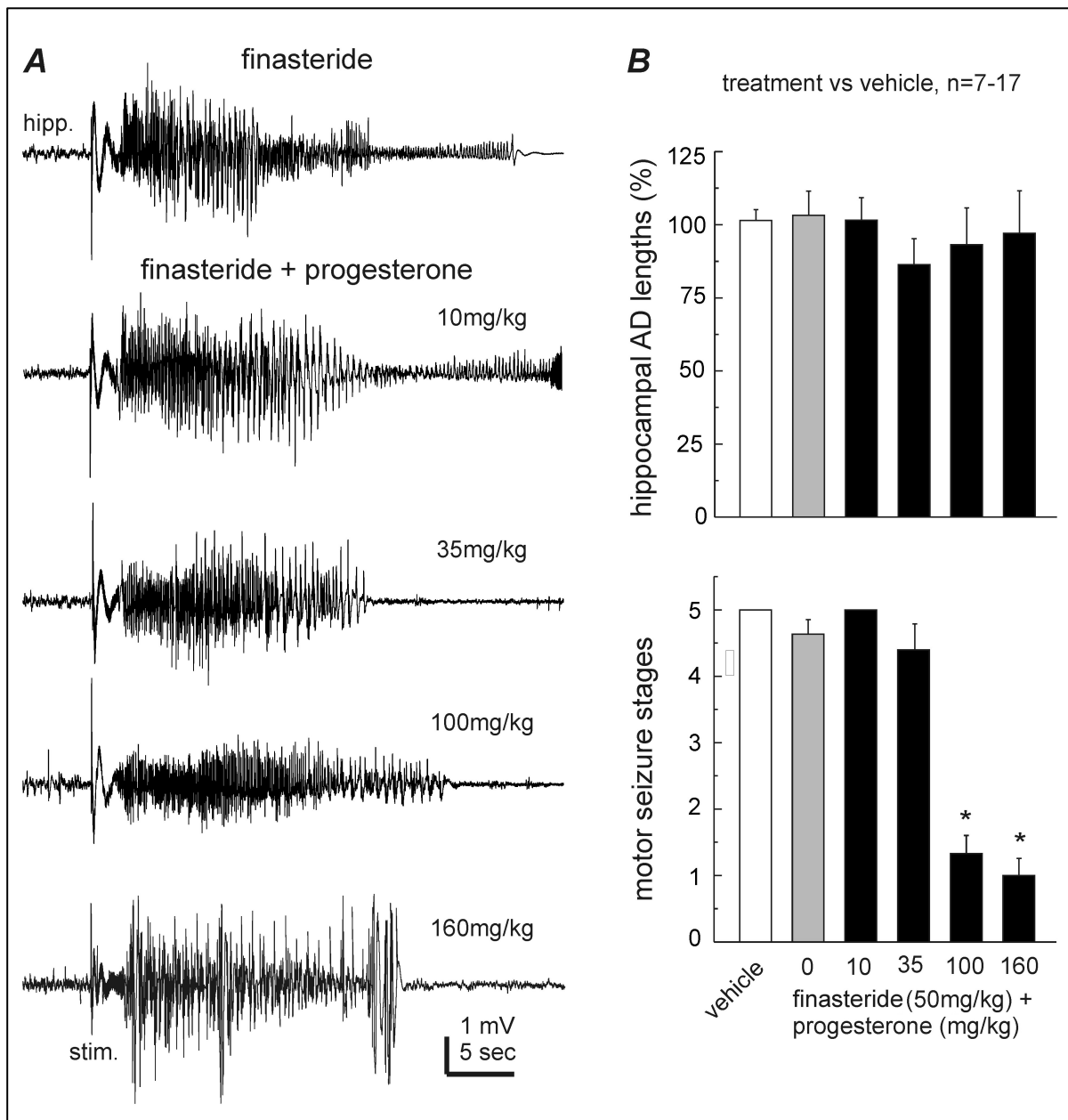


Figure 3.6 Effects of progesterone with finasteride pretreatment on hippocampal EEG afterdischarges and motor seizures. **A**, representative hippocampal EEG traces collected from a fully kindled mouse. **B**, mean $\pm$ SE hippocampal afterdischarge (AD) lengths and motor seizure stages. *, $p < 0.05$, progesterone vs. vehicle. AD, ANOVA followed by paired students t-test; Seizure stage, Related-samples Wilcoxon signed rank test. *, $p < 0.05$, treatments vs. vehicle. Mean $\pm$ SE.

To further explore how progesterone with finasteride pretreatment attenuated motor seizures but not “focal” hippocampal ADs, we recorded EEG signals from 5 hippocampal-kindled mice that had both hippocampal and cortical recording electrodes contralateral to the stimulating electrode (n=5). As indicated in **Fig 3.7**, hippocampal stimulations induced both hippocampal and cortical ADs in these animals (as with other subjects, hippocampal AD lengths were normalized as percentiles of fully kindled vehicle responses in individual animals).

A single dose of progesterone (100 mg/kg) was used in this experiment. As illustrated in **Fig 3.7A** (representative traces), finasteride alone did not affect the occurrence of hippocampal discharge, cortical discharge ($p>0.05$, **Fig 3.7A**, top traces), or seizure stage ($p>0.05$, **Fig 3.7B**, striped bars). As in our previous studies, after finasteride pre-treatment, injection of progesterone had little effect on the focal (hippocampal) discharges, but the cortical discharge was suppressed (**Fig 3.7A**). Figure **3.7B** presents the mean $\pm$ SE for the 5 cortically implanted subjects that were tested for hippocampal and cortical ADs and motor seizure stage. As indicated, 100 mg/kg of progesterone alone (grey bars, n=5) significantly reduced the hippocampal AD lengths ($55.97\pm6.53\%$ of vehicle control, 20-42 seconds long, $p=0.003$) and motor seizure stages (1.0 ± 0.54 , $p=0.038$) relative to vehicle controls. Cortical ADs were also significantly suppressed by 100 mg/kg of progesterone alone, and were barely detectable in 4 of 5 animals tested ($33.35\pm24.41\%$ of vehicle control, $p=0.012$). In the same 5 animals following finasteride pretreatment (50 mg/kg), progesterone at 100 mg/kg (**Fig 3.7B**, black bars) was still able to significantly reduce motor seizure stages (1.00 ± 0.55 , $p=0.004$) and attenuate cortical AD amplitudes ($47.81\pm6.34\%$ of vehicle control, $p=0.012$, **Fig 3.7**, black bars), but the

hippocampal AD lengths were not significantly reduced compared to vehicle controls ($p=0.937$; **Fig 3.7B**). Finasteride alone (striped bars) did not affect either cortical or hippocampal ADs, or motor seizure stages ($p>0.05$).

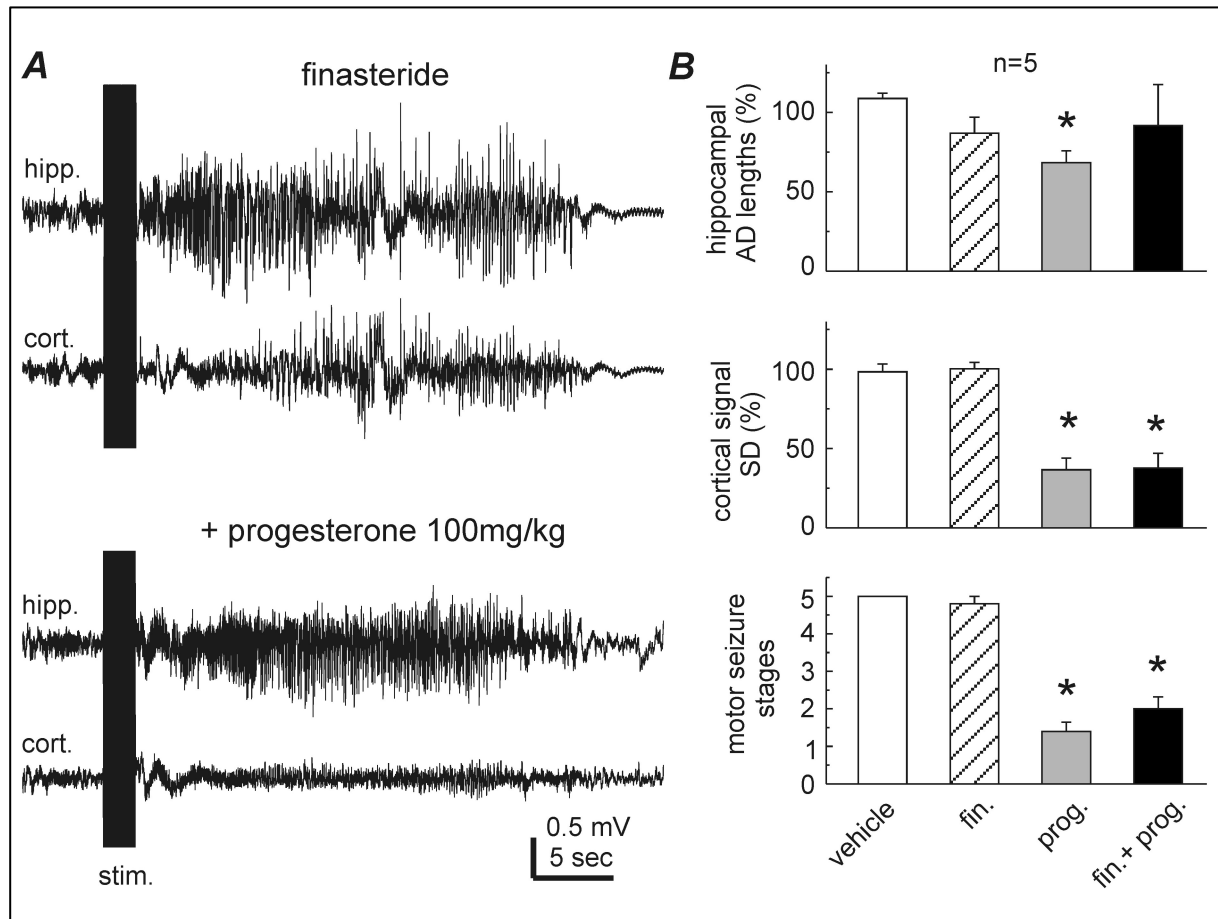


Figure 3.7 Effects of progesterone with finasteride pretreatment on cortical afterdischarges. **A**, representative hippocampal and cortical EEG traces were collected simultaneously from a fully kindled mouse. Illustrated traces were collected following finasteride injection alone (top) and progesterone (100 mg/kg) injection with finasteride (50 mg/kg) pretreatment (bottom). **B**, hippocampal afterdischarge (AD) lengths, standard deviation (SD) of cortical AD signals, and motor seizure stages following treatments. AD, ANOVA followed by paired students t-test; Seizure stage, Related-samples Wilcoxon signed rank test. *, $p<0.05$, treatments vs. vehicle. Mean $\pm$ SE.

3.3.3 Carbamazepine Produced Anticonvulsive Effects Similar to Progesterone With Finasteride Pretreatment, Whereas Midazolam Did Not

We next explored whether clinical anticonvulsive drugs with known mechanisms of action produced effects similar to that of progesterone with finasteride pretreatment. The five hippocampal-kindled mice with hippocampal and cortical recording electrodes were used for these experiments.

Midazolam is a fast-acting benzodiazepine and positive allosteric modulator of the GABA_A receptors that is recommended for the treatment of status epilepticus (Meierkord et al, 2010). Carbamazepine acts largely by suppressing the activity of voltage-gated sodium channels (Mula, 2013). These two drugs were injected 15 minutes before hippocampal stimulation in our kindled mice. The doses and injection-test interval were chosen based on previous kindling studies (Albright & Burnham, 1980; Higgins et al, 2010; Arai et al, 2012).

As indicated in **Fig 3.8**, midazolam at 2 mg/kg significantly reduced hippocampal AD lengths ($32.50 \pm 14.34\%$ of vehicle controls, 0-13.5 seconds, $p=0.037$, $n=9$) and motor seizure stages (0.12 ± 0.09 , $p=0.000$, $n=16$), together with a great attenuation of cortical AD amplitudes ($35.84 \pm 2.97\%$ of vehicle controls, $n=5$, $p<0.01$). In two of the five animals tested, midazolam abolished cortical ADs. Carbamazepine at 50 mg/kg significantly reduced motor seizure stages (1.79 ± 0.33 , $p=0.001$, $n=14$) and cortical AD amplitude ($47.01 \pm 6.04\%$ of vehicle controls ($p=0.01$, $n=4$), but not hippocampal AD lengths ($p>0.05$) as compared to vehicle control (**Fig 3.8B**). Overall, the anticonvulsive effects of 50 mg/kg of carbamazepine (**Fig 3.8**) were similar to those produced by progesterone (100 mg/kg) with finasteride (50 mg/kg) pretreatment (**Fig 3.7**).

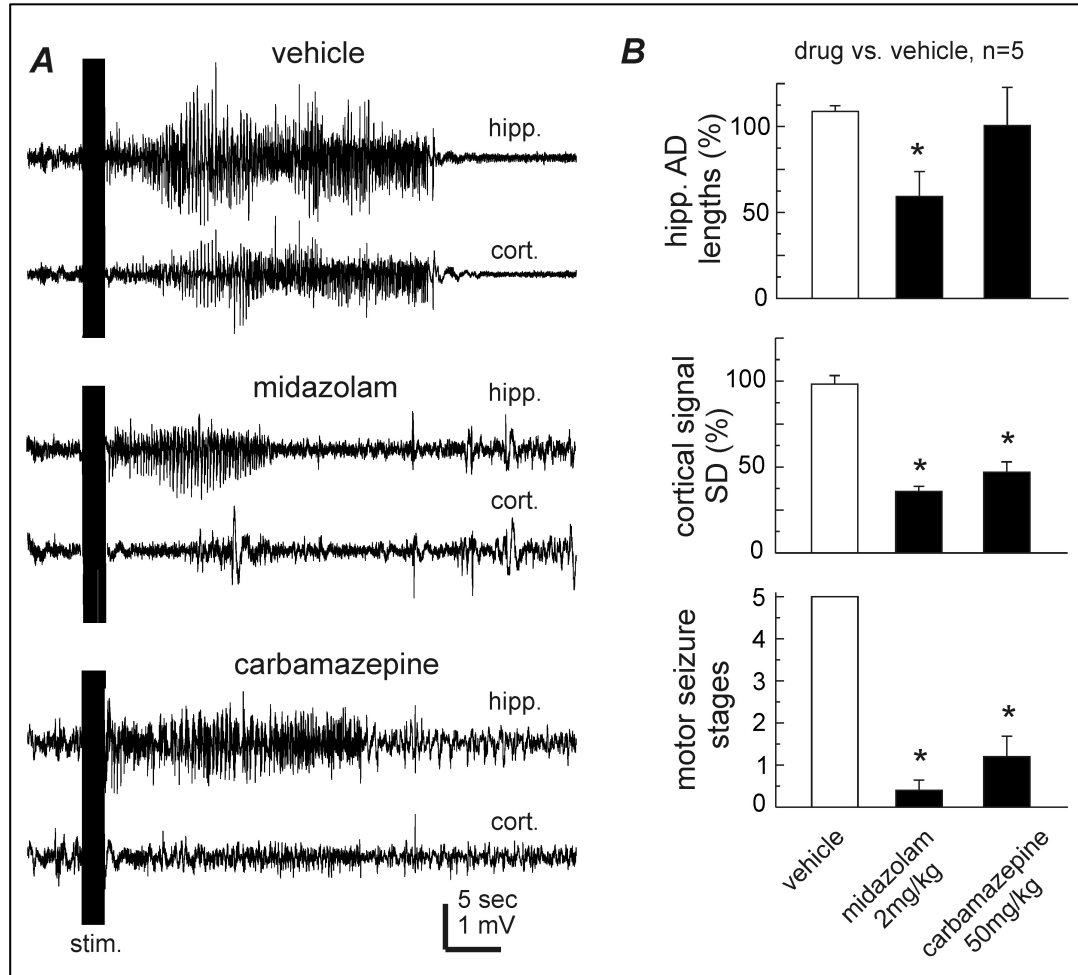


Figure 3.8 Effects of midazolam and carbamazepine on hippocampal/cortical afterdischarges and motor seizures. *A*, hippocampal and neocortical EEG traces collected simultaneously from a fully kindled mouse. Vehicle or indicated drugs were injected 15 min before the hippocampal stimulation. *B*, mean hippocampal afterdischarge (AD) lengths and motor seizure stages following vehicle or indicated drug treatments. *, $p < 0.05$, drug vs. vehicle, AD, Related-samples 2-way ANOVA, with related-samples Wilcoxon signed rank test; Seizure stage, Related-samples Wilcoxon signed rank test. Mean $\pm$ SE.

3.4 Chapter Discussion

Three main findings emerge from our current experiments. Firstly, at relatively high doses, progesterone and THP were able to reduce hippocampal ADs and generalized motor seizures in kindled mice (**Fig 3.3, 3.5**). Secondly, high doses of progesterone suppressed motor seizures in finasteride-pretreated mice, and these effects were associated with a strong suppression of cortical ADs, but not hippocampal ADs (**Fig 3.6-3.7**). Thirdly, carbamazepine mimicked the effects of progesterone with finasteride pretreatment in attenuating cortical ADs and motor seizures, whereas midazolam produced effects similar to progesterone and THP in suppressing hippocampal/cortical ADs and motor seizures (**Fig 3.8**).

3.4.1 Effects of Progesterone

High doses of progesterone (100 and 160 mg/kg) both suppressed the hippocampal AD by ~25%. To our knowledge, this is the first report of progesterone's effects on hippocampal AD duration. In addition, high doses of progesterone also suppressed generalized convulsions in kindled animals. The motor seizure suppression by high doses of progesterone we observed (**Fig 3.3**) is generally in keeping with previous studies (Lonsdale & Burnham, 2003; 2007; Lonsdale et al, 2006; Singh et al, 2010).

3.4.2 Effects of DHP

Our results with DHP were unexpected. These results contrast with previous results in amygdala-kindled male and female rats (Lonsdale & Burnham, 2003; Lonsdale

et al, 2006). In these previous rat studies, DHP reduced seizure stage and AD occurrence. In the present mouse studies, ADs and motor seizure stages were not affected by DHP.

There are important differences between previous kindling studies of DHP in rats and the present studies in mice. The most obvious difference is species. Rats and mice have differing levels of neuroactive steroids and their enzymes' mRNA in the brain (Korneyev et al, 1993). It is reasonable that certain compounds, particularly neuroactive steroids, might have different effects in laboratory rats and laboratory mice. Another important difference between the present studies and the Lonsdale (2003; 2006) rat studies relates to the kindling and recording sites. In Lonsdale's previous experiments, rats were kindled with a single bipolar stimulating electrode in the right basolateral amygdala, whereas in the current experiment, stimulations were delivered to the right CA3 hippocampal region. Animals kindled from different sites can have very different responses to anticonvulsant drugs (Albright & Burnham, 1980; Morimoto et al, 1987; Otsuki et al, 1998; Francis et al, 2002; Morimoto et al, 2004). We chose to use hippocampal kindled mice because they are increasingly used in epilepsy research, including molecular studies (Reddy & Mohan, 2011; Reddy & Ramanathan, 2012).

Although DHP's results in the present experiments in hippocampal-kindled mice conflict with previous amygdala kindling experiments in rats, we propose that these contrasting results may be attributable to differences in species and kindling sites.

3.4.3 Effects of THP

Seizure suppression by progesterone is generally thought to be a result of progesterone's metabolism to THP (Frye et al, 2002; Zheng, 2009; Reddy & Ramanathan

2012). THP is a positive allosteric modulator of GABA_A receptors (Lambert et al, 2009; Zheng 2009; King 2013). THP's anticonvulsant effects have been shown in a variety of previous experiments (Lonsdale et al, 2006; Lonsdale & Burnham, 2007; Reddy, 2010; Singh et al, 2010), including kindled mice (Reddy et al, 2012). Our present observations confirm that THP has anticonvulsant effects in our mouse kindling model.

We found that THP (10 and 30 mg/kg) effectively shortened hippocampal ADs and suppressed motor seizures (**Fig 3.5**). These data are in general agreement with results in many other models (above). Of interest, these effects were mimicked by 2 mg/kg of midazolam (**Fig 3.8**), a drug known to enhance the activity of GABA_A receptors (Reves et al, 1985; Lahat et al, 1992; Lambert et al, 2009).

3.4.4 Effects of Progesterone in the Presence of Finasteride

To explore progesterone's anticonvulsive actions independent of its metabolites DHP and THP, we pretreated animals with finasteride (50 mg/kg) to inhibit *5 α -reductase*. Previous studies have shown that finasteride pretreatment prevents or greatly reduces progesterone's metabolism to DHP/THP (Finn et al, 2006). In particular, Ciriza et al (2006) have reported that hippocampal levels of DHP and THP are elevated in rats following progesterone injection (1-4 mg/kg), and that such elevations are absent in animals pretreated with finasteride (50 mg/kg).

In our experiments, finasteride pretreatment diminished the effects of progesterone (at 100 and 160mg/kg) on hippocampal AD durations (**Fig 3.7**). Finasteride pretreatment did not, however, abolish progesterone's suppression of motor seizures. This was an unexpected result. It appeared that finasteride blocked progesterone's effects

on focal, but not generalized seizure activity. To further explore this phenomenon, we implanted mice with recording electrodes in the motor cortex as well as the bilateral hippocampi.

When the cortically impanted animals were treated with a high dose of progesterone alone (100 mg/kg), the hippocampal and cortical ADs were shortened, and motor seizure stage decreased dramatically (**Fig 3.7**). When the cortically impanted animals were treated with a high dose of progesterone (100 mg/kg) with finasteride (50 mg/kg) pretreatment, the hippocampal AD was not shortened, as it had been by progesterone alone, but both the cortical AD and generalized convulsion were reduced (**Fig 3.7**). In fact, progesterone with finasteride pretreatment had similar effects to the effects carbamazepine on seizure stage and cortical AD: Hippocampal AD was unaffected, but the cortical AD amplitude was significantly reduced, as was seizure stage (**Fig 3.8**). This suggested that when the 5α -reduction of high doses of progesterone is prevented, it has effects on seizure spread, but not focal-like ADs in the hippocampus.

3.5 Originality

Our experiments have some novelty. We have not found any other experiments studying the effects of progesterone with finasteride pretreatment in a kindling model at such high doses. Most studies use a maximum of 25 mg/kg (ie, Reddy & Ramanathan, 2012). Our data, therefore, are novel. They indicate that there are anticonvulsant effects of progesterone that are independent of THP. The anticonvulsant effects of progesterone independent of THP are not well characterized in the literature.

We are aware of one other study has found that supraphysiological doses of progesterone with finasteride pretreatment can suppress convulsions (Kokate et al, 1999). Kokate et al (1999) found anticonvulsant effects of very high doses of progesterone with finasteride (ED₅₀ 324 mg/kg with 100 mg/kg finasteride) in the MES model of generalized seizures. In our kindling experiments, high doses of progesterone (100 and 160 mg/kg) with finasteride suppressed motor seizures and decreased cortical ADs' amplitudes, but did not affect focal hippocampal ADs. In this respect, our results in the kindling model are consistent with the maximal electroshock results of Kokate et al (1999): High doses of progesterone with finasteride pretreatment suppress generalized seizures.

Apparent discrepancies seem to exist, however, between our present observations and some past studies (Herzog & Frye, 2003; Reddy et al, 2004; Reddy & Ramanathan, 2012), which investigated the anticonvulsive effects of up to 25 mg/kg of progesterone with finasteride pretreatment. In these studies, the anticonvulsant effects of progesterone were blocked by finasteride pretreatment. The dose of progesterone used in our experiments, however, was much higher (100 mg/kg). The anticonvulsive effects of progesterone with finasteride pretreatment observed in the present experiment may represent progesterone actions seen only at high doses, since progesterone substantially reduced motor seizures at 100 and 160mg/kg, but not at 10 or 35mg/kg (**Fig 3.4**). Also of note, injection-treatment intervals of finasteride vary from 1 minute to 6 hours in the literature. Our injection-treatment interval for finasteride was based on full brain 5 α -*reductase* receptor occupancy, as per previous studies (Ciriza et al, 2006).

Collectively, these results suggest that, at least in hippocampal-kindled mice, high doses of progesterone may attenuate the secondarily generalized component of seizures via THP- and/or GABA-independent mechanisms. Future experiments are necessary to determine a precise mechanism. Possibilities will be discussed in the General Discussion (chapter 5).

3.6 Limitations

There are several limitations to our study. Firstly, we were not able to measure levels of progesterone, DHP, and THP in the blood or brain of the mouse. We therefore can only infer that finasteride caused a complete blockade of *5 α -reductase*, and that progesterone levels were elevated in the brain following progesterone with finasteride pretreatment. Such measures were not available in the present study, although they might be added to future studies. Secondly, when the *5 α -reductase* enzyme is inhibited, the very high doses of progesterone used may shunt progesterone metabolism to its non-*5 α -reduced* metabolic pathways. These other metabolic pathways of progesterone produce neuroactive steroids (Wiebe et al, 2005) that may, in part, confer the anticonvulsant effects observed. To test this hypothesis, neurosteroid levels would need to be measured. Thirdly, other endogenous neurosteroids are also metabolized by *5 α -reductase*. Finsteride, therefore, may have affected other endogenous neurosteroid pathways. The effects of finasteride on endogenous neurosteroids, such as testosterone, may also have contributed to the anticonvulsant effects we observed. This seems unlikely, however, since the very high doses of progesterone used probably overwhelmed any effects of endogenous neurosteroids on seizures.

To remove these limitations it would be necessary to measure levels of a number of neurosteroids in animals after progesterone treatment with and without finasteride. These measures would need to be made in a region specific manner. We are currently collaborating with another laboratory to develop an appropriate technique for femtomolar level measurements for this purpose. It would also be useful to compare potential differences in neurosteroid levels between kindled and naïve (unkindled) brains alone and under differing treatments.

3.7 Conclusion

The above studies further demonstrate anticonvulsive effects of progesterone and THP in hippocampal kindled mice. The progesterone effects continued to occur in the presence of finasteride. Our study suggests, therefore, that progesterone may have THP/GABA_A independent anticonvulsive or inhibitory actions in a hippocampal kindled mouse model. It remains to be tested whether these actions are generalizable to other species and/or other seizure models.

Chapter 4: Electrophysiological Studies In Vitro

These studies were conducted in the laboratory of Dr. Liang Zhang, who served as my co-supervisor. Dr. Zhang and I conducted pilot studies in the hippocampal and entorhinal slices until a suitable in vitro protocol was established. During this time, I mixed ACSF (artificial cerebrospinal fluid) and prepared drug solutions. Subsequently, volunteer Edwin Chow prepared ACSF and drugs and conducted in vitro recordings under our supervision.

I conducted the statistical analyses and was lead author on the paper publishing these results combined with our in vivo results (Chapter 3):

M. Jeffrey, M. Lang, J. Gane, E. Chow, C. Wu and L. Zhang. 2014. Novel anticonvulsant effects of progesterone in a mouse model of hippocampal electrical kindling. *Neuroscience* 257: 65-75.
<http://dx.doi.org/10.1016/j.neuroscience.2013.1074>

As mentioned above, the published paper is in **Appendix 4** of this thesis.

4.1 Introduction

Analysis of data presented in chapter 3 suggested that progesterone could have anticonvulsant actions independent of the GABA_A receptor. In the present study, we examined the effects of progesterone on entorhinal epileptiform field potentials in the presence of the GABA_A receptor antagonist picrotoxin and the *5 α -reductase* inhibitor finasteride in vitro. We used horizontal brain slices, a standard in vitro preparation utilized in epilepsy research (Heinemann et al, 2006; Thompson et al, 2006). In vitro neurophysiology experiments are often done in brain slices prepared from the hippocampal formation (Heinemann et al, 2006; Thompson et al, 2006). We chose the entorhinal cortex as our recording site because it reliably produced epileptiform field potentials in our own in vitro studies, as well as in other studies in vitro (Wu et al, 2006; Buzsáki et al, 2012) and in vivo (Fountain et al, 1998; Lévesque et al, 2013).

Extracellular field potentials are a summation of all ionic processes in the network population measured, including excitatory and inhibitory post-synaptic processes (Buszáki et al, 2012). They are essentially EEG recordings measured through an extracellular microelectrode. The properties of the field potentials can be analyzed, including their durations, their waveforms, their amplitudes, and power. Comparing field potentials' appearance before and after treatment, and after treatment washout, can indicate effects of the treatment on neuronal communication (Buszáki et al, 2012).

4.2 Methods

4.2.1 Overview

In the present experiments, brain slices were perfused with ACSF containing the GABA_A receptor antagonist picrotoxin (100 μ M), and finasteride (1 μ M). Finasteride was used to inhibit the enzyme *5 α -reductase*, which is present in rodent brains (Reddy & Ramanathan, 2012). To induce repetitive epileptiform potentials, 4-aminopyridine (4-AP, 100 μ M) was used (D'Antuono et al, 2010). The appropriate concentrations of picrotoxin, 4-AP and finasteride were determined in pilot studies. Progesterone was applied at 0.1 or 1 μ M for 20 min together with the above pharmacological agents. An outline of the experimental protocol is presented in **Fig 4.1**.

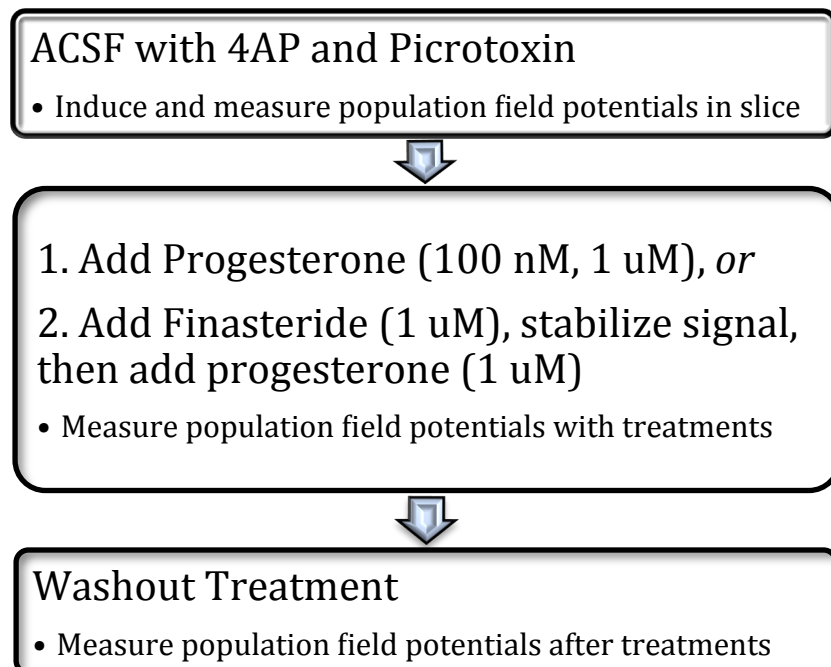


Figure 4.1 In vitro experimental protocol.

4.2.2 Brain slice preparation and extracellular recordings

Horizontal brain slices 0.5 mm thick were obtained from male C57 black mice 1–3 months old. All recordings were conducted in a submerged chamber at 36°C. Under our recording conditions, the slice was perfused with ACSF at a high rate (15 mL/min), with both the top and bottom surfaces of the slice exposed to the perfusate (given this high rate of perfusion, local metabolites of progesterone are likely washed away). Humidified gas of 95% O₂-5% CO₂ was allowed to pass over the perfusate to increase local oxygen tension (El-Hayek et al, 2011). The ACSF contained (in mM): NaCl 125, KCl 3.5, NaH₂PO₄ 1.25, NaHCO₃ 25, CaCl₂, MgSO₄ 1.3 and glucose 10 (pH of 7.4 when aerated with 95% O₂-5% CO₂).

Extracellular signals were recorded using a dual channel amplifier (700A or 700B, Molecular Devices) with the input frequency band set in the range of 0.1-1000 Hz, and the amplification gain at 1000×. The signals were digitized at 5000 Hz (Digidata 1440A, Axon Instruments/Molecular Devices, Union City, CA, USA). Pclamp software (Version 9 or 10; Axon Instruments/Molecular Devices) was used for data acquisition, storage and analyses.

4.2.3 Drugs

4-Aminopyridine (Sigma–Aldrich, Oakville, Ontario, Canada) was dissolved in distilled water as a stock solution of 0.5 M and then appropriately diluted in the ACSF. Picrotoxin (Sigma–Aldrich) was directly dissolved in the ACSF. Finasteride was dissolved in DMSO as a 10mM stock solution and then diluted in ACSF. Progesterone was dissolved in 100% alcohol as a stock solution of 50 mM and then diluted into the ACSF. All solutions

were mixed fresh daily.

4.2.4 Examination of epileptiform field potentials in vitro

The event detection function of the pClamp software was used to detect repetitive epileptiform potentials (El-Hayek et al, 2011). Original signals were treated with a 5-Hz high-pass filter to reduce DC drifts and to reveal rhythmic spike waveforms (peak to trough). Detected events were then visually inspected and artifacts were rejected. Fifteen to twenty events of epileptiform potentials were detected from each of 8 individual slices both before and after 20 minutes of progesterone application. These detected events were then averaged for measurements of duration and peak amplitude per epileptiform potential averaged across 15-20 events. The main frequency and corresponding power of the largest epileptiform spikes in the burst were determined from the spectral plots. The highest peak in the 0.5-5 Hz range was considered the main frequency. Time-frequency (power) analyses of spontaneous field potentials were made from extracellular data segments ~3 minutes long (time resolution 0.5 seconds).

4.2.5 Statistical analysis

Statistical tests were performed with SPSS (Version 21, SPSS Statistics, IBM) and SigmaStat (Systat Software Inc, San Jose, California, USA) software. Data are presented as mean and standard error of mean ($M \pm SE$) throughout the text and figures. Paired t-tests were used to compare measurements of epileptiform field potentials. Statistical significance was set at $p < 0.05$.

4.3 Results: Progesterone Decreased Entorhinal Epileptic Potentials in the Presence of a GABA_A Antagonist With and Without Finasteride Pretreatment

Repetitive epileptiform potentials were induced by ACSF containing the convulsive agent 4-AP (100 μ M; Salah & Perkins, 2011), and the GABA_A receptor antagonist picrotoxin (100 μ M; **Fig 4.2**). In these initial experiments, progesterone was applied for 20 minutes in the absence of finasteride. In subsequent experiments, finasteride (1 μ M) was added to the ACSF before progesterone was applied for 20 min (**Fig 4.1**). In all slices, the last 10-15 stable epileptiform field potentials before application of progesterone and first 10-15 stable epileptiform field potentials after $\sim$ 20 minutes of the application of progesterone were compared. Finasteride alone did not affect epileptiform field potentials.

Progesterone applied at 0.1 μ M (low dose) did not have any effects on either epileptiform population field potentials' waveforms (**Fig 4.3A**) or their properties (**Fig 4.3B**). Progesterone applied at 1.0 μ M, however, did have effects on both epileptiform field potential waveforms (**Fig 4.4A**) and their properties (**Fig 4.4B**). With 1.0 μ M of progesterone, the amplitude and power of rhythmic spike activity of field potentials were significantly reduced relative to the level of pre-treatment potentials ($p=0.007$ and $p=0.013$, $n=10$; **Fig 4.4A** and **4.4B**). It is possible that progesterone was metabolized to DHp and THP in the slice, but we are confident that our high perfusion rate washed any metabolites away from the slice. Interestingly, 45 minutes of washout did not reverse the effects of 1.0 μ M of progesterone (**Fig 4.2**, bottom trace).

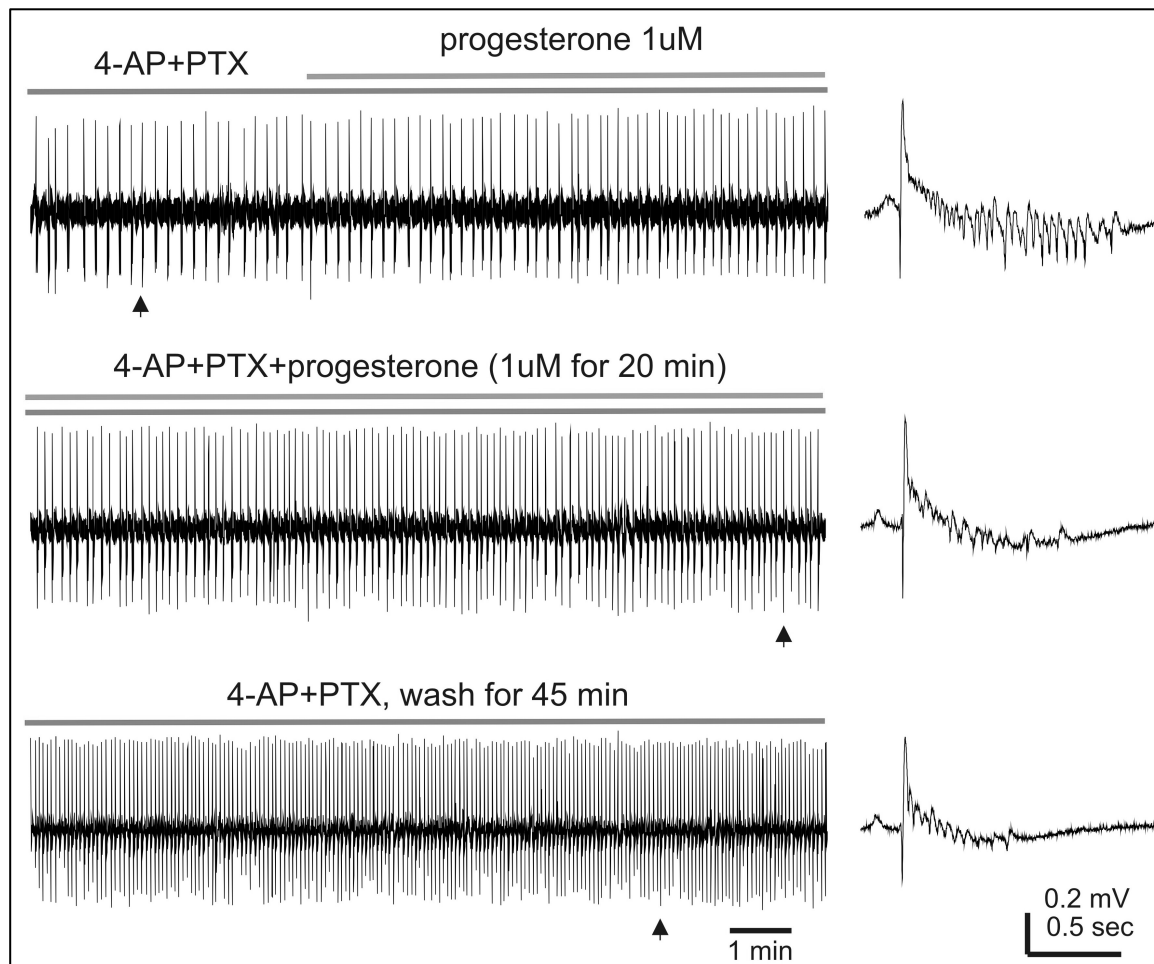


Figure 4.2 In vitro results for progesterone treatment alone. Each arrow indicates the expanded spike to the right of the recording. **Top trace:** ACSF containing 4-AP and PTX is applied to the slice until stable field potentials are elicited, top right, before progesterone is applied. **Middle trace:** 1 μ M Progesterone is applied for 20 minutes until its effects on field potentials have stabilized. Note the decreased amplitude and duration of the representative spike. **Bottom trace:** field potential recorded 45 minutes after progesterone application has ended. Note that the field potentials waveform is the same as in the middle trace during progesterone application. This indicates that the effect of progesterone in the slice is not reversible. Abbreviations: ACSF, artificial cerebrospinal fluid; 4-AP, 4-aminopyridine; PTX, picrotoxin.

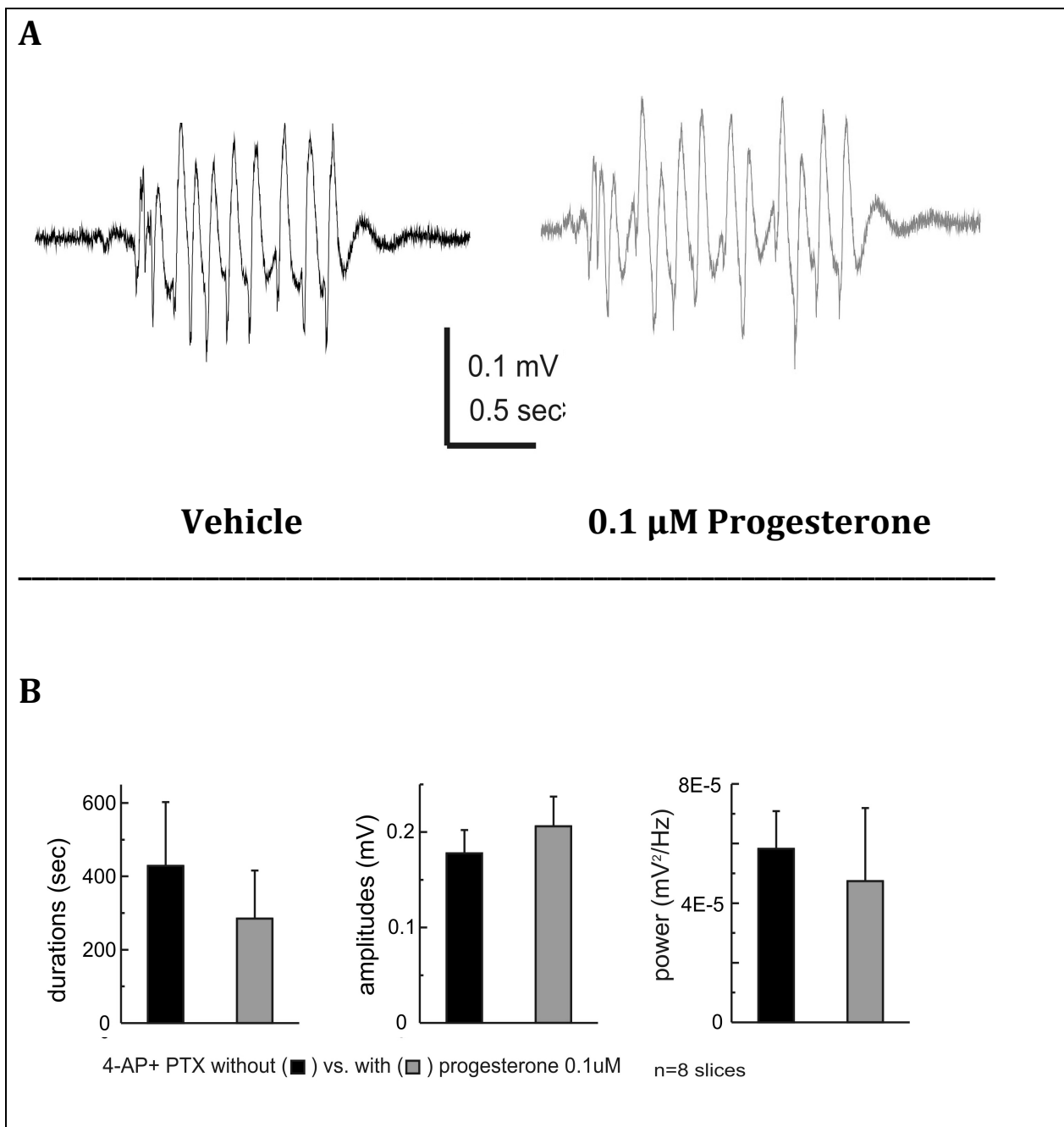


Figure 4.3 0.1 μ M progesterone (low dose) does not affect waveform properties. **A**, Black trace, vehicle; Grey trace, 0.1 μ M progesterone. **B**, Quantification of changes in waveform properties with 0.1 μ M progesterone as compared to vehicle. Left panel, duration; center panel, amplitude; right panel, power. Paired t-test, n=10 events before progesterone and 10 events before and after progesterone per each of 8 slices, $p > 0.05$ all measures. Abbreviations: 4-AP, 4-aminopyridine; PTX, picrotoxin. Mean $\pm$ SE.

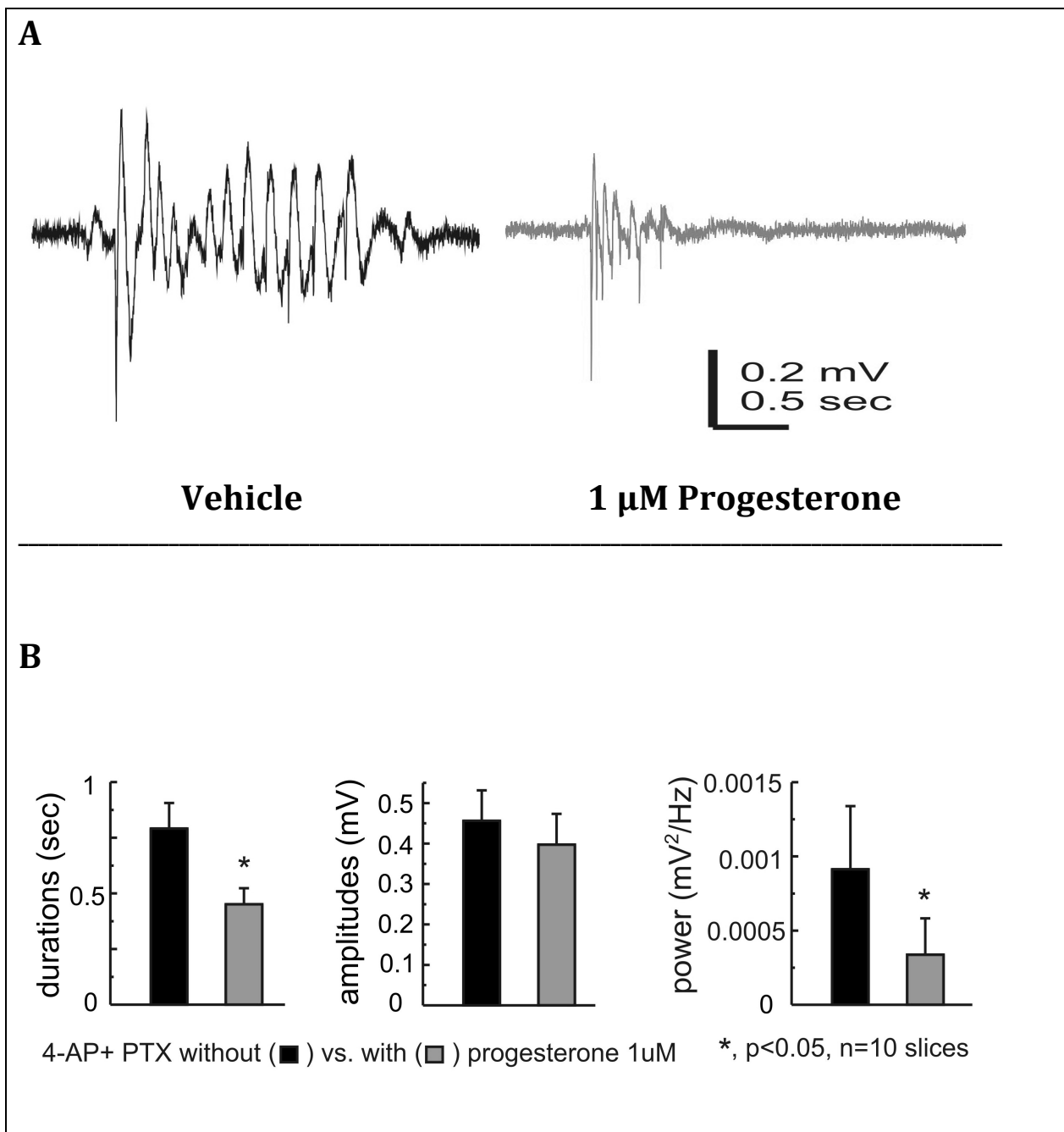


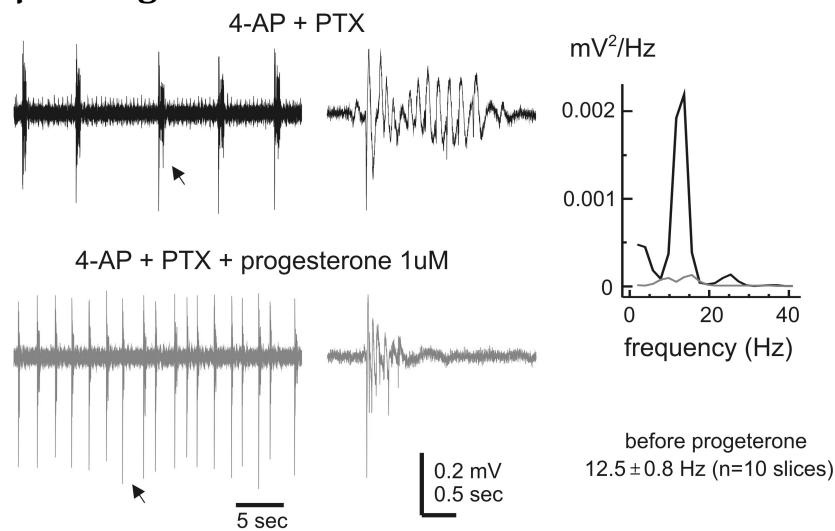
Figure 4.4 1.0 μ M progesterone significantly alters waveform properties. **A**, Black trace, vehicle; Grey trace, 1.0 μ M progesterone. **B**, Quantification of changes in waveform properties with 1.0 μ M progesterone as compared to vehicle. Left panel, duration; center panel, amplitude; right panel, power. Paired t-test, $n = 10$ events before progesterone and 10 events after progesterone per each of 8 slices, $* = p < 0.05$. Abbreviations: 4-AP, 4-aminopyridine; PTX, picrotoxin. Mean $\pm$ SE.

To further characterize electrophysiological effects of progesterone, progesterone was tested in the presence and absence of finasteride (**Fig 4.5B**, black traces). In the absence of finasteride, suppression of epileptiform activity was seen once again (**Fig 4.5A**). In the presence of 1 μ M of finasteride, 1 μ M of progesterone significantly reduced the amplitude and power of epileptiform field potentials (**Fig 4.5, 4.6**, right panels). Finasteride alone did not affect the population field potentials (**Fig 4.6**, black bars).

4.3 Major Findings

The above observations suggest that a high dose of progesterone is able to inhibit entorhinal epileptiform potentials in the presence of both the GABA_A receptor antagonist picrotoxin and finasteride. Since these effects of progesterone occurred in the presence of picrotoxin, they were clearly not mediated by a GABAergic mechanism. Since they continued to occur in the presence of finasteride, which prevents progesterone's metabolism to DHP (and subsequently THP), they were likely mediated by progesterone itself.

A: 1 μ M Progesterone



B: 1 μ M Progesterone + 1 μ M Finasteride

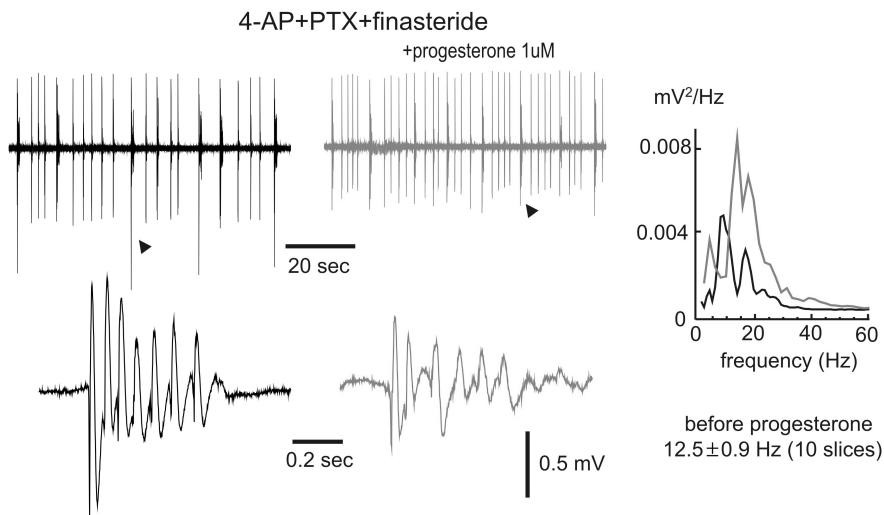


Figure 4.5 Progesterone retains its antiepileptiform properties in the presence and absence of 5α -reductase inhibitor finasteride. **A**, Progesterone 1 μ M added to ACSF. Black traces, ACSF before progesterone, grey traces, ACSF + 1 μ M progesterone. **B**, Progesterone with finasteride. Progesterone 1 μ M added to ACSF containing 1 μ M finasteride. Black traces, ACSF containing 1 μ M finasteride; grey traces, ACSF containing 1 μ M finasteride and 1 μ M progesterone.

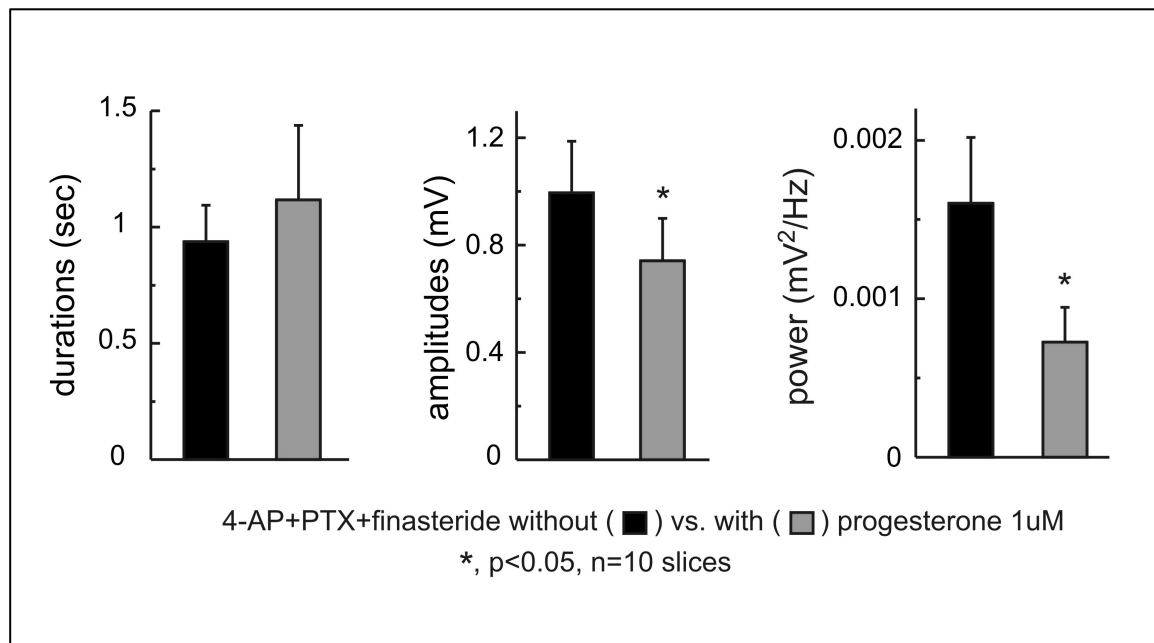


Figure 4.6 Quantification of waveform “power” changes induced by 1 μ M progesterone in the presence and absence of 1 μ M of finasteride. Left graph, ACSF (gray bar) + 1 μ M progesterone (black bar). Right graph, ACSF with 1 μ M finasteride (gray bar) + 1 μ M progesterone (black bar). Mean $\pm$ SE.

4.4 Originality

To our knowledge, the present study is the first study to report these effects.

Others have found different results with progesterone in vitro, but varying methodologies make comparisons difficult.

Edwards et al (2000a) found that in tetanized hippocampal slices, 10 nM progesterone, a physiological dose, reduced hippocampal epileptiform discharges. In non-tetanized slices, Edwards et al (2000a) found that 10 nM of progesterone had no effect. Higher doses were not tested. We did not find 10 nM of progesterone to be effective (data not shown), but there are important differences between our in vitro studies and those of Edwards et al (2000a). Our group used male mice, Edwards’ group used female ovariectomized rats that had or had not been given estrogen replacement therapy. Our

group used a pharmacologically induced spontaneous epileptiform in vitro model, whereas the epileptiform discharges in the Edwards et al (2000a) study were induced by a train of high frequency electrical stimulation. Finally, Edwards et al (2000a) recorded from the hippocampus, whereas we recorded from the entorhinal cortex.

Chapter 5: General Discussion

5.1 Overview

A significant number of persons with epilepsy do not have seizure control with the currently available anticonvulsant drugs (Mitchell et al, 2012; Mula, 2013). Lack of seizure control and the adverse side effects of existing drugs damage quality of life (Mitchell et al, 2012; Bootsma et al, 2009), including mental health and social wellbeing (Tellez-Zentano et al, 2007; Toledano & Gil-Nagel, 2008; Titlic et al, 2009; Adelöw et al, 2012; Quitas et al, 2012; Viteva, 2013; Gaitatzis & Sander, 2013). New anticonvulsant drugs are needed, but they should be drugs without the side effects that limit use and compliance (Bootsma et al, 2009).

Our laboratory had previously found progesterone metabolites to be effective anticonvulsants in amygdala kindled male and female rats (Lonsdale & Burnham, 2003; Lonsdale et al, 2006; Lonsdale & Burnham, 2007). This suggested that these progesterone metabolites, or analogs thereof, might be candidates for development as novel anticonvulsant compounds. Neuroactive steroids, however, have psychoactive properties (Morrell et al, 2005; Pack et al, 2011), which might limit their tolerability as anticonvulsant therapies. The purpose of our behavioral experiments, therefore, was to compare the psychotoxicity of anticonvulsant doses of DHP and THP in naïve (unkindled, non-epileptic) male and female rats. Minimal psychotoxic effects were found at anticonvulsant doses.

Next, we studied the effects of progesterone and its metabolites in electrophysiological models in vivo and in vitro, in male mice. After pilot studies, we suspected that progesterone could have THP/GABA independent anticonvulsant effects. Surprisingly, the GABA independent anticonvulsant effects of progesterone have not been rigorously assessed. Our general purpose was to investigate

progesterone's THP/GABA dependent and THP/GABA independent anticonvulsant effects in hippocampal kindled male mice in vivo, and in isolated entorhinal circuitry in vitro.

In kindled mice in vivo, we found anticonvulsant effects of progesterone and THP, but not DHP. Surprisingly, progesterone's anticonvulsant effects were still seen in the presence of finasteride. In vitro, we also saw anticonvulsant effects of progesterone both in the presence of finasteride and in the presence of the GABA_A blocker picrotoxin. These are novel results.

5.2 Behavioral Studies

5.2.1 Overview

We administered anticonvulsant doses of both DHP and THP to male and female rats, and tested the animals' performance in three behavioral tests.

5.2.1.1 The Forced Swim Test

We tested anticonvulsant doses of DHP (5 mg/kg) and THP (5 mg/kg) in the FST to assess depressant and/or antidepressant effects. Inhibition of progesterone's metabolism has been reported to have both depressant and antidepressant effects, suggesting that DHP and/or THP might have effects on mood as well as seizures (Frye & Walf, 2004; Uzunova et al, 2006; Walf & Frye, 2006; Frye et al, 2011; Frye 2010; Reddy, 2010; Schüle et al, 2011; Frye et al, 2012).

5.2.1.2 The Morris Water Maze

We used the Morris water maze (MWM) matching-to-place paradigm using the protocol of Schwabe et al (2007), with slight modifications, to assess the effects of anticonvulsant doses of DHP (5 mg/kg) and THP (5 mg/kg) on learning and memory. Previously it had been suggested that DHP and THP were associated with learning deficits in rats (Frye et al, 2007; Frye & Walf, 2008a, 2008b; Frye & Walf, 2009; 2010).

5.2.1.3 The Elevated Plus Maze

In the elevated plus maze (EPM), our goal was to determine whether anticonvulsant doses of DHP (5 mg/kg, 7.5 mg/kg, 10 mg/kg) and THP (5 mg/kg, 7.5 mg/kg, 10 mg/kg) are anxiogenic or anxiolytic. Anxiogenic properties might limit the potential value of these compounds, or their analogs, for development as drugs. We suspected that these metabolites would have an anxiolytic effect, as per the literature (Schüle et al, 2011).

5.2.1.4 Drug, Dose, and Injection-Test Interval Rationale

Few studies have used the combination of drugs and their dosages or the same treatment-test interval that we used in our behavioral studies. This likely accounts for results that seem inconsistent with other studies (below). Since our goal was to assess psychotoxic effects of acute treatment with DHP and THP at anticonvulsant doses, the rationale for our doses and injection/test interval for DHP and THP is based on previous studies.

5.2.2 Results

5.2.2.1 Forced Swim Test & Open Field (Section 2.4.1)

In our experiments, anticonvulsant doses of DHP and THP did not cause any significant changes in the FST (**Fig 2.1-2.2**). Thus, anticonvulsant doses of these progesterone metabolites did not affect depression-like behaviors in male and female rats. We also did not observe any significant effects in the OFT. Thus, DHP and THP treatments did not appear to cause sedative effects or locomotor impairment in rats (**Fig 2.3-2.6**).

We are not aware of any FST or OFT experiment testing the effects of DHP, at any dose. Our results with DHP, therefore, are novel. Other investigators have found THP to be antidepressant-like in the FST (for review, see Uzunova et al, 2006). We propose that differing methodologies account for these different results. In general, our results with THP are in agreement with other FST and OFT experiments using similar doses, test-injection intervals, and protocols.

5.2.2.2 The Morris Water Maze (Section 2.4.2)

We measured the acquisition of memory, working and reference memory, velocity and spatial memory in the MWM (**Fig 2.7-2.34**). In almost every measure of learning and memory, DHP and THP did not differ from vehicle control. We hypothesize that differences in species, definition of spatial/reference memory, MWM protocol, drug doses, routes of injections and administration protocols might explain our differing results from other investigators.

5.2.2.3 The Elevated Plus Maze (Section 2.4.3)

At a range anticonvulsant doses (5, 7.5, and 10 mg/kg), DHP and THP were neither anxiogenic nor anxiolytic, as compared to vehicle control in the EPM (**Figs 2.35-2.44**). Most other investigators have found THP to be a potent anxiolytic at doses of 5-10 mg/kg (Picazo & Fernández-Guasti, 1995; Rodgers & Johnson, 1998; Reddy et al, 2005; Kita et al, 2008). We propose that our handling protocol accounts, at least in part, for these differences. Our extended handling protocol may have reduced baseline levels of anxiety in the rats and affected our results (Whimby & Denenberg, 1967; Hogg 1996; see below).

5.2.3 Behavioral Studies: Limitations

There are three limitations to these behavioral experiments. The first is the lack of a positive control. It is difficult to know the relevance of our results with DHP and THP without comparison to an effective positive control, such as fluoxetine (antidepressant) in the FST, propranolol (impairs learning) in the MWM, and midazolam (anxiolytic) in the EPM. Another option would be to compare the behavioral effects of clinically relevant doses of anticonvulsant medications in each of these behavioral tests.

Secondly, differences between our behavioral experiments and the results observed in previous kindling studies may be due to neurobiological differences. The experiments that showed significant effects of DHP and THP were experiments with amygdala kindled male and female rats (Lonsdale & Burnham, 2003; Lonsdale et al, 2006; Lonsdale & Burnham, 2007). The behavioral

experiments that assessed potential psychotoxicities, however, were performed in rats that had not been kindled. Kindled brains differ significantly from non-kindled brains (Goddard et al, 1969; Morimoto et al, 1986; Burnham, 1989; Vreugdenhil & Wadman, 1992; Bernstein et al, 1999; Francis et al, 2002; Morimoto et al, 2004; Gavrilovici et al, 2006; Hum et al, 2009; Sloviter et al, 2010). Neurosteroids also affect kindled and non-kindled brains' GABA_A receptors differently (Kia et al, 2011). It may have been preferable to perform our toxicity tests in kindled animals. This might be done in future studies.

The third significant limitation to our experiments is the handling paradigm that was used. Subjects were handled for 5 minutes a day, 5 days a week for 4 weeks (20 sessions of 5 minutes each) in order to match previous kindling studies in our laboratory (Lonsdale & Burnham, 2003; Lonsdale et al, 2006; Lonsdale & Burnham, 2007). Although there is extended human contact in kindling studies, most rats are not handled so extensively before behavioral experiments (Khisti & Chopde, 2000; Rodríguez-Landa et al, 2007). Handling can have numerous effects on neurobiology and behavior (Whimby & Denenberg, 1967; Wade & Maier, 1986; Boix et al, 1990; Andrews & File, 1993; Hogg, 1996; Schmitt & Hiemke, 1998; van Praag et al, 2000; Moncek et al, 2004; Bayne, 2005; Hutchinson et al, 2005; McNair et al, 2007; VanElzakker et al, 2008; Bortolato et al, 2011; Bogdanova et al, 2013). The neurobiological effects of our handling protocol in non-kindled animals (the present behavioral studies), therefore, may differ from the neurobiological effects of human contact in previous kindling studies from our laboratory (Lonsdale & Burnham, 2003; Lonsdale et al, 2006; Lonsdale & Burnham, 2007).

The effects of GABAergic drugs, such as benzodiazepines, in behavioral tests, are also attenuated with extended rodent handling (Boix et al, 1990; Andrews & File, 1993). Our handling protocol may have obscured psychotoxic effects of DHP and THP. In particular, they may have obscured the possible anxiolytic effects of anticonvulsant doses of THP. Future studies might test DHP and THP in less well-handled animals.

5.2.4 Behavioural Studies: Summary

In male and female rats, anticonvulsant doses of DHP and THP are not associated with serious adverse effects in models of acute depression, locomotion, learning and memory, or anxiety (MWM in preparation; FST **Appendix 1**, submitted; EPM, **Appendix 2**, submitted).

5.3 Electrophysiological Studies

5.3.1 Electrophysiology In Vivo: Overview (Chapter 3)

To assess potential mechanisms of anticonvulsant action, we began to investigate the anticonvulsant effects and electrophysiological profiles of progesterone and its metabolites, as well as clinically used drugs in male mice. Male mice were used to avoid the endogenous estrous cycles in females that complicate the interpretation of experiments testing neurosteroids.

The purpose of the electrophysiological experiments in vivo was to characterize the neurophysiological and behavioral effects of progesterone and its metabolites, and to compare them to clinically used anticonvulsant drugs. We used hippocampal-kindled male mice with a novel surgical procedure that allowed for chronic EEG recordings with multiple intracranial electrodes (Jeffrey et al, 2013, **Appendix 3, Fig 3.1**). Our measurements, in acutely induced seizures, included properties of the AD (Racine, 1972a) and motor seizure stage (Racine, 1972b). We tested doses of the neuroactive steroids progesterone, DHP and THP. Progesterone was tested in the presence and absence of the *5 α -reductase* inhibitor finasteride, which inhibits the metabolism of progesterone to DHP. We also tested the anticonvulsant drugs midazolam and carbamazepine as positive controls. We used the vehicle, β -cyclodextrin, as a negative control; it did not show effects on hippocampal ADs or motor seizure stages.

5.3.2 Progesterone, DHP and THP: Hippocampal ADs and Behavioral Motor Seizures

5.3.2.1 Progesterone

We injected progesterone 30 minutes before hippocampal stimulation at doses of 10, 35, 100, and 160 mg/kg (n=6-17). We observed a dose-dependent reduction in hippocampal AD and motor seizure stage (**Fig 3.3B**). Progesterone doses of 100 and 160 mg/kg reduced AD length ($p<0.05$). Progesterone doses of 100 and 160 mg/kg also reduced motor seizure stages as compared to vehicle control ($p<0.05$).

5.3.2.2 DHP

We injected DHP 15 minutes before hippocampal stimulation at doses of 5 and 10 mg/kg. Higher doses could not be tested due to solubility problems. Unlike previous studies from our laboratory, which were done in amygdala-kindled rats, in our model of kindled mice, DHP was not anticonvulsant at doses of 5 and 10 mg/kg. At 5 mg/kg, neither AD duration nor motor seizure stage was reduced compared to vehicle control ($p>0.05$, n=10; **Fig 3.4B**). Similarly, at 10 mg/kg, neither AD duration nor motor seizure stage was reduced compared to vehicle control ($p>0.05$, n=14).

5.3.2.3 THP

We injected THP 15 minutes before hippocampal stimulation at doses of 1, 3.5, 10 and 30 mg/kg (n=5-8). At doses of 10 and 30 mg/kg, THP significantly reduced hippocampal AD length and motor seizure stage ($p<0.05$, n=7; **Fig 3.5B**).

THP at doses of 1 and 3.5 mg/kg had no effect on hippocampal ADs ($p>0.05$, $n=5-8$). THP significantly reduced motor seizure stage at doses of 3.5, 10, and 30 mg/kg ($p<0.05$, $n=6-8$). At 1 mg/kg, THP had no effect, but approached statistical significance compared to vehicle control in a reduction of motor seizure stage ($p=0.059$, $n=8$).

The results with progesterone and THP generally agree with previous studies (Bayenburg et al, 2001; Lonsdale & Burnham, 2003; Lonsdale et al, 2006; Ciriza et al, 2006; Singh et al, 2010). These results confirm the known anticonvulsant effects of progesterone and THP in kindled mice. In the above results, progesterone is likely metabolizing to THP. Therefore, in our next experiments, we tested the anticonvulsant action of progesterone independent of its metabolism to THP using the *5 α -reductase* inhibitor finasteride.

5.3.2.4 Anticonvulsant Effects of High Doses of Progesterone Were Not Diminished by Finasteride Pretreatment

In our next experiments, we tested the anticonvulsant action of progesterone independent of its metabolism to THP using the *5 α -reductase* inhibitor finasteride. Finasteride alone did not affect AD or seizure stage ($p>0.05$, $n=8-14$; **Fig 3.6**). To ensure blockade of the *5 α -reductase* enzyme, we pretreated animals with 50 mg/kg of finasteride 3 hours before hippocampal stimulation. Progesterone was injected 30 minutes before stimulation (2.5 hours after finasteride injection).

Interestingly, despite the lack of effect on hippocampal ADs, with finasteride pretreatment, progesterone decreased motor seizures compared to vehicle control at 35, 100, and 160 mg/kg ($p > 0.05$, $n = 5-10$; **Fig 3.6B**) in mice with CA3 recording electrodes. Since there was no reduction of hippocampal AD, but significant reductions in motor seizure stage, we hypothesized that progesterone (with finasteride) was suppressing seizure generalization from the hippocampal focus. To test this hypothesis, we recorded hippocampal and cortical ADs in five hippocampal-kindled mice.

5.3.3 Mice with Recording Electrodes in the Hippocampus and the Motor Cortex: Progesterone and Finasteride

Hippocampal and cortical ADs were induced by contralateral hippocampal stimulation. Since the hippocampal ADs preceded the cortical ADs (3.37 ± 0.64 seconds), the cortical ADs observed may represent an electroencephalographic measure of seizure spread from the stimulated hippocampus to the motor cortex.

Finasteride alone did not significantly affect hippocampal AD, cortical AD or seizure stage ($p > 0.05$, $n = 5$, **Fig 3.6**) as compared to vehicle control in the cortically implanted mice. Finasteride pre-treatment, however, prevented the reduction of hippocampal ADs that had been observed when animals were treated with 100 and 160 mg/kg of progesterone alone (**Fig 3.6, 3.7**). With and without finasteride (50 mg/kg) pretreatment, progesterone at 100 mg/kg reduced motor seizure stage ($p < 0.05$).

5.3.4 Mice with Recording Electrodes in the Hippocampus and the Motor Cortex: Midazolam Resembles THP, and Carbamazepine Resembles Progesterone with Finasteride

We next tested the anticonvulsant drugs carbamazepine and midazolam, which have known mechanisms of anticonvulsant action. Carbamazepine suppresses voltage-gated sodium channel activity as its primary mechanism of action (Mula, 2013; Rogawski & Löscher, 2004). Midazolam, a benzodiazepine, is a positive allosteric modulator of the GABA_A receptors with a rapid onset of action (Meierkord et al, 2010). These two drugs were injected 15 minutes before hippocampal stimulation in kindled mice (**Fig 3.8**).

Like progesterone with finasteride, carbamazepine at 50 mg/kg did not suppress the hippocampal AD ($p=0.507$, $n=13$) compared to vehicle (**Fig 3.8B**). Also like progesterone with finasteride, carbamazepine did suppress seizure stage ($p=0.001$, $n=13$). Motor seizure suppression by carbamazepine was associated with significant reduction in cortical AD amplitude ($47.01 \pm 6.04\%$ of vehicle controls, $n=5$, $p<0.01$).

Midazolam at 2 mg/kg resembled THP. Midazolam significantly suppressed the hippocampal AD length and cortical AD amplitude ($p<0.05$, $n=14$ and 5, respectively) and reduced seizure stage ($p<0.05$, $n=12$) compared to vehicle controls (**Fig 3.8B**).

5.3.5 Electrophysiology In Vitro: Overview (Chapter 4)

In vitro experiments were conducted to assess whether progesterone could have THP and GABA_A independent inhibitory effects in isolated entorhinal circuitry. The enzyme inhibitor finasteride was used to inhibit the enzyme *5 α -reductase*, hence blocking the metabolism of progesterone to DHP and THP (**Fig 1.3**).

The effects of the treatments were measured in conventional entorhinal cortex brain slices in vitro. We measured incidence, frequency, and power (rhythmicity) of seizure-like events induced in the entorhinal slice by the GABA_A antagonist picrotoxin. We observed epileptiform field potentials as rhythmic events with incidences of 10-15 events per minute, with peak amplitudes of up to 1.5 mV and durations of up to 2 seconds. Spectral analysis determined that rhythmic spike activities in individual epileptiform potentials had dominant frequencies of 12.5 ± 0.8 Hz.

5.3.6 In vitro results: Progesterone Retains its Anticonvulsant Effects in the Presence of Picrotoxin and Finasteride

With 1 μ M of progesterone, the amplitude and power of the rhythmic spike activity in epileptiform field potentials were significantly reduced as compared to baseline ($p=0.007$ and $p=0.013$, $n=10$; **Fig 4.4**). Since these effects of progesterone occurred in the presence of picrotoxin, they were clearly not mediated by a GABAergic mechanism. Since they continued to occur in the presence of

finasteride, which prevents progesterone's metabolism to DHP (and subsequently THP), this mechanism is also independent of these metabolites, and is likely mediated by progesterone itself.

Our in vitro results show that a supraphysiological concentration of progesterone, in the presence or absence of finasteride, is able to inhibit epileptiform population field potentials in the presence of a GABA antagonist. We suggest these in vitro results concur with our in vivo results (above), and confirm that progesterone may have an anticonvulsant mechanism of action independent of THP and the GABA_A receptor.

5.3.7 Electrophysiology Experiments: Originality

In vivo, progesterone's anticonvulsant effects persisted in the presence of finasteride. Progesterone (100 mg/kg) with finasteride (50 mg/kg) pretreatment was associated with a strong suppression of the cortical AD, but, unlike progesterone alone, progesterone with finasteride did not suppress of the hippocampal AD. This was an unexpected and novel result, which we subsequently explored in vitro.

Our in vitro experiment measuring field potentials in the entorhinal cortex confirmed our in vivo results. In the presence of the GABA_A receptor antagonist picrotoxin, 1.0 μ M of progesterone inhibited population field potentials in mouse entorhinal slices. This confirmed that progesterone's high-dose anticonvulsant effects were not GABAergic. This effect persisted when slices were pre-treated with finasteride, which ensured that metabolism of progesterone to

DHP and THP was not occurring in our in vitro protocol. This confirmed that progesterone's high-dose anticonvulsant effects were not dependent on its metabolites, DHP and THP.

5.4 Disparities Between Past and Present Kindling Studies

Our results in electrophysiological experiments in mice did not entirely concur with our laboratory's previous experiments in rats. In particular, DHP was not an effective anticonvulsant drug in our mouse model. We hypothesize that differences in species and kindling site contribute to our differing results in the present studies.

5.4.1 Neurosteroids and Their Receptors Differ in Mice and Rats

There are substantial differences in neurosteroids between the mouse and rat brains (Korneyev et al, 1993; Morimoto et al, 2004; Bonthuis et al, 2010). This includes both levels of neurosteroids and their receptors in the brain (Brinton et al, 2008; Do Rego et al, 2009). Species differences may have contributed to DHP's lack of efficacy in mice compared to its anticonvulsant efficacy in rats.

5.4.2 Kindling the Hippocampus

The kindling site and recording method in our experiments also differed from previous experiments in our laboratory. Previous experiments stimulated and recorded from a single basolateral amygdala in the rat (Lonsdale & Burnham, 2003; Lonsdale et al, 2006; Lonsdale & Burnham, 2007). In the present experiments, we

stimulated the hippocampal CA3 area and recorded ADs from the contralateral CA3 and the motor cortex in the mouse.

The hippocampus and amygdala may also differ in their response to neurosteroids. Frye & Walf (2008a; 2008b; 2010), for instance, found that progesterone affected hippocampus- and amygdala-dependent tasks differently. Progesterone improved water maze performance, a hippocampal-dependent task, but not cued fear conditioning, an amygdala-dependent task. It is possible that amygdala-kindled animals may have had different responses to progesterone and its metabolites than hippocampal-kindled animals.

5.5 Potential Mechanisms for Progesterone's Antiepileptiform Effects

5.5.1 THP-Mediated GABAergic Inhibition

In many animal models, seizure suppression by progesterone is lost or weakened when the animals are pre-treated with finasteride (Kokate et al, 1999; Herzog & Frye, 2003; Reddy et al, 2004; Reddy & Ramanathan, 2012). Many of these experiments, however, used doses of progesterone lower than those used in the present experiments. In Kokate et al's (1999) experiments, however, very high doses of progesterone did not lose efficacy in the MES with finasteride (Kokate et al, 1999). Kokate et al's (1999) results, therefore, suggest that progesterone may have a mechanism of anticonvulsant action independent of THP and GABAergic

inhibition. This hypothesis is supported by the results of the present studies, both in vivo and in vitro.

5.5.2 Action on Ion Channels

Recently, progesterone has been reported to regulate brain activity via a number of GABA_A receptor independent mechanisms (Zhu et al, 2008; Hwang et al, 2009; Zheng 2009; Johannessen et al, 2011; Kelley & Mermelstein, 2011; Luoma et al, 2011; Singh & Su 2013; King, 2013). There are, therefore, many possibilities.

Progesterone can inhibit voltage-dependent calcium, sodium and potassium channels with high specificity in vitro (Luoma et al 2011; Kelly & Mermelstein, 2011). Increasingly, progesterone's effects on voltage-gated calcium channels in particular are becoming a focus of research (Luoma et al, 2011; Luoma et al, 2012). The activation of calcium channels can in turn activate potassium and sodium channels, and subsequently, NMDA receptors.

Inhibition of calcium signaling could prevent other excitatory signaling that is dependent on calcium channel activation (Turner et al, 2011). Single cell in vitro neuroprotection studies have shown that micromolar (supraphysiological) levels of progesterone block calcium currents and channels, and the channels' gene expression, reducing excitotoxicity (Maurice & Su, 2009; Johannessen et al, 2011).

5.5.3 Action on Sigma Receptors

Sigma receptors are widely distributed in the CNS, and are localized to intracellular membranes (van Waarde et al, 2011). Sigma (σ) receptors have been

known to modulate NMDA receptor-mediated glutamatergic signaling for over 20 years (Monnet et al, 1995). Intriguing recent reports in vitro suggest that progesterone could exert its anticonvulsant effects by antagonizing the σ receptor, whereas the excitatory neurosteroids pregnenolone sulfate and dehydroepiandrosterone are σ receptor inverse agonists and agonists, respectively (Monnet et al, 1995; Ahmed et al, 2012; King, 2013). The progesterone metabolite THP is not an antagonist for σ receptors (Monnet et al, 1995). Sigma receptors, therefore, might mediate progesterone's non-THP mediated inhibition of downstream excitatory signaling (Vilner & Bowen, 2000; Ahmed et al, 2012).

Recent experiments have shown the specific progesterone binding to σ receptors antagonizes voltage-gated sodium channels and blocks calcium channels (Johannessen et al, 2011; Luoma et al, 2012). Progesterone at a concentration of 239-441 nM displaced other ligands in human embryonic kidney cells, and has a binding affinity for the receptor in the micromolar range (Zamanillo et al, 2007; Johannessen et al, 2011). These data agree fairly well with our own results in the hippocampus in vitro: effects of progesterone were insignificant at ≤ 100 nM progesterone, but we found significant effects when 1 μ M of progesterone was applied to the slices.

The role of σ receptors with respect to epilepsy is not clear. The broader physiological role(s) of progesterone binding σ receptors is unknown (Ahmed et al, 2012). The σ receptors are known, however, to be involved in many CNS disorders, some of which are common comorbidities of epilepsy: depression, anxiety, psychosis, memory deficits, and endocrine disorders (Schmitz et al, 1999; Tellez-Zenteno et al, 2007; Bootsma et al, 2009; Titlic et al, 2009; Collina et al, 2013).

5.6 Clinical Meaning

5.6.1 Clinical Meaning: Behavioral Studies

Acute anticonvulsant doses of DHP and THP did not cause behavioral toxicity in models of depression, anxiety, or learning and memory (Appendices 1,2; Jeffrey et al, in preparation). These progesterone metabolites, or their analogs, might have beneficial effects in treating drug-refractory epilepsy without exacerbating common mental health comorbidities of epilepsy: depression, anxiety, and problems with learning and memory (Kanner, 2009).

5.6.2 Clinical Meaning: Electrophysiological Studies

Our electrophysiology studies suggest a possible target for future drug development. We are the first group to report that high doses of progesterone may have non-THP-mediated, non-GABAergic mechanisms of anticonvulsant action in kindling and field potential models of seizures. If our work can be confirmed, the target of progesterone may be useful in the development of novel anticonvulsant compounds. The σ receptors could be promising drug targets for investigation. It is possible that the pharmacology of σ receptors has relevance to the comorbidities of epilepsy as well.

5.7 Future Studies

Our in vivo results suggest different responses to progesterone with and without finasteride pretreatment in the hippocampus (no AD suppression) and neocortex (complete AD suppression). Our in vitro results in the entorhinal cortex

demonstrated clear inhibition of epileptiform field potentials with supraphysiological doses of progesterone. Both in vitro and in vivo results suggest differential responses by brain region. To investigate behavioral effects and identify potential mechanisms, we suggest the following in vivo and in vitro experiments.

For all future studies, the ability to measure levels of progesterone, DHP, THP, *5 α -reductase* and σ receptors in different regions of the brain following treatments would potentially yield insights into site of action. Our collaboration with a biochemical laboratory to measure these compounds is ongoing.

5.7.1 Future Behavioral Studies

The present experiments examined the possible psychotoxic effects of DHP and THP at anticonvulsant doses. Almost none were found. It is possible, however, that these compounds might have significant effects at higher doses, and that some of these might have therapeutic applications (ie, as anxiolytics). The forced swim test, open field test, elevated plus maze, and Morris water maze experiments, therefore, might be repeated in rats in dose-response studies, which would include higher doses. Efforts should be made to dissolve higher doses of DHP (>10 mg/kg) so they might be included in the dose-response studies.

It would be important to have positive controls that act as comparators in these studies. As mentioned earlier, in future experiments could use positive control drugs such as fluoxetine (antidepressant) in the FST, propranolol (impairs learning) in the MWM, and midazolam (anxiolytic) in the EPM. The use of a non-benzodiazepine anticonvulsant drug, such as carbamazepine, would also be

important to understanding any relative psychotoxicity of DHP and THP compared to clinically used compounds.

It would also be important to modify our handling technique to avoid the long period of handling used in the present experiments, which we suspect may have compromised the present experiments. Since the proposed future dose-response studies would include our anticonvulsant doses, they would allow a confirmation of our conclusions in less-handled animals.

5.7.2 Future Electrophysiology Studies In Vivo

Our experiments in kindled mice revealed high dose, non-GABAergic anticonvulsant effects of progesterone. Future genetic experiments might reveal mechanism(s) involved. Genetically modified animals could be tested in the kindling model with high doses of progesterone. They could investigate whether the effect of progesterone persists in kindled homozygous and heterozygous *5 α -reductase* knock out mice, as compared to wild type mice (tests would be done with finasteride).

An important future study would attempt to resolve the differences between the effects of neurosteroids in rats (Lonsdale & Burnham, 2003; Lonsdale et al, 2006; Lonsdale & Burnham, 2007) and mice (the present studies, Jeffrey et al, 2014). A first experiment might involve dose-response studies of progesterone, DHP and THP in amygdala-implanted (Lonsdale studies) and hippocampal implanted (present studies) kindled rats. The same study could then be repeated in mice.

5.7.3 Future Studies In Vitro

We have proposed that progesterone's high-dose effects may be mediated by σ receptors. Future in vitro experiments might examine the effects of progesterone, with and without finasteride, in cell cultures with genetically modified σ receptors.

The σ -1 receptors have been somewhat characterized. Unfortunately, σ -2 receptors are not yet well characterized; they have not been cloned or sequenced. Transgenic knock out animals are not yet available. The σ -2 receptors can be overexpressed or knocked down, however, using short interfering RNA (siRNA) in HeLa cells in vitro. This is done in cancer research (Xu et al, 2011). We suggest an experiment with neuronal cells using similar techniques for σ -2 and σ -1 receptor genetic manipulation. These would assess excitability differences when progesterone is applied. If, in the presence of high doses of progesterone, excitability increases when σ -receptors are knocked down and/or decreases when σ -receptors are overexpressed, progesterone's mechanism of anticonvulsant action at the cellular level would be strongly associated with the antagonism of σ -receptors.

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Appendix 1: Forced Swim Test and Open Field,
Submitted

Anticonvulsant Doses of Progesterone Metabolites Are Not Toxic in the Forced Swim or Open Field Tests.

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Abstract

In previous studies from our laboratory, the pregnane neurosteroids 5 α -dihydroprogesterone (DHP) and 3 α ,5 α -tetrahydroprogesterone (THP, also called allopregnanolone) were effective anticonvulsants in the amygdala-kindling model in rats. Amygdala-kindling models drug-refractory complex partial seizures with secondary generalization. The purpose of the present study was to examine the potential psychotoxic side effects of anticonvulsant doses of DHP and THP. Anticonvulsant doses of DHP, THP or vehicle were injected, and the forced swim test and the open field test were conducted in adult male and female Wistar rats. No differences were observed between experimental and control subjects on any behavioral measure. These data suggest that DHP and THP, or their analogs, might be effective against partial or secondarily generalized seizures without adverse psychotoxic side effects.

1. Introduction

Progesterone has anticonvulsant properties in both humans and in animal seizure models (Pack *et al*, 2011; Finocchi & Ferrari, 2011; Herzog *et al*, 2012). Most of these anticonvulsant effects appear to relate not to progesterone itself, but to its active metabolites, 5 α -dihydroprogesterone (DHP) and 3 α ,5 α -tetrahydroprogesterone (THP, also known as allopregnanolone). Progesterone is metabolized to DHP by the enzyme 5 α -*reductase* (5 α R), and DHP is further metabolized to THP by the reversible enzyme 3 α , 5 α -*hydroxysteroid oxidoreductase* (3 α HSOR, Figure 1).

Previous experiments in our laboratory have found the pregnane neurosteroids - progesterone, DHP, and THP - to be effective anticonvulsants in the amygdala-kindling model, a model of drug-refractory, partial seizures that secondarily generalize (Albright

& Burnham, 1980; Lonsdale & Burnham, 2003; Lonsdale *et al*, 2006; Lonsdale & Burnham, 2007). The focal amygdala component of amygdala-kindled seizures models complex partial seizures in humans, whereas the generalized convulsive component models human tonic-clonic attacks (Albright & Burnham, 1980).

In our kindling experiments, progesterone partially suppressed the focal and generalized seizures at somewhat toxic (ataxic) doses, whereas THP suppressed generalized (but not the focal) seizures at much lower, less toxic doses. Remarkably, DHP completely suppressed both the partial and the generalized components of the seizures at low, non-toxic doses. The mechanism of DHP is unknown, whereas THP is known to enhance GABAergic inhibition (Belelli *et al*, 2009; Akk *et al*, 2007)..

Despite the variety of anticonvulsant drugs available today, many patients do not achieve complete seizure control (Bialer & White, 2010; Bialer *et al*, 2010), especially for seizures of partial onset (Nasreddine *et al*, 2010). Given our promising results with DHP and THP, we hypothesize that these compounds, or their analogs, might be developed as effective treatments for partial seizures or partial seizures that secondarily generalize. An analog of THP, ganaxolone, is already in development, although it has dose-limiting side effects of sedation (Nohria & Giller, 2007; Steven & Brodie, 2011).

If new anticonvulsants are to be developed, it is important that they have low toxicity. All the anticonvulsant treatments currently available have side effects, limiting use and compliance (Bootsma *et al*, 2009). While neither DHP nor THP produced obvious sedation or ataxia at anticonvulsant doses in our kindling studies, it is possible that they might have subtle, neurotoxic effects that would not be observed using standard sedation/ataxia scales. In particular, progesterone and its metabolites have been associated with mood changes in the human population (Schüle *et al*, 2011). To

investigate the possible subtle psychotoxic effects of anticonvulsant doses of DHP and THP, therefore, we conducted the forced swim test and the adjunctive open field test in male and female rats.

The forced swim test (FST), also known as the “Porsolt test,” is a drug-validated animal model of depression-like behavior in rodents (Porsolt *et al*, 1977 & 1978). It is a model commonly used in the screening of novel antidepressant compounds (Cryan *et al*, 2002). It responds to a variety of antidepressant compounds with diverse mechanisms of action (Borsini & Meli, 1988; Cryan *et al*, 2005).

The FST is usually performed in conjunction with the open field test (OFT). The OFT confirms that the changes in active and passive behaviors observed in the FST do not result from changes in the subjects’ basic activity levels.

2. Material and Methods

All experimental protocols were approved by the Animal Care Committee at the Faculty of Medicine, University of Toronto, and were in accordance with the guidelines of the Canadian Council for Animal Care.

2.1 Subjects

Adult male and female Wistar rats, 60 days of age (Charles River, Quebec, Canada) were used in this experiment. All animals were housed individually in 24 x 24 x 45 cm transparent plastic cages. Food and water were available *ad libitum*. Temperature (21° C) and a 12-hour light/dark cycle (light on at 7:00 h) were kept constant in the vivarium. Male and female sample sizes were n=36 with equal numbers in each treatment group (n=12) for each test. Female animals’ estrous cycles were not tracked in

this experiment. The OFT was conducted using different groups of male and female animals than those used in the FST.

2.2 Drugs

β -cyclodextrin, DHP and THP were obtained from Sigma Chemical Company. The vehicle injections consisted of 45% β -cyclodextrin in physiological saline mixed thoroughly with a stir bar. DHP and THP were dissolved in the same vehicle, shaken vigorously, and then gently heated and sonicated until the compounds had dissolved. Fresh solutions were made for each day of drug testing. Drugs were injected intraperitoneally (i.p.). The vehicle control injections were matched in volume to the DHP and THP injections.

DHP was administered i.p. at a dose of 5 mg/kg, and THP was administered i.p. at a dose of 5 mg/kg. The doses selected (5 mg/kg) are within the anticonvulsant dose range, but not the ataxic range, for males and females in our previous experiments. As in previous experiments, the interval between injection and testing was 15 minutes (Lonsdale & Burnham, 2003; Lonsdale *et al*, 2006; Lonsdale & Burnham, 2007).

2.3 Handling Procedure

Before testing in the FST and OFT, animals were handled for five minutes a day, 5 days a week for four weeks. This was done to match the handling done in our previous kindling studies. Male and female animals were handled (and subsequently tested) in separate groups on different days.

2.4 The Forced Swim Test.

At the end of the handling period, the animals (~90 days old) began testing in the Forced Swim test (FST). On each experimental day, the animals were allowed to acclimatize to the experimental room in their home cages for at least thirty minutes before experimental procedures began. The experimental room had low light levels, from indirect sources, and was kept quiet. All tests were conducted between 9 am and 4 pm.

The Forced Swim Test (FST) apparatus consisted of a clear plastic cylindrical apparatus 45 cm high and 20 cm wide (Geneq Inc., Quebec, Canada). The apparatus was filled with water (25° C) to a level of 27 cm, such that animals could not either touch the bottom or escape.

The FST procedure took place over two days. On the first day, the untreated animal was placed in the swimming apparatus for ten minutes without any treatment (training day). On the following day (test day), the animal was injected i.p. with 5 mg/kg of DHP, THP or with vehicle and – after 15 minutes - was placed in the swimming apparatus for five minutes. Active (swimming) and passive (immobile or floating) behaviors were measured using a stopwatch by an experimenter who was blinded to the treatment groups.

Following each exposure to the apparatus, animals were gently dried and placed under a heat lamp before being returned to their home cages.

2.5 The Open Field Test

At the end of the handling period, a different group of animals (~90 days old) began testing in the Open Field test (OFT). The OF apparatus was constructed of plywood with a 100 x 100 cm base and 60 cm high walls. It was illuminated by a 60-

Watt light bulb, positioned 1.5 meters above the apparatus floor. The interior of the apparatus was painted white with 10 x 10 cm squares outlined in black on the floor. The apparatus was varnished for easier cleaning. The open field apparatus was cleaned between subjects with dilute Virox.

On the day of the test, the animals were allowed to acclimatize to the experimental room in their home cages for at least thirty minutes before testing began. The experimental room was different from the room used for the FST, and none of the animals had been exposed to the experimental room or the OF apparatus before the test. Apart from the 60-Watt light bulb, the experimental room had low ambient light levels from indirect sources, and was kept quiet.

Before testing, animals were injected (i.p.) with DHP, THP or with vehicle and - after 15 minutes - were placed in the northwest corner of the OF apparatus. Using a Labview program and video tracking, the following data were collected: horizontal speed, distance, and velocity, vertical activity (rearing), time grooming, and fecal boli. Labview recordings of the OFT were subsequently scored by an experimenter blinded to treatment. All tests were conducted between 9 am and 4 pm.

2.6 Data Analysis

SPSS Statistics version 20 was used for all data analyses. The effects of treatment were analyzed with one-way ANOVAs by an experimenter blinded to the treatment groups.

3. Results

The FST and OFT results for male animals are presented in Figure 2 and the results for females are presented in Figure 3. As indicated, FST measures of active and

passive behaviors were very similar in THP-, DHP-, and vehicle-treated animals in both the male and the female groups (Figure 2A and 3A). Similarly, the OFT measures of grooming time, horizontal time, rearing time, immobile time and their ratios were similar in the different treatment groups in both male or female animals (horizontal and vertical activity are represented by Figure 2B-C, 3B-C). The different treatment groups also did not spend differing amounts of time in the perimeter of the maze (risk assessment, data not shown). Analyses of variance showed no significant differences among any of the treatment groups in either male or female subjects ($p>0.05$).

4. Discussion

The kindled-amygdala focus is a model of drug-refractory complex partial seizures that secondarily generalize (Albright & Burnham, 1980). Previous studies in our laboratory have shown that progesterone, and especially its metabolites DHP and THP, have anticonvulsant activity in this model (Lonsdale & Burnham, 2003; Lonsdale *et al*, 2006; Lonsdale & Burnham, 2007).

A significant number of people with seizures resist the currently available anticonvulsant drugs. New drugs are needed for these people, but they should be drugs without the side effects that limit use and compliance (Bootsma *et al*, 2009). Analogs of DHP or THP might be developed for use in humans if it can be shown that these compounds are free of significant side effects.

Inhibition of progesterone's metabolism has been reported to have both depressant and antidepressant effects, suggesting that DHP and/or THP may have effects on mood as well as seizures (Schüle *et al*, 2011; Reddy, 2010; Walf *et al*, 2006).

Therefore we tested DHP and THP in the forced swim test (FST) and open field test (OFT) to look for potential psychotoxic side effects related to mood.

The FST is a model that is widely used to assess pharmacologically antidepressant-like effects (Porsolt *et al*, 1977, 1978; Cryan *et al*, 2002, 2005). It has impressive predictive validity for clinically effective antidepressants with differing mechanisms (Cryan *et al*, 2005, 2002; Borsini & Meli, 1988), and it is generally agreed that the FST can be a marker of behavioral states in rodents that resemble human depression.

The present study is the first study in which the direct effects of DHP in the FST have been measured in rats. In the present study, anticonvulsant doses of DHP did not cause any significant changes in the FST, suggesting that anticonvulsant doses of this compound are not associated with changes in mood, at least in rats. THP has been previously tested in the FST and might have been expected to have antidepressant-like effects. There is a complex relationship between stress, neurosteroids, and depression (Pack *et al*, 2011). Major depressive disorders are hypothesized to be a consequence of endogenous GABAergic deficits (Luscher *et al*, 2011), and THP is known to enhance GABAergic inhibition (Belelli *et al*, 2009; Akk *et al*, 2007). Also, THP levels are lower in depressed patients, and normalize with successful antidepressant treatment (Zorumski *et al*, 2012). This change in THP has been attributed to an alteration in 3α -HSD enzyme activity caused by the selective serotonin re-uptake inhibitors (SSRIs) (Schüle *et al*, 2011), increasing endogenous THP. Consequently, GABAergic conductance should increase, hence reducing immobility in the FST (Luscher *et al*, 2011). In the present study, however, we did not find significant effects of THP in the FST, at least at the anticonvulsant dose that was used.

Contrary to our findings, other investigators have found THP to have antidepressant-like effects in rodents in the FST (Khisti & Chopde, 2000; Rodríguez-Landa *et al*, 2007). Rodríguez-Landa *et al* reported that THP reduced immobility in the FST at a dose of 1 mg/kg, which is lower than the dose used in the present experiment. Khisti & Chopde found that SSRIs potentiated THP's antidepressant-like effects when THP was administered *via* intracerebroventricular (i.c.v.) injection. Different routes of injection limit comparison between the present study (i.p.) and Khisti & Chopde's (i.c.v.).

It is not immediately clear why different results have been obtained in the different studies, but one possible difference may relate to differences in handling. Our handling protocol was intended match the amount of handling rats received in our previous kindling studies (5 minutes per weekday for 1 month). This was more handling than rats received in the studies of Khisti & Chopde and Rodríguez-Landa *et al*. Handling can be a source of environmental enrichment for rats, affecting behavioural outcomes and responses to drugs (Andrews & File, 1993; Boix *et al*, 1990; Biggio *et al*, 2007). Environmental enrichment has many effects on the brain. It reduces spontaneous seizures (Manno *et al*, 2011; Young *et al*, 1999), changes the hippocampal proteome (McNair *et al*, 2007), is neuroprotective (Young *et al*, 1999), and has other behavioral and neurobiological effects (von Praag *et al*, 2000).

Others have found that social isolation stress (being singly housed) increases the immobility time on the FST (Yates *et al*, 1991; Evans *et al*, 2012). Despite the animals in the current study being singly housed, our handling protocol may have reduced social isolation stress, and changed many outcomes compared to animals that have not been handled so extensively. The handling and downstream effects may have obscured

antidepressant or depressant effects of DHP and THP in the FST, by preventing stress associated with social isolation.

The OFT is often done in conjunction with animals naïve to the FST to show that changes in activity in the FST are not due to drug-induced changes in the animals' general levels of activity (Porsolt *et al*, 1978). Changes in motor behavior in the OFT were not observed following administration of 5 mg/kg THP or DHP to either male or female rats. These results are consistent with the absence of sedation or ataxia following administration of 5 mg/kg doses of THP or DHP in our previous experiments (Lonsdale and Burnham, 2003; Lonsdale *et al*, 2006; Lonsdale and Burnham, 2007). Once again, this suggests that DHP and THP can have anticonvulsant effects at doses that are not psychotoxic.

5. Conclusion

In summary, in the FST and OFT, we did not find altered immobility or locomotion with DHP or THP at a dose of 5 mg/kg. The results of the present study suggest that DHP and THP do not have antidepressant/depressant or locomotor effects at anticonvulsant doses. DHP, THP, or their analogs, might be effective treatments for drug-refractory seizures without psychotoxic side effects on mood or locomotion. Experiments on the potential side effects of DHP and THP on learning and memory are currently being conducted in our laboratory.

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8. Figure legends

Figure 1. Metabolism of progesterone to metabolites 5 α -dihydroprogesterone (DHP) and 3 α ,5 α -tetrahydroprogesterone (THP, also called allopregnanolone). Progesterone is metabolized to DHP via *5 α -reductase (5 α -R)* and reversible metabolism of DHP to THP via *3 α ,5 α -hydroxysteroid oxidoreductase 3 α HSOR*.

Figure 2. Male Rats. **A**, Effects of DHP and THP on FST immobility time in male rats. **B**, Effects of DHP and THP on vertical activity (number of rearing motions) in the OFT in male rats. **C**, Effects of DHP and THP on horizontal activity (number of 10x10 cm squares crossed) in the OFT in male rats. Abbreviations: 5 α -dihydroprogesterone (DHP); 3 α ,5 α -tetrahydroprogesterone (THP, also called allopregnanolone); Forced Swim Test (FST); Open Field Test (OFT).

Figure 3. Female Rats. **A**, Effects of DHP and THP on FST immobility time in female rats. **B**, Effects of DHP and THP on vertical activity (number of rearing motions) in the OFT in female rats. **C**, Effects of DHP and THP on horizontal activity (number of 10x10 cm squares crossed) in the OFT in female rats. Abbreviations: 5 α -dihydroprogesterone (DHP); 3 α ,5 α -tetrahydroprogesterone (THP, also called allopregnanolone); Forced Swim Test (FST); Open Field Test (OFT).

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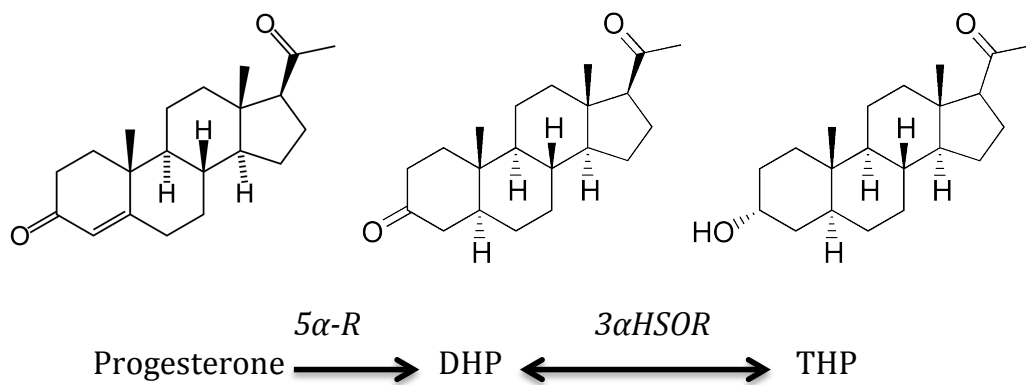
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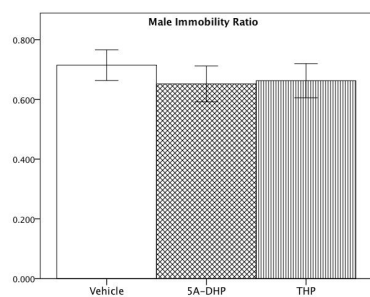
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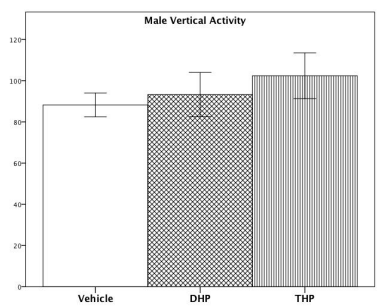
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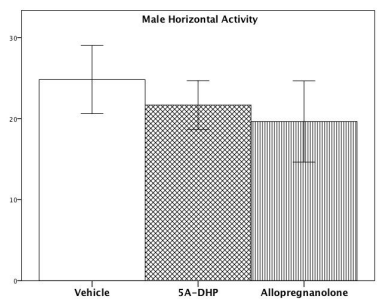
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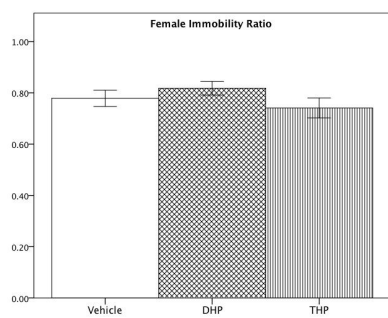
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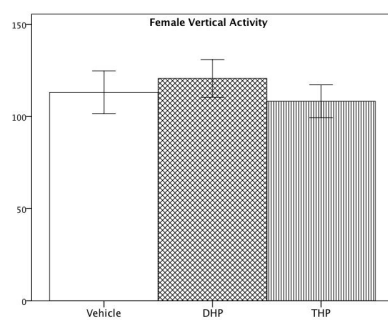
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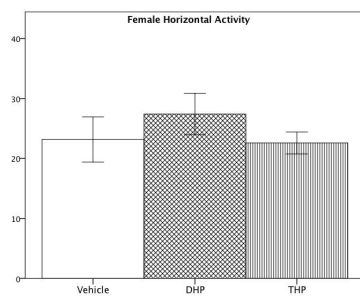
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Appendix 2: Elevated Plus Maze Paper, Submitted

Antiseizure Doses of Progesterone Metabolites Are Not Anxiogenic in the Elevated Plus Maze

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Abstract

The progesterone metabolites 5alpha-dihydroprogesterone and allopregnanolone have antiseizure effects against amygdala-kindled seizures in male and female rats. The amygdala kindling model is an animal model of human complex partial seizures secondarily generalized. Compounds that have antiseizure effects in this model, or their analogs, may be suitable for development as antiseizure medications. If a compound is to be developed as a drug, it is important to determine whether the compound has adverse behavioral effects. The purpose of this study was to determine whether antiseizure doses of 5alpha-dihydroprogesterone or allopregnanolone are anxiogenic when administered to adult male and female rats prior to testing in the elevated plus maze. Rats were randomly assigned to receive an injection of vehicle or one of the doses of 5alpha-dihydroprogesterone or allopregnanolone. Fifteen minutes following the injection, each rat was placed in the center of the maze and allowed to explore the maze for five minutes. Maze behavior was recorded by a videocamera and computer assisted tracking. The percent of time spent on open arms and closed arms, and the number of open and closed arm entries were recorded. Data analysis indicated that administration of antiseizure doses of 5alpha-dihydroprogesterone and allopregnanolone did not produce anxiogenic effects in male or female rats in the elevated plus maze. This favorable behavioral outcome provides further support for investigation of these compounds, or their analogs, for clinical use as antiseizure drugs.

Keywords: 5alpha-dihydroprogesterone, allopregnanolone, rats, elevated plus maze, anxiogenic, anxiolytic

1. Introduction

The epilepsies are a group of heterogeneous neurological disorders that are characterized by recurrent seizures [1,2]. The most common treatment for epilepsy is antiseizure drug therapy. Approximately 30% of patients with epilepsy, however, have seizures that are resistant to treatment with the currently available anticonvulsant drugs [3,4].

Many patients with drug-resistant seizures experience complex partial seizures, which may secondarily generalize to tonic-clonic seizures. Complex partial seizures are often difficult to control with medications that are currently available [5]. More effective medications for the treatment of complex partial seizures are needed.

Progesterone is a steroid with well-known reproductive effects. Progesterone is produced by the ovaries, testes, and adrenal glands. It is also synthesized *de novo* in glia and neurons [6,7]. Steroids that are synthesized in the nervous system, including progesterone and its metabolites, are classified as neurosteroids [8].

Anticonvulsant effects of progesterone have been demonstrated in several rodent seizure models *in vivo* [9,10] and *in vitro* [11]. In clinical studies, progesterone has also been shown to reduce the frequency of seizures in female patients, including the frequency of complex partial seizures [12,13].

It seems probable, however, that the antiseizure effects of progesterone are not mediated by progesterone itself, but by its metabolites. Several studies have indicated that the metabolites have more potent antiseizure effects than progesterone, and that progesterone loses most of its antiseizure effects in the presence of metabolic blockers [10,14].

The major metabolic pathway of progesterone in the rodent brain is the two-step reduction pathway [15], which is illustrated in Figure 1. Progesterone is first metabolized to 5 α -dihydroprogesterone by the enzyme 5 α -reductase. 5 α -dihydroprogesterone is then reduced to allopregnanolone by 3 α -hydroxysteroid dehydrogenase. When the activity of 5 α -reductase is blocked by finasteride, or when rodents are deficient in the enzyme, the antiseizure properties of progesterone are significantly reduced [14,16]. This suggests that 5 α -dihydroprogesterone and/or allopregnanolone are, at least partially, responsible for the antiseizure effects of progesterone.

In previous experiments, we have tested the antiseizure effects of both 5 α -dihydroprogesterone and allopregnanolone in male and female rats in the amygdala kindling model, a pharmacologically validated model of human complex partial seizures with secondary generalization [17]. The generalized component of the kindled seizure models human tonic-clonic seizures, while the amygdala focal component of the seizure models complex partial seizures. In

this model, 5alpha-dihydroprogesterone suppressed the amygdala focal seizures—as well as the secondarily generalized seizures—at low, non-toxic doses. These data suggest that 5alpha-dihydroprogesterone, or an analog, might be effective against complex partial seizures in humans [18].

Allopregnanolone was also effective against the generalized component of the kindled seizures, but it did not suppress the amygdala focal seizure, even at high, toxic doses [19,20]. An analog of allopregnanolone is already being developed as an anticonvulsant and is currently in clinical trials [21].

If a compound is to be developed for clinical use, toxicity is a major concern. Toxicity in our kindling experiments was assessed using Loscher's observational scales for sedation and ataxia [22]. 5alpha-dihydroprogesterone did not produce sedation or ataxia at the doses used. Allopregnanolone did produce sedation and/or ataxia, but only in some subjects and at higher doses.

These negative toxicological results were promising, but the tests used to assess toxicity in our previous experiments were not powerful enough to detect subtle effects on anxiety, mood, memory, or cognition. Prior to developing analogs of progesterone metabolites as potential anticonvulsant drugs, it will be important to determine whether these metabolites have psychotoxic side effects that could limit their therapeutic use.

The major objective of the present experiment was to determine whether antiseizure doses of 5alpha-dihydroprogesterone (5 mg/kg, 7.5 mg/kg, 10 mg/kg) and allopregnanolone (5 mg/kg, 7.5 mg/kg, 10 mg/kg) are anxiogenic when administered to adult male and female rats. Anxiogenic properties might limit the potential value of these compounds, or their analogs, for development as drugs.

A secondary objective was to determine whether 5alpha-dihydroprogesterone and/or allopregnanolone, administered at antiseizure doses, have anxiolytic effects compared to the vehicle control. If either steroid produces anxiolytic effects relative to the vehicle control, extended dose response experiments using both a positive and negative control will be performed. Thus behavioral studies might reveal potential beneficial effects as well as toxic effects.

In this study, anxiogenic/anxiolytic effects were tested using the elevated plus maze. The elevated plus maze is a standard behavioral test used to evaluate the anxiogenic/anxiolytic effects of compounds in rodents [23]. It consists of two open arms and two closed arms that are elevated to a height of about 80 cm above the ground. Rats normally fear high, open spaces and display a preference for the enclosed arms. Rats, however, are exploratory by nature and will explore the open arms if they become less anxious. A drug is considered to be anxiolytic when the drug-treated animals spend more time in the open arms and make more open arm entries than animals that receive only the vehicle control [24,23]. A drug is considered to be anxiogenic when the drug-treated animals spend more time in the closed arms and make more closed arm entries than animals that receive only the vehicle control [25].

The doses of 5 α -dihydroprogesterone and allopregnanolone selected for the present experiment were antiseizure doses that caused no sedation and/or ataxia, or minimal sedation and/or ataxia, in our previous studies. A 15 minute injection-test interval was selected to match the injection-test interval that had been used in previous studies.

Species, strain, and handling of animals have been demonstrated to affect behavior in the elevated plus maze [26,27]. Therefore, the same species (rat) and strain (Wistar) of rats that had been used previously were also used in the present study. Animals were handling-habituated to mimic conditions in earlier experiments.

2. Methods

2.1. Subjects

Adult male and female rats (Charles River, Quebec, Canada) were housed individually in 24 x 24 x 45-cm. transparent, plastic cages. Subjects were allowed free access to food and water. The vivarium was kept at a constant temperature of 21°C and maintained on a 12-h light/dark cycle (lights on at 7:00 a.m.). Rats were 60 days old on the date of delivery to the vivarium and approximately 90 days old on the day of behavioral testing. The Animal Care Committee (Faculty of Medicine) of the University of Toronto approved all protocols.

2.2. Drugs

Allopregnanolone, 5 α -dihydroprogesterone, and the vehicle beta-cyclodextrin were obtained from Sigma-Aldrich Canada Ltd (Oakville, Ontario). Drugs were dissolved in a 45% solution of beta-cyclodextrin in physiologic (0.9%) saline. Fresh solutions of vehicle and drugs were made on each day of drug testing. All injections were administered intraperitoneally (i.p.).

Allopregnanolone and 5 α -dihydroprogesterone were injected i.p. at doses of 5 mg/kg, 7.5 mg/kg, or 10 mg/kg for each drug. All vehicle control injections were volume-matched to drug injections. Each rat received only one injection of drug or vehicle.

2.3. Procedure for handling

In order to replicate handling in previous dose-response studies, all rats were handled five days a week for a month prior to testing in the elevated plus maze.

2.4. Elevated Plus Maze

The elevated plus maze apparatus is shaped like a plus sign, with four arms of equal length and width (50 cm. long x 10 cm. wide). Two of the opposite facing arms are open and consist of black Plexiglas runways which have slightly raised (0.5 cm.) edges. The other two opposite facing arms consist of black Plexiglas runways that are enclosed by 40 cm. high black opaque Plexiglas walls and uncovered at the top. The apparatus is 80 cm. in height from the floor to the horizontal plane of the runway surfaces.

All rats were transported in their home cages to the behavioral testing room 30 minutes prior to testing. Subjects were tested in a quiet room under dim incandescent light. All behavioral testing took place between 9:00 a.m. and 6:00 p.m.

Rats were randomly assigned to receive an injection of either beta-cyclodextrin vehicle, one of the doses of 5alpha-dihydroprogesterone, or one of the doses of allopregnanolone (n = 11-12 rats per drug/vehicle group). Fifteen minutes after injection of vehicle or a drug treatment, each rat was placed in the center of the maze, facing an open arm. Subjects were allowed to explore the maze for 5 minutes and behavior was recorded by a video camera mounted on the ceiling above the elevated plus maze. The signal was relayed to a computer and monitor. Computer assisted tracking was used to trace the path of each rat over the five minute period. Percent of time spent on open arms, percent of time spent on closed arms, the number of open arm and closed arm entries, and total distance travelled were recorded. An arm entry was defined as the entry of all four paws of the rat into an arm. The elevated plus maze was cleaned thoroughly with disinfectant spray (Virox[®]) before each rat was tested.

This study was performed using male rats and then repeated as above using female rats as subjects.

2.5. Statistics

Behavioral data were analysed by single factor (treatment) ANOVA. When appropriate, multiple comparison tests were performed using Tukey's post-hoc tests.

3. Results

3.1. The effects of 5alpha-dihydroprogesterone and allopregnanolone on performance in the elevated plus maze (male rats)

Analysis of variance showed no significant differences between any of the treatment groups and the control group in the percent of time spent on the open arms, the percent of time spent on

the closed arms, the number of open arm entries, the number of closed arm entries, or the total number of arm entries ($p>0.05$). Data are presented in Figure 2.

There were no significant differences in spontaneous motor activity across treatment conditions as determined by the number of closed arm entries and the total number of arm entries.

3.2. The effects of 5alpha-dihydroprogesterone and allopregnanolone on performance in the elevated plus maze (female rats)

Analysis of variance revealed no significant differences between any of the treatment groups and the control group in the percent of time spent on the open arms, the percent of time spent on the closed arms, the number of open arm entries, the number of closed arm entries, or the total number of arm entries ($p>0.05$). Data are presented in Figure 3.

There were no significant differences in locomotor activity across treatment groups as determined by the number of closed arm entries and the total number of arm entries.

4. Discussion

Although 5alpha-dihydroprogesterone and allopregnanolone have antiseizure effects in the kindling model [18,19,20], the administration of progesterone metabolites may be associated with undesirable behavioral effects [28]. Prior to developing analogs of progesterone metabolites for clinical use, it is important to determine whether antiseizure doses of these metabolites produce significant behavioral impairment in animals tested in standard animal behavioural models. The purpose of the present study was to determine whether antiseizure doses of 5alpha-dihydroprogesterone and allopregnanolone produce anxiogenic/anxiolytic effects in rats tested in the elevated plus maze.

At antiseizure doses, 5alpha-dihydroprogesterone was not anxiogenic when administered 15 minutes prior to testing in the elevated plus maze. To our knowledge, there are no reports of anxiogenic effects of 5alpha-dihydroprogesterone in the literature.

5alpha-dihydroprogesterone also did not have anxiolytic effects when compared to vehicle control. This result is consistent with the absence of 5alpha-dihydroprogesterone anxiolytic activity in the mouse elevated plus maze test as reported by Rodgers and Johnson [29].

In contrast, administration of 5alpha-dihydroprogesterone (4 mg/kg) was reported to be anxiolytic in the rat defensive burying test [30], which seems to conflict with our data. The anxiolytic effects observed in this 1995 study may have been the result of the much longer injection-test interval used in the defensive burying behavior test (4 hours in comparison to 10-15 minutes in the elevated plus maze tests). It is possible that 5alpha-dihydroprogesterone may have been converted to allopregnanolone or other anxiolytic metabolites during this prolonged injection-test interval.

At antiseizure doses, allopregnanolone was not anxiogenic when administered 15 minutes prior to testing in the elevated plus maze. Although acute administration of allopregnanolone has not been reported to have anxiogenic effects, chronic administration of allopregnanolone may be anxiogenic [28].

Allopregnanolone also did not produce anxiolytic effects at the doses tested in our protocol. This was an unexpected result. Acute i.p. or s.c. administration of allopregnanolone at 5 mg/kg doses, 10-15 minutes prior to elevated plus maze testing, has been reported to be anxiolytic in previous rodent studies [29,31].

The difference in results between the present study and previous studies may relate to the long period of pre-test handling used in the present study and designed to duplicate the handling experienced by our kindled subjects. The behavioral effects of drugs in the elevated plus maze test are affected by the handling history of the subjects. It has been demonstrated that benzodiazepines are ineffective as anxiolytics in handling-habituated rats [32]. Allopregnanolone may not have produced anxiolytic effects in our study due to the extensive handling procedure that we used to mimic conditions in our previous experiments.

In summary, in the present study, acute administration of antiseizure doses of 5alpha-dihydroprogesterone and allopregnanolone did not produce anxiogenic effects in rats in the elevated plus maze. This is a favorable behavioral outcome and supports further investigation of these compounds or their analogs for potential clinical use. Anxiety is a common comorbidity in patients with epilepsy [33] and it is important to develop new medications that do not, themselves, provoke anxiety.

Further studies are needed to determine if chronic administration of antiseizure doses of 5alpha-dihydroprogesterone or allopregnanolone produces anxiogenic effects in rats tested in the elevated plus maze. In order to also determine possible anxiolytic effects of chronic administration of these progesterone metabolites, the rats should not be handled extensively prior to testing in the elevated plus maze and a positive control (diazepam) should be added to the experimental protocol.

Conflict of Interest

The authors of this article have no conflicts of interest to report.

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Figure Legends

Figure 1. Progesterone metabolic pathway. The reduction of progesterone to 5alpha-dihydroprogesterone by 5α-reductase, followed by the reduction of 5alpha-dihydroprogesterone to allopregnanolone by 3α-hydroxysteroid dehydrogenase. Abbreviations: 3alpha-hydroxysteroid dehydrogenase (3α-HSD).

Figure 2. Effects of 5alpha-dihydroprogesterone and allopregnanolone on the performance of male rats in the elevated plus maze (n = 11-12 per group). Effects of 5alpha-dihydroprogesterone and allopregnanolone on the percent of time on the open arms (A), the percent of time on the closed arms (B), the number of open arm entries (C), the number of closed arm entries (D), and the total number of arm entries (E).

There were no significant differences between any of the treatment groups for either 5alpha-dihydroprogesterone or allopregnanolone when compared to the control group ($p>0.05$).

Abbreviations: 5alpha-dihydroprogesterone (DHP), allopregnanolone (THP).

Figure 3. Effects of 5alpha-dihydroprogesterone and allopregnanolone on the performance of female rats in the elevated plus maze (n = 11-12 per group). Effects of 5alpha-dihydroprogesterone and allopregnanolone on the percent of time on the open arms (A), the percent of time on the closed arms (B), the number of open arm entries (C), the number of closed arm entries (D), and the total number of arm entries (E).

There were no significant differences between any of the treatment groups for either 5alpha-dihydroprogesterone or allopregnanolone when compared to the control group ($p>0.05$).

Abbreviations: 5alpha-dihydroprogesterone (DHP), allopregnanolone (THP).

Figure 1

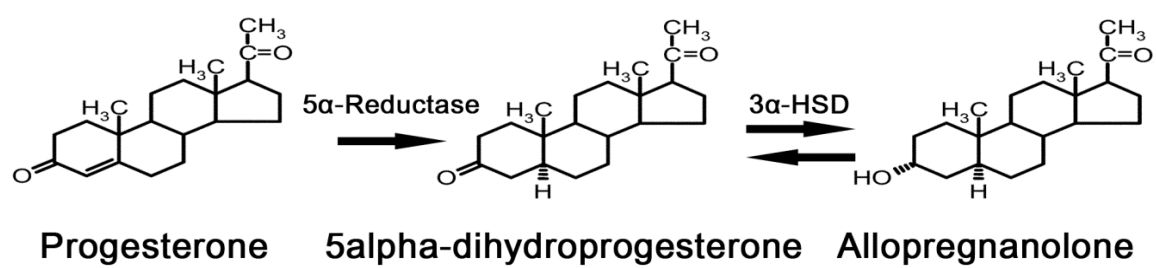
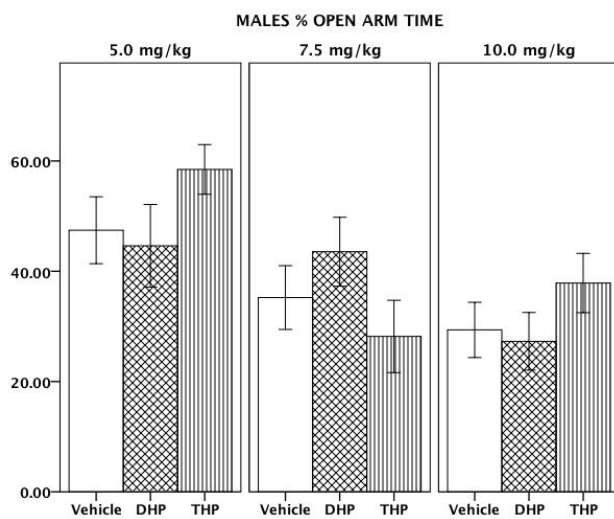
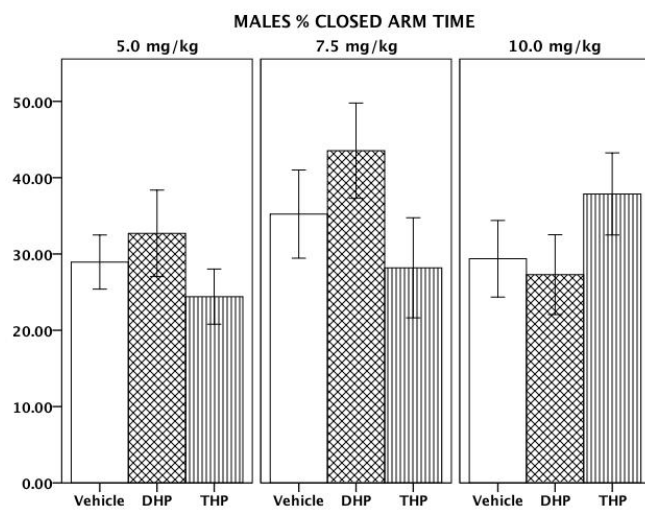


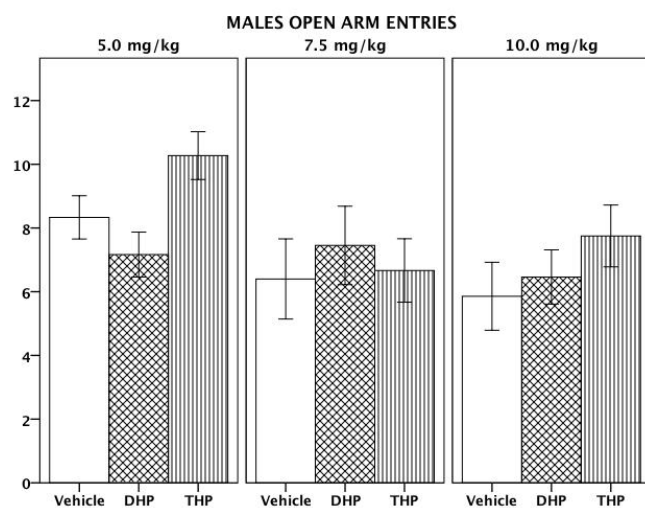
Figure 2A



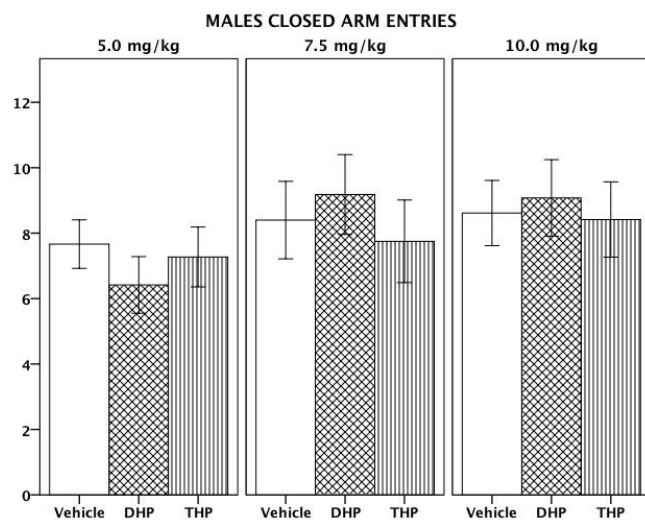
2B



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2D



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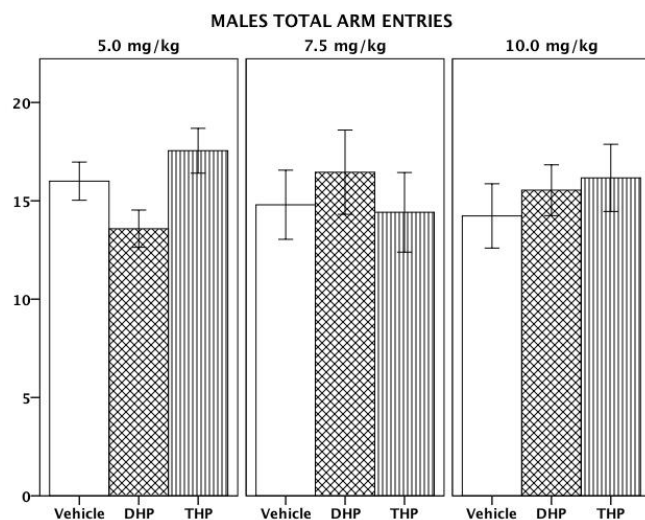
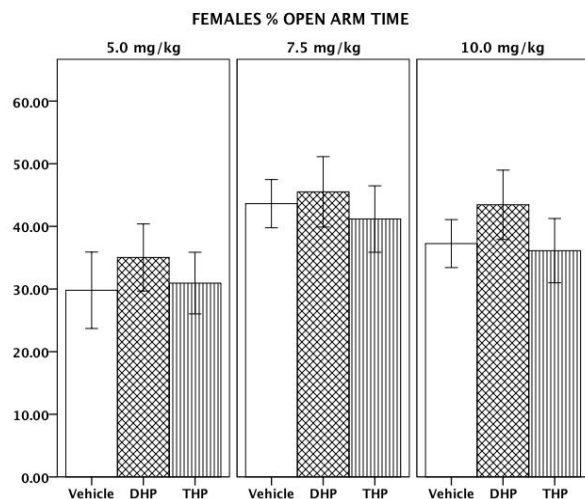
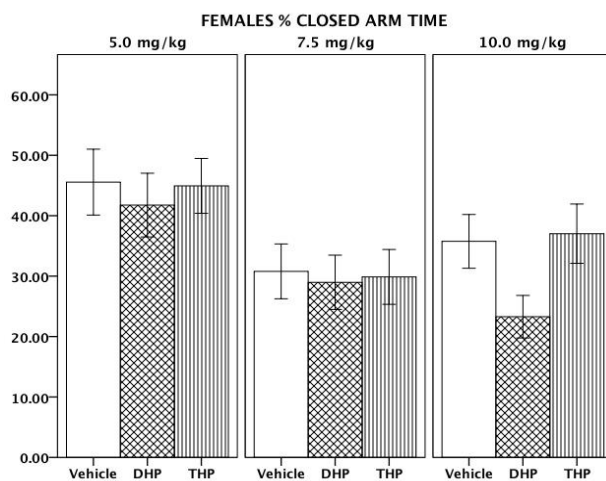


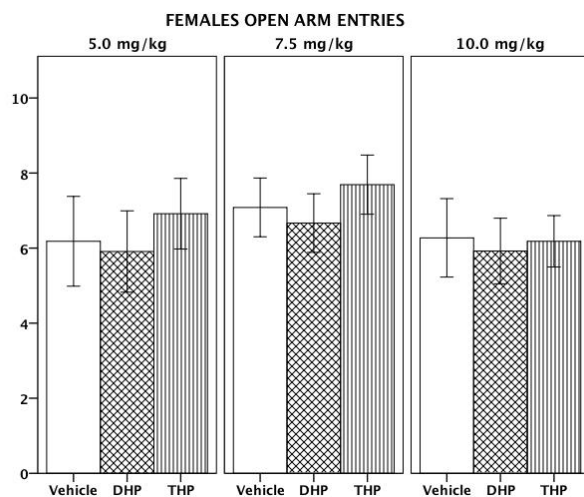
Figure 3A



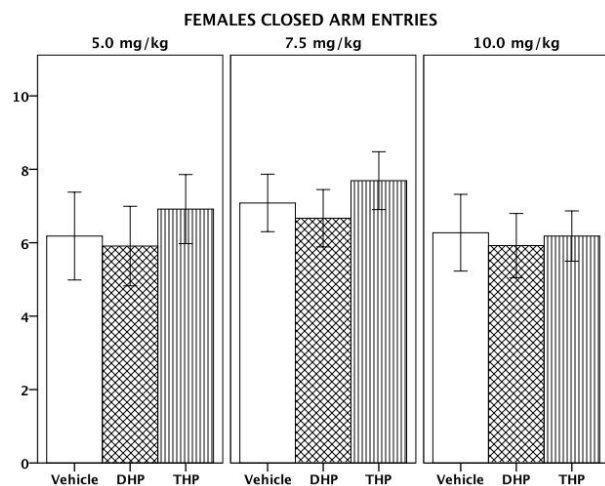
3B



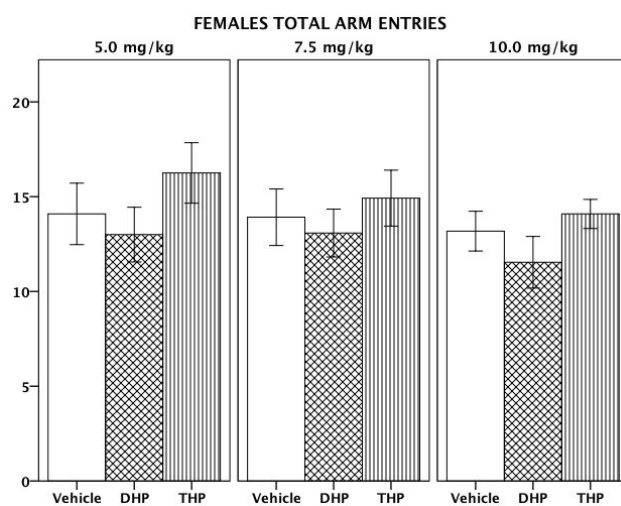
3C



3D



3E



Appendix 3: Novel Surgical Methods, Accepted

<http://www.biomedcentral.com/1471-2202/14/82>

METHODOLOGY ARTICLE

Open Access

A reliable method for intracranial electrode implantation and chronic electrical stimulation in the mouse brain

Melanie Jeffrey^{1,2,4†}, Min Lang^{1†}, Jonathan Gane¹, Chiping Wu^{1,4}, W McIntyre Burnham^{2,4} and Liang Zhang^{1,3,4*}

Abstract

Background: Electrical stimulation of brain structures has been widely used in rodent models for kindling or modeling deep brain stimulation used clinically. This requires surgical implantation of intracranial electrodes and subsequent chronic stimulation in individual animals for several weeks. Anchoring screws and dental acrylic have long been used to secure implanted intracranial electrodes in rats. However, such an approach is limited when carried out in mouse models as the thin mouse skull may not be strong enough to accommodate the anchoring screws. We describe here a screw-free, glue-based method for implanting bipolar stimulating electrodes in the mouse brain and validate this method in a mouse model of hippocampal electrical kindling.

Methods: Male C57 black mice (initial ages of 6–8 months) were used in the present experiments. Bipolar electrodes were implanted bilaterally in the hippocampal CA3 area for electrical stimulation and electroencephalographic recordings. The electrodes were secured onto the skull via glue and dental acrylic but without anchoring screws. A daily stimulation protocol was used to induce electrographic discharges and motor seizures. The locations of implanted electrodes were verified by hippocampal electrographic activities and later histological assessments.

Results: Using the glue-based implantation method, we implanted bilateral bipolar electrodes in 25 mice. Electrographic discharges and motor seizures were successfully induced via hippocampal electrical kindling. Importantly, no animal encountered infection in the implanted area or a loss of implanted electrodes after 4–6 months of repetitive stimulation/recording.

Conclusion: We suggest that the glue-based, screw-free method is reliable for chronic brain stimulation and high-quality electroencephalographic recordings in mice. The technical aspects described this study may help future studies in mouse models.

Keywords: Glue, Kindling, Mice

Background

Electrical kindling of limbic structures in rodents is a widely used model for complex partial seizures with secondary generalization [1–4]. This model requires implantation of intracranial electrodes and subsequent brain stimulation for several weeks. The majority of

previous studies have employed this model in rats, using anchoring screws and dental acrylic to secure the implanted electrodes [5–9]. Recent studies have increasingly applied the kindling model in mice largely due to the availability of transgenic mouse strains [10–13].

In general, it is a technical challenge to firmly mount intracranial electrodes in mice with the anchoring screw/dental acrylic approach, since the mouse's thin skull may not be strong enough to accommodate the anchoring screws. As an alternative approach, we have previously developed a screw-free, glue-based method for implanting intracranial electrodes in mice [14]. Using

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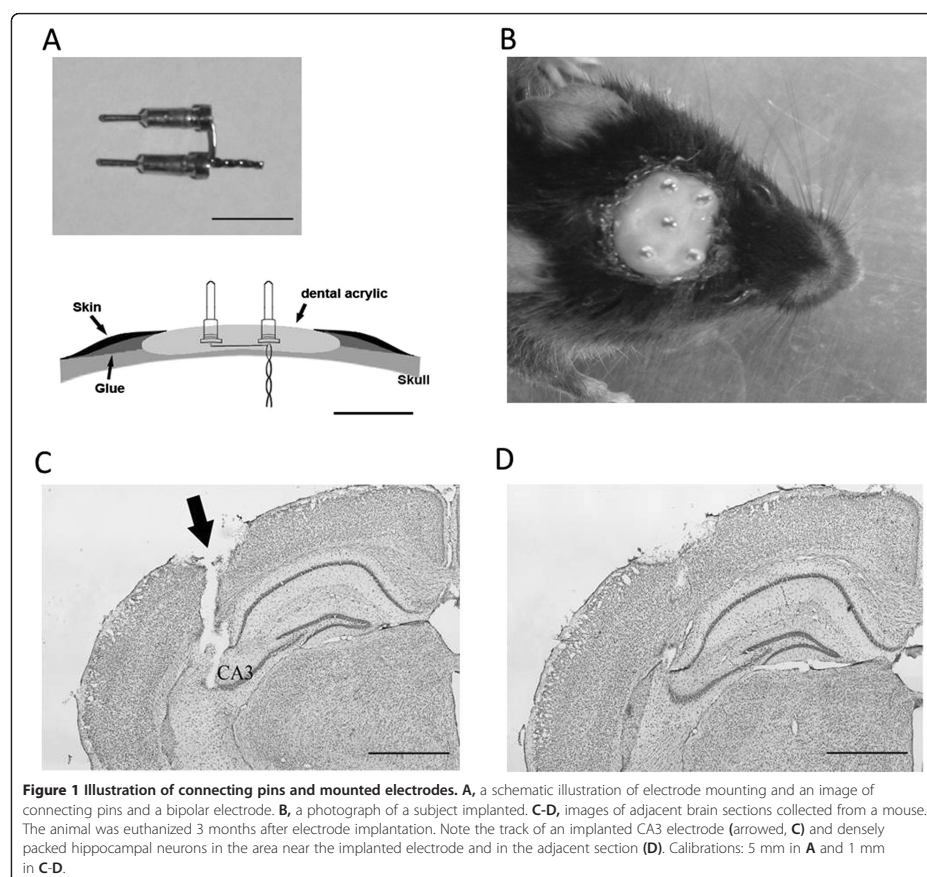
this method, we have carried out chronic intracranial electroencephalographic (EEG) recordings in several mouse models [15-20]. In these studies, a pre-assembled electrode array was used. While the glue-based method can be used for implanting bipolar stimulating electrodes in the mouse brain [21], its validity for the chronic brain stimulation in mice that is required for kindling has not been vigorously assessed. The aim of our present study is to address this issue. We thus further modified the glue-based implantation method and conducted hippocampal electrical kindling in adult mice. EEG discharges and behavioral seizures were consistently observed in kindled mice. Importantly, no animal encountered infection in the implanted area or a loss of implanted electrodes after 4-6 months of repetitive stimulation/

recording. We thus suggest that the glue-based method is reliable for chronic brain stimulation and high-quality EEG recording in mouse models.

Results

Long-term stability of implanted electrodes and animal behavioral outcomes

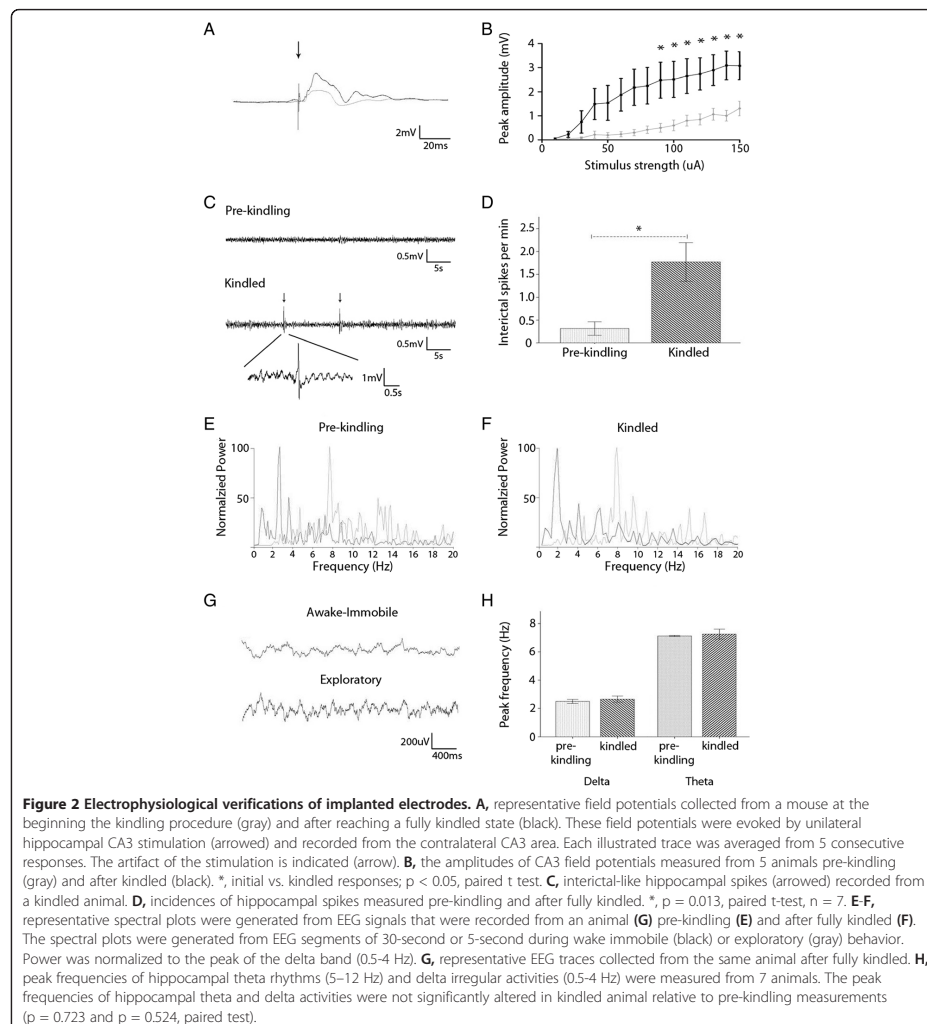
25 adult male mice (initial ages of 6-8 month-old) underwent implantation of bipolar electrodes in bilateral hippocampal CA3 (Cornu Ammonis area 3). These electrodes were secured onto the skull with a modified version of the screw-free and glue-based method [14,21]. Electrode construction, assembly and mounting are schematically illustrated in Figure 1A. The surgical procedure for electrode implantation is detailed in the



Methods. Of the 25 animals implanted, we observed no evident behavioral abnormalities or spontaneous seizures in the absence of kindling stimulations. Importantly, no animal encountered local infection in the implanted area or a loss of implanted electrodes after 4–6 months of repetitive stimulation and recordings.

The locations of implanted electrodes were initially verified by *in vivo* electrographic activities and later,

histological assessments. In response to a single stimulation of the unilateral CA3 area, synaptic field potentials were reliably recorded from the contralateral CA3 area (Figure 2A). The amplitudes of these field potentials increased with strong stimuli and reached a near plateau level at the stimulation intensity of $\geq 100 \mu\text{A}$ (Figure 2B). No animals showed epileptic responses (such as long-lasting multi-spike waveforms) following a single stimulation. In



addition, the animals exhibited hippocampal “physiological” activities similar to those we and others have previously observed in mice [15-20,22]. Specifically, CA3 recordings revealed rhythmic activities in the theta band (5–12 Hz) while the animals moved/explored local environments and irregular activities in the delta band (0.5-4 Hz) during immobility or sleep (Figure 2E-H). The peak frequencies of hippocampal theta and delta activities were not significantly changed in kindled animals ($n = 7$ mice; Figure 2H). As bilateral hippocampi are strongly interconnected via ventral and dorsal hippocampal fissures and the CA3 area is critical for the generation of hippocampal EEG rhythms and epileptiform activities [23], the above data provide electrographic evidence for accurately implanted CA3 electrodes.

The locations of implanted CA3 electrodes were histologically confirmed in 6 animals. The tracks of implanted electrodes were recognizable in brain sections that corresponded to the desired stereotaxic coordinates (Figure 1C). There was no evident loss of hippocampal neurons in the areas near the implanted electrodes or in adjacent brain sections (Figure 1D). Taken together these observations with the electrographic data above, we suggest that the screw-free and glue-based method is suitable for reliable implantations of bipolar stimulating/recording electrodes in adult mice.

Seizure activities induced by hippocampal kindling

We used a standard kindling protocol in the present experiments [24,25]. The animals were considered fully kindled if they exhibited stage 5 behavioral seizures on 3 consecutive days. Of 25 animals tested, 21 animals were fully kindled between 6 and 26 stimulations (mean $\pm$ SD 12.14 ± 6.41). Two of the remaining 4 animals failed to reach a fully kindled state due to contamination of implanted electrodes (see Discussion) and the other two died spontaneously with underlying causes presently unknown.

In addition to consistent expression of the stage 5 behavioral seizures, the kindled animals showed significant changes in hippocampal electrographic activities. First, the evoked field potentials were greatly enhanced in the kindled animals as compared to their own baselines (Figure 2A). Such enhancements were recognizable at all stimulation intensities examined (Figure 2B), suggesting an overall increase of hippocampal synaptic strength induced by the kindling process. Secondly, the afterdischarges (ADs) were greatly increased in amplitude and duration in the kindled animals (Figure 3A, C). Finally, interictal-like hippocampal spikes, similar to those previously described [26-29], were frequently observed in the kindled animals but were either absent or barely detectable in the same animals during baseline monitoring (Figure 2C-D).

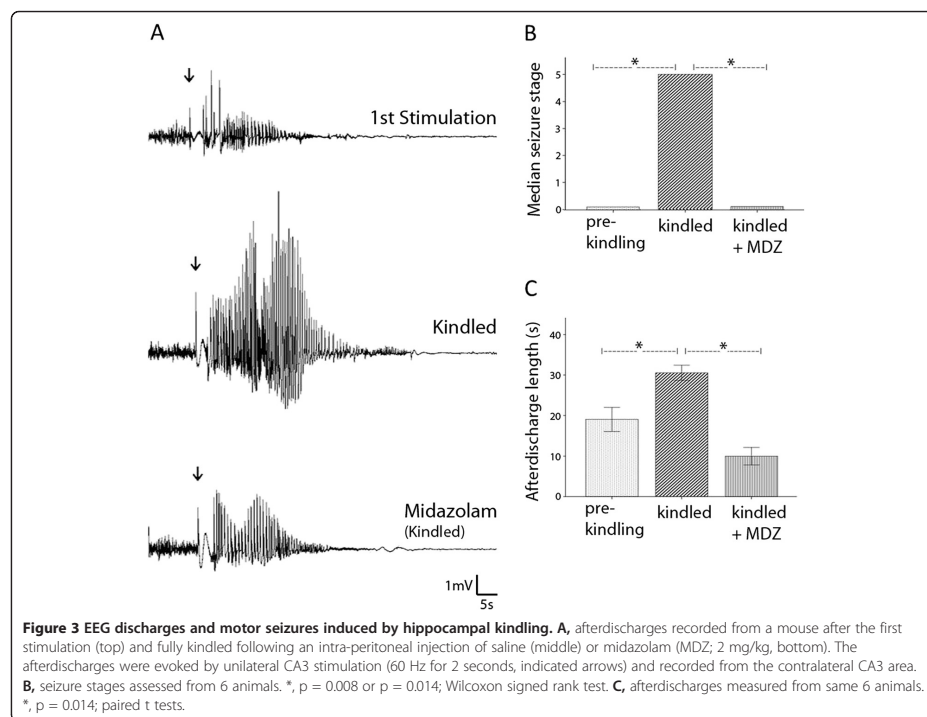
The behavioral seizures and ADs observed from the kindled animals were sensitive to inhibition by midazolam, a fast acting benzodiazepine analogue recommended for the treatment of status epilepticus in clinical practice [30,31]. Midazolam was administered via intra-peritoneal injections at a dose of 2 mg/kg, 15 minutes prior to the hippocampal stimulation. In the 6 kindled animals tested, ADs and behavioral seizures were significantly attenuated following midazolam injections compared to those observed following the saline injections (Figure 3A-C). Together, the above observations suggest that a robust epileptic process is established in the kindled mice.

Discussion

Using a modified version of the screw-free and glue-based method, we implanted bipolar electrodes in bilateral hippocampal CA3 areas. In our experiments, no animal had adverse health-related events, spontaneous seizures, or abnormal behavior in the absence of electrical stimulation. There was no local infection in the implanted area and no loss of implanted electrodes after repetitive stimulations and recordings for 4–6 months. The kindled animals exhibited consistent stage 5 behavioral seizures, robust ADs (Figure 3A-D), and frequent interictal-like spikes (Figure 2C-D). The AD and behavioral seizures were sensitive to suppression by midazolam (Figure 3A-C). We thus suggest that the modified glue method is reliable for intracranial electrode implantation, chronic brain stimulation and high-quality EEG recordings in mice. Specific technical issues of the modified method are discussed below.

In our previous studies [14-20], we used a pre-constructed electrode array with a plastic base and applied glue between the plastic base and skull surface. Because the glue bonds the plastic base strongly to the skull, the electrode array can be firmly mounted onto the skull without using anchoring screws. This method is convenient for implanting the electrode array with 2–3 monopolar electrodes, and allows stable intracranial EEG recordings in mouse models [15-20]. This method, however, is limited for implanting bipolar electrodes that are aimed to stimulate and/or record from spatially separated brain structures, since these electrodes are difficult to assemble in a compact array. In addition, due to the curvature of the skull, these electrodes need to be individually inserted into the brain for accurate positioning.

We thus used a modified approach in the present experiments. We first glued a plastic base onto the skull surface and drilled small holes through the plastic base and underneath skull. We then individually positioned bipolar electrodes in the brain and overlaid dental acrylic onto the plastic base covering the implanted electrodes.



When hardened, the dental acrylic and the plastic base beneath the acrylic were strongly bonded; this secured the implanted electrodes (Figure 1A-B). This modified approach is successful because dental acrylic denatures the plastic base before hardening and then bonds to the plastic base after hardened. The implanted electrodes are secured onto the skull when the dental acrylic has hardened. Experience is needed, however, to promptly overlay the dental acrylic onto the plastic base, and to carefully cover the implanted electrode bases, before the acrylic hardens. We used a dental acrylic with hardening time of 6–9 minutes in the present experiments to have sufficient time to apply the dental acrylic.

In the present experiments, the pins used to connect electrodes were detached from standard IC sockets (Figure 1A). We chose these pins because the IC sockets are widely available from commercial sources at a low cost. In addition, these pins are relatively strong and offer good contacts when connected. Thus, although animals frequently push or press these pins against their cages or metal food racks, the implanted electrodes maintain integrity for chronic stimulation and/or

recordings. Care should be taken, however, when detaching these pins from wire sockets, to preserve a gasket-like piece in the female end of individual pins.

We implanted bipolar electrodes in bilateral hippocampal CA3 areas in the present experiments. This experimental design was used to test the possibility of stimulating two brain regions, and/or to increase the probability of electrical kindling in individual animals in the event that one bipolar electrode fails. The latter case was encountered in our initial experiments, likely due to electrode contamination that occurred during and/or after implantation. Such a problem did not occur in our later experiments, by visual inspection of electrode tips under a microscope and measurements of electrode conductivity before implantation. Therefore, a careful examination of the constructed electrodes prior to surgical implantation may greatly increase the success rate of chronic brain stimulation or recording.

Several previous studies have used cyanoacrylate-type glue alone or together with dental acrylic to secure EEG recording as well as stimulating electrodes in mouse models [32–34]. However, the underlying procedures

were not detailed. It is possible that the electrode implantation methods used in these previous studies and our present experiments may share some common features. By detailing the glue-based implantation method, our present study may support the previous studies and provide complementary technical aspects for further employment of this approach in mouse models.

Conclusion

We suggest that the glue-based and screw-free implantation method is reliable for chronic brain stimulation and high-quality EEG recordings in mice. The method may also be used, with some modifications, to implant other probes or infusion tubes in the mouse brain.

Methods

Animals

Male C57 black mice (initial ages of 6–8 months, Charles River Laboratory, Quebec, Canada) were used in the present experiments. These animals were housed in a vivarium that was maintained at 22°C with 12-hour light and dark cycles. Food and water were available *ad libitum*. Stimulations and recording experiments were done between 12–5 pm. All experimental procedures described below have been reviewed and approved by the Animal Care Committee of the University Health Network in accordance with the guidelines of the Canadian Council on Animal Care.

Electrode construction

All electrodes were made of polyamide-insulated stainless steel wires (outer diameter 200 μ m, Plastics One, Ranoake, VA). Twisted bipolar wires were used for stimulation and recording, with their intracranial tips ~100 μ m apart. The extracranial tips of the twisted wire assembly were soldered to the female ends of two connecting pins, with one wire bent into an L-shape to separate the connecting pins (Figure 1A). Care was taken to fully remove the insulation layer before soldering. A liquid solder (Soldering Liquid Flux, Certanium Alloys and Research Company, Cleveland OH, USA) or weak phosphoric acid was used to ensure good contact between the stainless steel wire and the connecting pin. A single wire was similarly soldered to a single pin for reference. The resistance of each constructed wire electrode was $\leq 1.0 \Omega$. After being soldered to the connecting pins, the bipolar wires were then cut to ~3 mm in length to target the desired hippocampal CA3 area (see below). The single monopolar wire was cut to ≤ 0.5 mm for epidural position of the reference electrode. These electrodes were cleaned with 75% alcohol and stored in a sterilized glass vial until use. The connecting pins were detached from standard IC sockets (Samtec series SS socket strips, SS-132-G2, Electrosonic,

Toronto, Ontario, Canada). These pins are 7.5 mm long in total; the male end of the pin is 3.2 mm long. The outer diameter is 1.8 mm or 0.5 mm for female or male end of the pin respectively (Figure 1A). Measured after being dissected out from implanted animals ($n = 3$), the total weight of implanted electrode assembly, including electrode wires, plastic base and dental acrylic (see below), was 0.50–0.52 grams. As adult mice were used in the present experiments, the weight of implanted electrode assembly was $\leq 2\%$ of animal body weight.

Surgery for electrode implantation

Surgical procedures were modified from those we previously described [14,21]. Briefly, the animal was anaesthetized with 2% isoflurane and then placed on a stereotaxic frame. After skin incision and exposure of the skull surface, the tip of a mini drill bit (see below) was aimed to bregma via a micromanipulator. After determining the bregma position, the drill bit was moved up but its X-Y position was unchanged, and a thin plastic base then was glued onto the skull surface. After the glue had cured, three small holes were then drilled through the plastic base and the skull according to the stereotaxic coordinates of the hippocampal CA3 area (bregma -2.5 mm, lateral ± 1.3 mm, and a depth of 3.2 mm [35]). The reference electrode was positioned at bregma 1 mm, lateral 2.0 mm and a depth of 0.7 mm. The electrode depths were adjusted to accommodate for the thickness (200 μ m) of the plastic base.

The plastic base was cut from a curved part of plastic weighting boats (polystyrene antistatic weighting dishes, Fisher Scientific, cat#08-732-115). The weighting boats were 140x140 mm in length-width and 25 mm in depth, with thickness of ~200 μ m. The plastic base was soft and could be gently pressed to accommodate curvature of the skull, and thus bound tightly with the skull surface after being glued. We used a cyanoacrylate-type glue (Insta-cure+, cure time 5–15 seconds, made in U.S.A., cat# BSI-106C; obtained from Canadian Hobbycraft, Concord, Ontario, Canada). A motorized drill (model FM3545, Foredom Electric, Bethel, CT, USA) and a mini drill bit (part 115603, Ball Mills Carbide, CircuitMedic, Haverhill, MA, USA) were used to drill small holes (≤ 0.5 mm) through the skull. These holes were large enough for inserting the electrodes, but small enough to prevent dental acrylic leakage into the brain (see below).

Micromanipulators were also used to individually insert the bipolar electrodes into bilateral hippocampal CA3 areas. After positioning and holding these electrodes with the micromanipulators, dental acrylic was overlaid onto the plastic base such that the bases of the connecting pins were covered by the dental acrylic (Figure 1B). Care should be taken to apply acrylic so as not to interfere with electrode positions and to

contaminate connecting pins. We used a dental acrylic with hardening time of 6–9 minutes (Jet Tooth Shade, Reference No. 1404; Lang Dental Mfg. Co., Inc., Wheeling, IL, USA) to carefully cement the implanted electrodes. After the dental acrylic had hardened, the electrodes were released from the micromanipulators. The incised skin was then glued to the dental acrylic (Figure 1A,1B), which prevents infection in the implanted area. The animals were released from the stereotaxic frame and allowed to recover for at least one week before further experimentation.

Electrical stimulation

Unilateral CA3 stimulation was conducted in all present experiments. Constant square-wave current pulses (duration of 0.5 ms, intensities of 10–150 μ A) were generated from a Grass stimulator and delivered through an isolation unit (model S88H, Grass Medical Instruments, Warwick RI, USA). To establish an input–output plot for evoked CA3 field potentials, a single stimulation was applied every 30 seconds at intensities of 10–150 μ A (10 μ A increments; 5 consecutive responses at a given intensity). A standard kindling protocol [24,25] was used. Animals were considered fully kindled after consecutive stage 5 seizures on 3 consecutive days.

Recordings and measurements

All recordings were from the CA3 area contralateral to the CA3 stimulation site. Signals were recorded via a 2-channel microelectrode AC amplifier (model 1800, A-M Systems, Carlsborg, WA, USA), with the input frequency band set in the range of 0.1–1000 Hz, and the amplification gain at 1000 $\times$. The signals were digitized at 5000 Hz (Digidata 1440A, Axon Instruments/Molecular Devices, Union City, CA, USA). Pclamp software (Version 9 or 10; Axon Instruments/Molecular Devices) was used for data acquisition, storage and analyses.

The amplitudes of evoked field potentials were measured from averaged traces (5 consecutive responses) at a given stimulation intensity. To measure hippocampal rhythmic activities associated with “active” and “inactive” behaviors, stable data segments of 5–10 seconds or 30–60 seconds were selected while the animals were moving/exploring or immobile/asleep. Spectral plots were generated from these data segments and peak frequencies were measured from the spectral plots for individual animals. To detect interictal-like spikes, individual animals were recorded for up to four hours before kindling was initiated. After animals were fully kindled, interictal spikes were recorded for 4–6 hours after the most recent ADs. A spike was only counted if its amplitude was large (≥ 2 times the amplitude of the background signal) and its waveform was similar to those previously described [26–29]. To minimize interference of movement-related

artifacts, the rates of interictal spikes were measured in the periods (1–2 hours) while the animals were immobile/asleep. To measure AD durations, corresponding EEG data were treated with a 0.5 Hz high-pass (Bessel) filter to reduce slow drifts in EEG signals.

Behavioral assessments

Animal's behaviors were recorded with a high definition camera and analyzed by blinded experimenters. Behavioral seizures were scored using a modification of the Racine scale for the mouse [25]: stage 0, no response or behavioral arrest; stage 1, chewing or head-nodding; stage 2, chewing and head nodding; stage 3, single or bilateral forelimb clonus; stage 4, bilateral forelimb clonus and rearing; stage 5, loss of righting reflex (falling).

Brain histology

Histological experiments were conducted as we previously described [15,17]. Briefly, the animals were anesthetized with pentobarbital (70 mg/kg, i.p.) and transcardially infused with saline and then with 10% phosphate-buffered formalin solution before decapitation. Cryostat coronal sections 30 μ m thick were obtained throughout the brain, stained with cresyl violet, and examined under a light microscope.

Statistical analysis

Statistical tests were performed with SPSS software (Version 20, SPSS Statistics, IBM). Data are presented as mean and standard error of mean ($M \pm SE$) throughout the text and figures except where indicated.

Abbreviations

AD: After-discharges; CA3: Cornu ammonis 3 area; EEG: Electroencephalography.

Competing interests

The authors of this study declare that they have no competing interests.

Authors' contributions

MJ and ML made equal contributions to this study. All authors were involved in experimental design, background/literature search and conceptualizations of this study. Specifically, MJ, ML and JG conducted experiments and data analysis. CW constructed electrodes and conducted surgeries for electrode implantation. MJ, ML and LZ assembled manuscript. MB edited the manuscript. All authors have read and approved the final manuscript.

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in experimental design, data collection and interpretation, analysis, writing of the report, or the decision to submit for publication.

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Appendix 4: Electrophysiological Studies In Vivo
and In Vitro, Accepted

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**Novel Anticonvulsive Effects of Progesterone
in A Mouse Model of Hippocampal Electrical Kindling**

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Summary

Progesterone is a known anticonvulsant, with its inhibitory effects generally attributed to its secondary metabolite, $5\alpha,3\alpha$ -tetrahydroprogesterone (THP), and THP's enhancement of GABA_A receptor activity. Accumulating evidence, however, suggests that progesterone may have non-genomic actions independent of the GABA_A receptor. In this study, we explored THP/GABA_A-independent anticonvulsive actions of progesterone in a mouse model of hippocampal kindling and in mouse entorhinal slices *in vitro*. Specifically, we examined the effects of progesterone in kindled mice with or without pretreatments with finasteride, a 5α -*reductase* inhibitor known to block the metabolism of progesterone to THP. In addition, we examined the effects of progesterone on entorhinal epileptiform potentials in the presence of a GABA_A receptor antagonist picrotoxin and finasteride.

Adult male mice were kindled via a daily stimulation protocol. Electroencephalographic (EEG) discharges were recorded from the hippocampus or cortex to assess “focal” or “generalized” seizure activity. Kindled mice were treated with intra-peritoneal injections of progesterone (10, 35, 100 and 160mg/kg) with or without finasteride pretreatment (50 or 100mg/kg), THP (1, 3.5, 10 and 30mg/kg), midazolam (2mg/kg) and carbamazepine (50mg/kg). Entorhinal cortical slices were prepared from naïve young mice, and repetitive epileptiform potentials were induced by 4-aminopyridine (100μM), picrotoxin (100μM) and finasteride (1μM).

Pretreatment with finasteride did not abolish the anticonvulsant effects of progesterone. In finasteride pretreated mice, progesterone at 100 and 160mg/kg decreased cortical but not hippocampal afterdischarges. Carbamazepine mimicked the effects of progesterone with finasteride pretreatments in decreasing cortical discharges

and motor seizures, whereas midazolam produced effects similar to progesterone alone or THP in decreasing hippocampal afterdischarges and motor seizures. In brain slices, progesterone at 1 μ M inhibited entorhinal epileptiform potentials in the presence of picrotoxin and finasteride.

We suggest that progesterone may have THP/GABA_A-dependent and independent anticonvulsive actions in the hippocampal-kindled mouse model.

Introduction

Epilepsy is characterized by recurrent seizures. Drug-refractory seizures affect one third of adults with epilepsy, and remain a significant challenge in the pharmacotherapy of epilepsy (Theodore *et al.*, 2006). Uncontrolled seizures have significant impacts on quality of life and adverse effects of anticonvulsant drugs can be just as damaging (Viteva *et al.*, 2013). It is therefore imperative to understand the pathological processes underlying epilepsy and to develop treatments for patients who cannot achieve seizure control with the currently available medications.

In both men and women, complex neuroendocrine interactions are implicated in seizure occurrence, co-morbidities, and the etiology of epilepsy (Pack *et al.*, 2011). Progesterone and its metabolites in particular have a strong influence on seizure propensity. In catamenial epilepsy and relevant animal models, when progesterone levels are high, seizures are less likely to occur (Verrotti *et al.*, 2010; Stevens & Harden, 2011; Pack *et al.*, 2011). Progesterone is first metabolized to 5 α -dihydroprogesterone (DHP) by the unidirectional enzyme *5 α -reductase* and subsequently to 5 α ,3 α -tetrahydroprogesterone (THP) by the bidirectional enzyme *3 α ,5 α -hydroxysteroid oxidoreductase*. The anticonvulsant effects of progesterone are generally attributed to its

metabolism to THP, a powerfully inhibitory neurosteroid that enhances GABA_A receptor activity at an unknown binding site distinct from the benzodiazepine and barbiturate binding sites (Reddy *et al.*, 2004; Lambert *et al.*, 2009; Finocchi & Ferrari, 2011). In many animal models, seizure suppression and anti-epileptogenesis effects of progesterone are lost or weakened when the animals are pretreated with finasteride, a competitive *5 α -reductase* inhibitor that blocks the metabolism of progesterone to DHP, and, therefore, to THP (Kokate *et al.*, 1999; Herzog & Frye, 2003; Finn *et al.*, 2006; Mukai *et al.*, 2008; Chaudhary & Turner, 2010; Reddy & Ramanathan, 2012). Several lines of evidence, however, suggest that progesterone may regulate brain function independent of its metabolite THP (Zhu *et al.*, 2008; Hwang *et al.*, 2009; Zheng 2009; Johannessen *et al.*, 2011; Luoma *et al.*, 2011). In this study, we explored whether progesterone offers THP/GABA_A-independent anticonvulsive or inhibitory actions in hippocampal-kindled mice.

Methods

Animals

Male C57 black mice (Charles River Laboratory, Quebec Canada), between 6-10 months of age, were used in the present study. Male animals were used to avoid complications from the cyclic fluctuation of progesterone and its metabolites. Animals were housed in a vivarium that was maintained at 22° C with a 12 hour light/dark cycle (lights on at 6:00 am). Food and water were available *ad libitum*. All experiments were conducted between 10am-5pm. All experimental procedures were reviewed and approved by the Animal Care Committee of the University Health Network, in accordance with the guidelines of the Canadian Council on Animal Care.

Electrode Implantation

Surgical procedures were modified from those previously described (Wu *et al.*, 2008; El-Hayek *et al.*, 2011). A modified version of a screw-free, glue-based method was used to secure implanted electrodes (Jeffrey *et al.*, 2013). Briefly, the animal was anaesthetized with 2% isoflurane and then placed in a stereotaxic frame. After a skin incision and exposure of the skull surface, a thin plastic base (~200µm thick, cut from plastic weighing boats) was glued onto the skull surface with cyanoacrylate glue (Insta-cure+, Canadian Hobbycraft, Concord, Ontario, Canada). After the glue had set, three small holes (<0.5 mm in diameter) were drilled through the plastic base and the skull in order to insert the stimulation and recording electrodes. The inserted electrodes were then cemented onto the skull with dental acrylic.

Two bipolar electrodes were inserted into the right and left hippocampal CA3 areas, with coordinates relative to bregma: 2.5 mm posterior, 1.3 mm lateral and 3.0 mm down from brain surface. A reference electrode was positioned, relative to bregma, at: 1.0 mm anterior, 2.0 mm lateral, and 0.5 mm down from brain surface. In some experiments, a bipolar electrode was implanted in CA3 on one side, and two monopolar recording electrodes were implanted contralaterally, one in CA3 (coordinates as above) and the other in the neocortex. The coordinates for cortical implantation, relative to bregma, were: 0.6 mm posterior, 1.5 mm lateral and 1.0 mm down from brain surface. All electrodes were made of polyamide-insulated stainless steel wires (outer diameter 125 µm; Plastics One, Ranoake, VA).

Hippocampal kindling

Unilateral CA3 stimulation was conducted in all experiments. Constant unipolar square-wave current pulses (duration of 0.5ms, intensities of 10-150 μ A base to peak) were generated by a Grass stimulator and delivered through a stimulus isolation unit (model S88H, Grass Medical Instruments, Warwick RI, USA). A standard kindling protocol (Albright & Burnham, 1980; Reddy & Rogawski, 2010) was used in the present experiments. Initially, daily stimuli (60 Hz for 2 seconds) were applied at an intensity of 150 μ A until an afterdischarge (AD) event of ≥ 5 seconds was elicited, as $>90\%$ of the cohort did not elicit evident AD on the first two days when stimulated with intensities from 10-150 μ A. An ascending stimulation series was then used to determine each animal's individual AD threshold. In the ascending series, stimulation was increased from 10 to 150 μ A with 10 μ A increments, spaced 5 minutes apart, until an AD event of ≥ 5 seconds was observed. The stimulation intensity at which an AD event of ≥ 5 second was elicited was considered the AD threshold. The mice were stimulated daily at 125% of their AD threshold and were considered fully kindled when three consecutive stage 5 events (see below) were elicited. All animals were kindled within a month after receiving their first stimulation. The animals were stimulated at 125% of their AD threshold on all non-trial days to ensure the stability of ADs ($<10\%$ fluctuation in duration) and consistent stage 5 motor seizures during the course of drug testing.

EEG recordings and data analysis

EEG signal was recorded from the CA3 site contralateral to the CA3 stimulation site. Local differential recordings - between the adjacent tips of the contralateral recording electrode - were used in order to reduce artifacts and common-ground EEG signals. Monopolar recordings were done if the local differential recordings were

unsuccessful, presumably due to contaminated electrode tips of the twisted bipolar electrodes. In some experiments, two monopolar electrodes were implanted in the contralateral CA3 and neocortical areas for simultaneous recordings of hippocampal and neocortical EEG.

Signals were recorded via a 2-channel microelectrode AC amplifier (model 1800, A-M Systems, Carlsborg, WA, USA), with the input frequency band set in the range of 0.1-5000 Hz, and the amplification gain at 1000x. The signals were digitized at 5000 Hz (Digidata 1440A, Axon Instruments/Molecular Devices, Union City, CA, USA). PClamp software (Version 10; Axon Instruments/Molecular Devices) was used for data acquisition, storage and analyses (El-Hayek *et al.*, 2011). EEG afterdischarges (ADs) were recognized as repetitive single spike and poly-spike waveforms with amplitudes 2 times of baseline signals and durations of ≥ 5 seconds. For measurements of AD durations, original EEG signals were treated with a 0.5 Hz high-pass (Bessel) filter to minimize slow drifts. In some experiments, standard deviations of cortical EEG signals were measured to assess AD amplitude changes. AD lengths or standard deviations were normalized as percentiles of kindled untreated responses in individual animals.

Behavioral assessments

Animal behaviors were recorded with a high definition camera and analyzed by experimenters blinded to treatments. Motor seizures were scored using a modification of the Racine (1972) scale for the mouse (Reddy & Rogawski, 2010): stage 0, no response or behavioral arrest; stage 1, chewing or head-nodding; stage 2, more chewing and head nodding; stage 3, single or bilateral forelimb clonus; stage 4, bilateral forelimb clonus and rearing; stage 5, loss of righting reflex.

Drug treatments

Progesterone, THP, carbamazepine, finasteride and β -cyclodextrin were obtained from Sigma-Aldrich (Oakville, Ontario, Canada). Midazolam was obtained in clinically injectable form from Sandoz Canada Inc. (Quebec, Canada).

Progesterone, THP, and finasteride were freshly prepared on the day of treatment and dissolved in physiological saline containing 45% β -cyclodextrin. These solutions were sonicated at $\sim 40^{\circ}\text{C}$ until the compounds had completely dissolved. Carbamazepine was initially dissolved in DMSO and then diluted into the β -cyclodextrin solution. The final amount of DMSO was $\leq 30\ \mu\text{L}$ in individual animals with body weights ranging from 29-35g.

All drugs were administered via intra-peritoneal injections, with a total volume 0.2-0.3 mL. Vehicle injections were made with 0.3 mL β -cyclodextrin solution. Neurosteroids or anticonvulsant drugs were injected at one dose per test day. The same group of animals was used for drug testing, thus treatments on animals were spaced 2-3 days apart to allow complete elimination of the compounds between tests. Each animal served as its own control.

Progesterone at 10, 35, 100 or 160 mg/kg was injected 30 minutes before hippocampal stimulation. THP at 1, 3.5, 10 or 30 mg/kg was injected 15 minutes before hippocampal stimulation. These doses and injection protocols were based on previous studies (Lonsdale *et al.*, 2006; Lonsdale & Burnham, 2007; Reddy & Ramanathan 2012; Singh *et al.*, 2010). Finasteride at 50 or 100 mg/kg was injected 3 or 0.5 hours before hippocampal stimulation as these injection protocols were shown to significantly reduce THP levels in the rat brain (Finn *et al.*, 2006; Ciriza *et al.*, 2006; Mukai *et al.*, 2008).

Midazolam (2mg/kg) and carbamazepine (50mg/kg) were injected 15 min before hippocampal stimulation (Higgins *et al.*, 2010; Dihr & Rogawski, 2012).

Brain slice preparation and extracellular recordings

Horizontal brain slices of 0.5 mm thickness were obtained from male C57 black mice 1-3 months old. All recordings were conducted in a submerged chamber at 36°C. Under our recording conditions, the slice was perfused with an artificial cerebrospinal fluid (ACSF) at a high rate (15 mL/min), with both the top and bottom surfaces of the slice exposed to the perfusate. Humidified gas of 95%O₂-5%CO₂ was allowed to pass over the perfusate to increase local oxygen tension (El-Hayek *et al.*, 2011). The ACSF contained (in mM): NaCl 125, KCl 3.5, NaH₂PO₄ 1.25, NaHCO₃ 25, CaCl₂ 2, MgSO₄ 1.3 and glucose 10 (pH of 7.4 when aerated with 95%O₂-5%CO₂). Extracellular signals were recorded using a dual channel amplifier (700A or 700B, Molecular Devices). Data acquisition, storage and analysis were done using the digitizer and PClamp software as described above.

Examination of epileptiform field potentials *in vitro*

Brain slices were perfused with ACSF containing the GABA_A receptor antagonist picrotoxin (100 µM), and finasteride (1 µM). 4-aminopyridine (100 µM) was used to induce repetitive epileptiform potentials (D'Antuono *et al.*, 2010). Progesterone was applied at 1 µM for 20 min together with the above pharmacological agents.

4-aminopyridine (Sigma-Aldrich, Oakville, Ontario, Canada) was dissolved in distilled water as a stock solution of 0.5 M and then appropriately diluted in the ACSF. Picrotoxin (Sigma-Aldrich) was directly dissolved in the ACSF. Finasteride was dissolved in DMSO as a 10 mM stock solution and then diluted in ACSF. Progesterone

was dissolved in 100% alcohol as a stock solution of 50 mM and then diluted into the ACSF.

The event detection function of the Pclamp software was used to detect repetitive epileptiform potentials (El-Hayek *et al.*, 2011). Original signals were treated with a 5Hz high-pass filter to reduce DC drifts and to reveal rhythmic spike waveforms. Detected events were then visually inspected and artifacts were rejected. 15-20 events of epileptiform potentials were detected from individual slices before and at the end of progesterone application. These detected events were then averaged for measuring duration and peak amplitude. The main frequency and corresponding power of the epileptiform rhythmic spikes were determined from the spectra plots.

Statistical analysis

Statistical tests were performed with SPSS (Version 21, SPSS Statistics, IBM) and SigmaStat (Systat Software Inc, San Jose, California, USA) software. Data are presented as mean and standard error of mean ($M \pm SE$) throughout the text and figures. Friedman's test was used to compare EEG ADs with Wilcoxon signed rank *post hoc* correction. Changes in motor seizure stages were also analyzed using the related-samples Wilcoxon signed rank test. Paired t-tests were used to compare measurements of epileptiform field potentials. Statistical significance is set as $p < 0.05$.

Results

Progesterone and THP reduced hippocampal ADs and motor seizures

We first verified anticonvulsive actions of progesterone and THP in our hippocampal kindled mice. Vehicle (β -cyclodextrin control) injections did not produce anticonvulsive effects as the lengths of hippocampal ADs and motor seizure stages were

not significantly different with or without vehicle injections. Progesterone was injected 30 minutes before hippocampal electrical stimulation at doses of 10, 35, 100 and 160 mg/kg (n=6-17). Progesterone caused dose-dependent reductions in hippocampal AD lengths and motor seizure stages (**Fig 1B**). Specifically, both hippocampal AD lengths and motor seizure stages were significantly reduced from vehicle controls by progesterone at 100 and 160 mg/kg. Reductions in hippocampal AD lengths and motor seizure stages were noticeable at 35 mg/kg of progesterone, but these changes were not statistically different from vehicle controls. Representative EEG traces showing shortened hippocampal ADs by progesterone are illustrated in **Fig 1A**.

THP was injected 15 minutes before hippocampal stimulation and at doses of 1, 3.5, 10, and 30mg/kg (n=5-8). THP at 10 and 30mg/kg significantly reduced hippocampal AD lengths relative to vehicle controls and abolished motor seizures (**Fig 2B**). THP at 3.5 mg/kg caused a significant reduction in motor seizure stages but not hippocampal AD lengths (**Fig 2B**). There were no evident anticonvulsive effects by THP at 1 mg/kg. Representative EEG traces showing shortened hippocampal ADs by THP are illustrated in **Fig 2A**.

Anticonvulsant effects of high dosage progesterone were not diminished by finasteride pretreatment

To test whether progesterone exerts anticonvulsant effects independent of its metabolism to THP, we treated animals with finasteride prior to progesterone injections and electrical stimulation. Finasteride (50mg/kg) was injected 2.5 hours before subsequent progesterone injection. Finasteride alone did not produce anticonvulsive effects, but finasteride pretreatment diminished progesterone's effects on hippocampal AD lengths. At all 4 doses of progesterone examined with finasteride pretreatment,

hippocampal AD lengths were not significantly different from vehicle controls (n=7-14; **Fig 3A, B**). Interestingly, finasteride pretreatment did not abolish progesterone's suppression of motor seizures. In finasteride-pretreated mice, progesterone at 100 and 160mg/kg significantly reduced motor seizure stages relative to vehicle controls (**Fig 3B**).

To further explore how progesterone with finasteride pretreatment attenuated motor seizures but not “focal” hippocampal ADs, we recorded hippocampal and cortical EEG signals from 5 hippocampal-kindled mice. Hippocampal stimulations induced both hippocampal and cortical ADs in these animals, and the onset of hippocampal ADs preceded the onset of neocortical ADs by 3.37 ± 0.64 seconds (**Fig 4A**), suggesting that the cortical ADs may represent electrographic seizure activity spread from the stimulated hippocampus. Similar to the results described above (**Fig 1**), 100 mg/kg of progesterone significantly reduced the hippocampal AD lengths and motor seizure stages relative to vehicle controls (**Fig 4A, B**). The motor seizure suppression was associated with attenuated cortical ADs, as cortical ADs were barely detectable in 4 of the 5 animals examined and the amplitudes (as measured by standard deviations of underlying EEG signals) were significantly reduced ($36.62 \pm 7.40\%$ of vehicle cortical AD signal, n=5, $p < 0.01$; **Fig 4B**). In the same 5 animals following finasteride pretreatment (50m/kg), progesterone at 100 mg/kg was still able to reduce motor seizure stages and attenuate cortical AD signals ($37.77 \pm 9.23\%$ of vehicle controls, n=5, $p < 0.01$), but the hippocampal AD lengths were not significantly reduced compared to vehicle controls (**Fig 4A, B**).

We also examined the effects of finasteride pretreatment at a higher dose (100 mg/kg) in an attempt to attain stronger inhibition of *5 α -reductase*. These experiments

were conducted in another 5 kindled mice that underwent hippocampal-cortical EEG recordings. Finasteride injections (100 mg/kg) were made 30 minutes before subsequent progesterone injections (160 mg/kg). The progesterone injections significantly reduced motor seizures (1.8 ± 0.48 vs. stage 5 in vehicle controls) and cortical AD signals ($47.81 \pm 6.34\%$ of vehicle controls, $p < 0.05$), but not hippocampal AD lengths ($95.07 \pm 16.05\%$ of vehicle controls). Taking these and the above observations together, we suggest that finasteride used in our experiments (at 50 or 100 mg/kg) may be effective at inhibiting *5 α -reductase* and that DHP/THP independent pathways may mediate the anticonvulsive effects of progesterone in finasteride pretreated kindled mice.

Carbamazepine produced similar anticonvulsive effects as progesterone with finasteride pretreatment

We next explored whether clinical anticonvulsive drugs with known mechanisms of action produced effects similar to that of progesterone with finasteride pretreatment. Midazolam is a fast-acting benzodiazepine and positive allosteric modulator of the GABA_A receptors (Reves et al., 1985; Lahat et al., 1992; Lambert et al., 2009), recommended for treatment of status epilepticus (Meierkord *et al.*, 2010). Carbamazepine acts largely by suppressing the activity of voltage-gated sodium channels (Mula, 2013). These two drugs were injected 15 minutes before hippocampal stimulation in kindled mice.

Midazolam at 2 mg/kg significantly reduced hippocampal AD lengths and motor seizure stages, together with a great attenuation of cortical AD amplitudes ($35.84 \pm 2.97\%$ of vehicle controls, $n=5$, $p < 0.01$; **Fig 5A, B**). Carbamazepine at 50 mg/kg significantly reduced motor seizure stages but not hippocampal AD lengths compared to vehicle controls (**Fig 5A, B**). Motor seizure suppression by carbamazepine was associated with

significant reduction in cortical AD amplitudes ($47.01 \pm 6.04\%$ of vehicle controls, $n=5$, $p>0.01$). Overall, the anticonvulsive effects of carbamazepine were similar to those produced by progesterone with finasteride pretreatment.

Progesterone decreased entorhinal epileptic potentials in the presence of a GABA_A antagonist and finasteride

Since the entorhinal cortex is critical for hippocampal-neocortical communication and for generation of epileptic seizures (Spencer & Spencer, 2009), we tested whether progesterone has inhibitory effects on entorhinal epileptiform potentials in mouse brain slices. Repetitive epileptiform potentials were induced by ACSF containing the convulsive agent 4-animopyridien ($100 \mu\text{M}$; D'Antuono *et al.*, 2010), the GABA_A receptor antagonist picrotoxin ($100 \mu\text{M}$) and finasteride ($1 \mu\text{M}$). Progesterone was applied at $1 \mu\text{M}$ for 20 min together with these pharmacological agents.

Monitored via extracellular recordings, the epileptiform potentials were recognized as rhythmic events with incidences of 10-15 events per min, peak amplitudes up to 1.5 mV and durations of up to 2 seconds. Rhythmic spike activity was evident in individual epileptiform potentials with dominant frequencies of $12.5 \pm 0.8 \text{ Hz}$ as determined via spectral analysis. The amplitude and power of rhythmic spike activity of epileptiform field potentials were significantly reduced after progesterone application relative to those measured before ($p=0.007$ and $p=0.013$, $n=10$; **Fig 6**). However, the incidences of these epileptiform potentials were increased following progesterone application, and this change might be partly due to weakened individual epileptiform events and hence shorter periods of post-event inhibition.

Discussion

Four main findings emerge from our current experiments. Firstly, at relatively high doses, progesterone and THP were able to reduce hippocampal ADs and generalized motor seizures in kindled mice. Secondly, progesterone retained its anticonvulsive effects in finasteride-pretreated mice, and these effects were associated with a strong suppression of cortical ADs, but not hippocampal ADs. Thirdly, carbamazepine mimicked the effects of progesterone with finasteride pretreatment in attenuating cortical ADs and motor seizures, whereas midazolam produced effects similar to progesterone and THP in suppressing hippocampal/cortical ADs and motor seizures. Lastly, progesterone inhibited population epileptiform potentials in mouse entorhinal slices in the presence of the GABA_A receptor antagonist picrotoxin and finasteride.

Kindling is a model of partial (focal) seizures with secondary generalization (Albright & Burnham, 1980; McIntyre & Poulter 2001; Morimoto *et al.*, 2004; Bialer & White, 2010; Löscher, 2011). In the present experiments, we stimulated the hippocampal CA3 area and recorded ADs from the contralateral CA3 and cortex. This protocol was used to avoid the switch artifacts that result from stimulating and recording at the same site. Bilateral hippocampi communicate efficiently through the dorsal and ventral hippocampal fissures (Witter, 2007); the contralateral CA3 ADs, therefore, may closely resemble the EEG seizure activity of the stimulated CA3 site. Thus we consider the contralateral CA3 ADs to represent “focal” or “near focal” seizure activity. As the hippocampus does not directly initiate motor activity, the generalized motor seizures that follow hippocampal stimulation result from the spread of the hippocampal ADs and associated electrographic signals to cortical and/or subcortical structures that initiate

motor activity (Löscher, 2011). In this context, we consider cortical ADs to represent EEG signals closely associated with generalized motor seizures in our model.

The motor seizure suppression by high doses of progesterone alone we observed (**Fig 1**) is generally in keeping with previous studies (Lonsdale & Burnham, 2003; 2007; Lonsdale *et al.*, 2006; Singh *et al.*, 2010). Seizure suppression by progesterone is generally thought to be a result of progesterone's metabolism to THP (Frye *et al.*, 2002; Zheng, 2009; Reddy & Ramanathan 2012) and the subsequent THP-mediated enhancement of the GABA_A receptor activity (Lambert *et al.*, 2009; Zheng 2009; King 2013). Our present observations are consistent with this view. We found that progesterone at 100 and 160 mg/kg reduced hippocampal AD lengths (**Fig 1**) and such effects were diminished by finasteride pretreatments (**Fig 3**). In addition, THP was effective at reducing hippocampal AD lengths (**Fig 2**) and this effect was mimicked by midazolam (**Fig 5**). Thus, progesterone, in the absence of finasteride pretreatments, could be exerting its anticonvulsant effects largely via its metabolism to THP and THP-mediated enhancement of GABA_A receptor activity. We noted that at 3.5mg/kg THP did not significantly shorten hippocampal AD lengths but reduced motor seizure stages (**Fig 2**). These effects appear qualitatively similar to those induced by 100 or 160mg/kg of progesterone in finasteride pretreated animals (**Fig 3**). It remains to be examined whether the motor seizure reduction by THP at 3.5mg/kg is mediated by GABA_A receptor dependent or independent actions. Nevertheless, previous studies have shown that THP can modulate glutamate and dopamine releases via interacting with the NMDA receptors (Cabrera *et al.* 2002; Giuliani *et al.* 2011).

To explore progesterone's anticonvulsive actions independent of its metabolites DHP and THP, we pretreated animals with finasteride (50 or 100 mg/kg) in an attempt to

inhibit *5 α -reductase*. Previous studies have shown that finasteride pretreatments (25-50 mg/kg) prevent or greatly reduce progesterone's metabolism to DHP/THP (Finn *et al.*, 2006). In our experiments, finasteride pretreatment diminished the effects of progesterone (at 100 and 160mg/kg) on hippocampal AD durations (**Fig 3A, B**). Unexpectedly, however, finasteride pretreatment did not abolish progesterone's suppressive effects on motor seizures. Progesterone at high doses (100 and 160 mg/kg) was still able to decrease motor seizure stages in finasteride-pretreated animals, and such motor seizure suppression was associated with attenuation of cortical ADs (**Fig 3A, B**). Carbamazepine produced similar anticonvulsive effects by attenuating cortical ADs and motor seizures (**Fig 4**). Collectively, these results suggest that at least in hippocampal-kindled mice, progesterone may attenuate the secondarily generalized component of seizures via DHP/THP-independent mechanisms, perhaps through voltage-gated ion channel mechanisms (Johannessen *et al.*, 2011; Luoma *et al.*, 2011).

Discrepancies seem to exist between our present observations and some past studies (Herzog & Frye, 2003; Reddy *et al.*, 2004; Reddy & Ramanathan, 2012) regarding anticonvulsive effects of progesterone with finasteride pretreatment. However, the anticonvulsive effects of progesterone with finasteride pretreatment we observed represent progesterone's actions at high doses, as progesterone substantially reduced motor seizures at 100 and 160mg/kg but without effects at 10 or 35mg/kg (Fig 3). Interestingly, Kokate *et al.* (1999) found that pretreatment with finasteride prevented the anticonvulsant activity of progesterone in the pentylenetetrazol model, but not the maximal electroshock model. In their maximal electroshock experiments, high doses of progesterone (ED₅₀ 324 mg/kg) with finasteride suppressed motor seizures. In our kindling experiments, high doses of progesterone (100 and 160 mg/kg) with finasteride

suppressed motor seizures and cortical ADs, but not focal hippocampal ADs. In this respect, our results in the kindling model are consistent with the maximal electroshock results of Kokate et al. (1999).

The exact mechanism(s) by which progesterone confers its anticonvulsive or inhibitory actions in finasteride-pretreated mice remain to be determined. While progesterone metabolism to DHP via *5 α -reductase* and then to THP via *3 α ,5 α -hydroxysteroid oxidoreductase* is widely recognized, it can also be metabolized via other enzymatic pathways (Wiebe *et al.*, 2005; King, 2013). Thus progesterone may exert anticonvulsive or inhibitory actions independent of its metabolism to DHP/THP. In addition, progesterone has been reported to regulate brain activity via GABA_A receptor-independent mechanisms (Zhu *et al.*, 2008; Hwang *et al.*, 2009; Zheng 2009; Johannessen *et al.*, 2011; Kelley & Mermelstein, 2011; Luoma *et al.*, 2011; Singh & Su 2013; King, 2013). Our *in vitro* observations are in keeping with these previous findings. We found that in mouse entorhinal slices treated with the GABA_A receptor antagonist picrotoxin and finasteride, progesterone at a high concentration (1 μ M) was able to decrease entorhinal epileptiform potentials.

In summary, we provide *in vivo* and *in vitro* data suggesting that progesterone may have THP/GABA_A independent anticonvulsive or inhibitory actions. However, the above notion is based on the utilization of finasteride as a pharmacological tool to inhibit *5 α -reductase* and hence progesterone's metabolism to DHP/THP. Although finasteride's inhibition of progesterone metabolism has been repeatedly demonstrated in previous studies (Finn *et al.*, 2006), brain levels of progesterone and its metabolites remain to be measured in our models. Correlation of these measurements with progesterone's anticonvulsive actions in the absence or presence of finasteride pretreatments is crucial to

confirm the proposed DHP/THP-independent pathways. In addition, progesterone can produce rapid responses by activating G-protein coupled membrane receptors, which are expressed abundantly in both the brain and peripheral tissues (Thomas & Pang 2012). The anticonvulsive effects of progesterone we observed from the kindled mice might be largely mediated by its actions in the brain, but the activity of peripheral membrane progesterone receptors and resultant influences on brain activity may also be involved. Overall, the exact mechanisms that underlie progesterone's anticonvulsive or inhibitory actions in our model remain to be elucidated.

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Disclosure

None of the authors has any conflict of interest to disclose. We confirm that we have read the Journal's policies on issues involving ethical publications and affirm that this report is consistent with those guidelines.

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Figure legends

Fig 1. Effects of progesterone on hippocampal EEG afterdischarges and motor seizures

A, representative hippocampal EEG traces collected from a fully kindled mouse. EEG signals were recorded via a bipolar electrode. Intra-peritoneal injections of vehicle or progesterone were made 30 min before the hippocampal stimulation. Original signals were treated with a high-pass filter (0.5 Hz) for illustrative purpose. **B**, mean hippocampal (hipp.) afterdischarge (AD) lengths and motor seizure stages following vehicle or progesterone treatments. The animals were allowed to recover for 2 days between progesterone treatments. AD lengths were normalized as percentiles of fully kindled no-treatment responses in individual animals. *, $p < 0.05$, progesterone vs. vehicle, Friedman's test with Wilcoxon signed rank post hoc correction.

Fig 2. Effects of THP on hippocampal EEG afterdischarges and motor seizures

A, representative hippocampal EEG traces collected from a fully kindled mouse. EEG signals were recorded via a mono-polar electrode. THP was injected 15 min before the hippocampal stimulation. **B**, mean hippocampal afterdischarge (AD) lengths and motor seizure stages following vehicle or THP treatments. The animals were allowed to recover for 2 days between THP treatments. AD lengths were normalized as percentiles of fully kindled no-treatment responses in individual animals. *, $p < 0.05$, THP vs. vehicle, Friedman's test with Wilcoxon signed rank post hoc correction.

Fig 3. Effects of progesterone with finasteride pretreatment on hippocampal EEG afterdischarges and motor seizures

A, representative hippocampal EEG traces collected from a fully kindled mouse. EEG signals were recorded via a bipolar electrode. Finasteride at 50mg/kg was injected

2.5 hours before progesterone treatment and 3 hours before the hippocampal stimulation.

B, mean hippocampal afterdischarge (AD) lengths and motor seizure stages post treatments. The animals were allowed to recover for 3 days between progesterone with finasteride treatments. AD lengths were normalized as percentiles of fully kindled no-treatment responses in individual animals. *, $p < 0.05$, progesterone vs. vehicle, Friedman's test with Wilcoxon signed rank post hoc correction.

Fig 4. Effects of progesterone with finasteride pretreatment on cortical afterdischarges

A, hippocampal and cortical EEG traces were collected simultaneously via monopolar recordings from a fully kindled mouse. Illustrated traces were collected following finasteride injection alone (top) and progesterone injection with finasteride pretreatment (bottom). **B**, mean hippocampal afterdischarge (AD) lengths, standard deviation (SD) of cortical AD signals, and motor seizure stages following treatments. Hippocampal AD lengths and cortical signal SD were normalized as percentiles of fully kindled no-treatment responses in individual animals. SD was used as a measure of cortical AD amplitudes. The animals were allowed to recover for 3 days between finasteride or progesterone plus finasteride treatments. *, $p < 0.05$, treatments vs. vehicle, Friedman's test with Wilcoxon signed rank post hoc correction.

Fig 5. Effects of midazolam and carbamazepine on hippocampal/cortical afterdischarges and motor seizures

A, hippocampal and neocortical EEG traces collected simultaneously via monopolar recordings from a fully kindled mouse. Vehicle or indicated drugs were injected 15 min before the hippocampal stimulation. **B**, mean hippocampal afterdischarge (AD) lengths and motor seizure stages following vehicle or indicated drug treatments.

The animals were allowed to recover for 2 days between drug treatments. AD lengths were normalized as percentiles of fully kindled no-treatment responses in individual animals. *, $p < 0.05$, drug vs. vehicle, Friedman's test with Wilcoxon signed rank post hoc correction.

Fig 6. Effects of progesterone on entorhinal epileptiform potentials in vitro

A, representative epileptiform potentials collected via extracellular recordings from an entorhinal slice. Original signals were treated with a 5 Hz high-pass filter for purpose of event detection. Arrowed events are expanded at right. Spectral plots at far right were generated from 10 events of epileptiform potentials including those illustrated. The slice was treated with 100 μ M 4-aminopyridine, (4-AP,) 100 μ M picrotoxin and 1 μ M finasteride, and progesterone was applied at 1 μ M for 20 min together with these pharmacological agents. **B**, averages from 15-20 epileptiform potentials were obtained before and at the end of progesterone application, and measurements were made from the averaged events and pooled together from 10 slices. *, $p < 0.05$, before vs. after progesterone application, paired t-test.

Figure 1

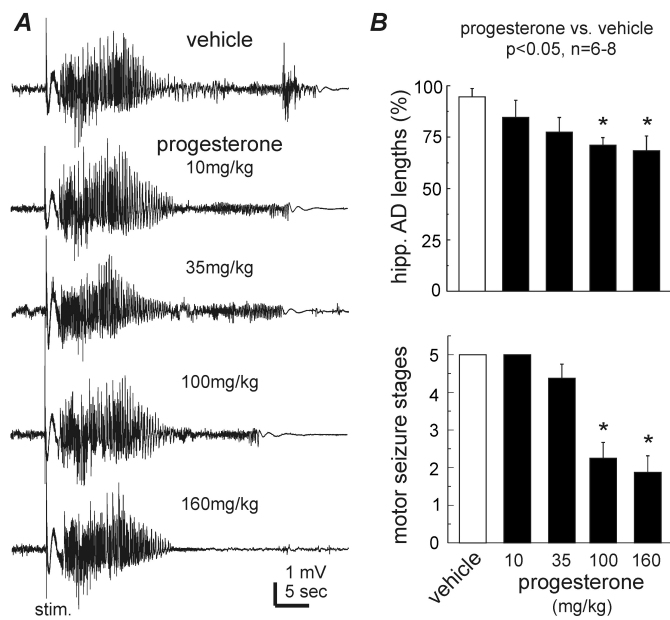


Figure 2

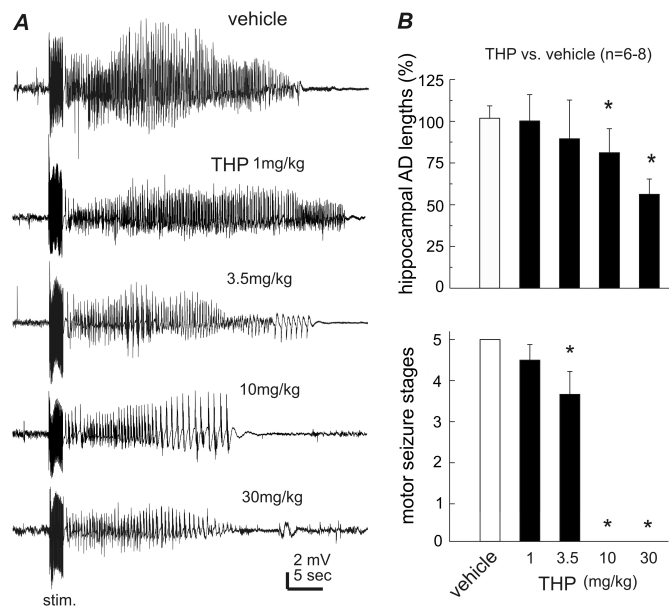


Figure 3

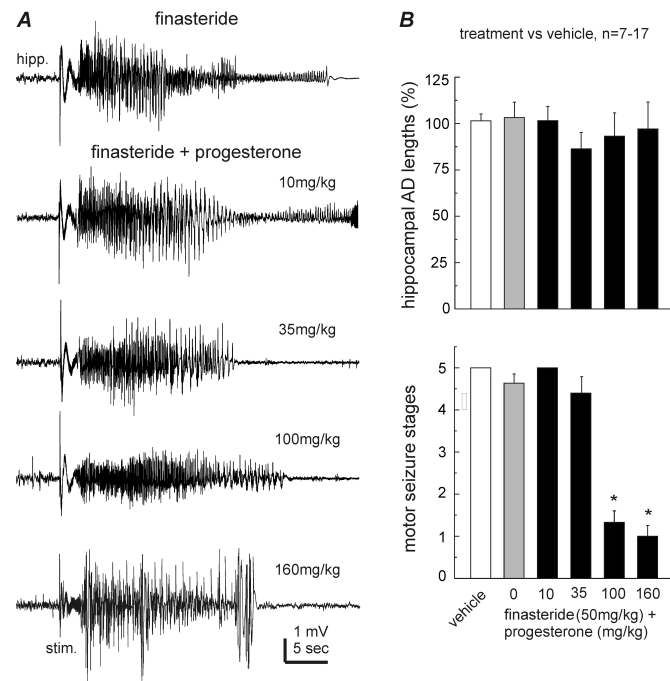


Figure 4

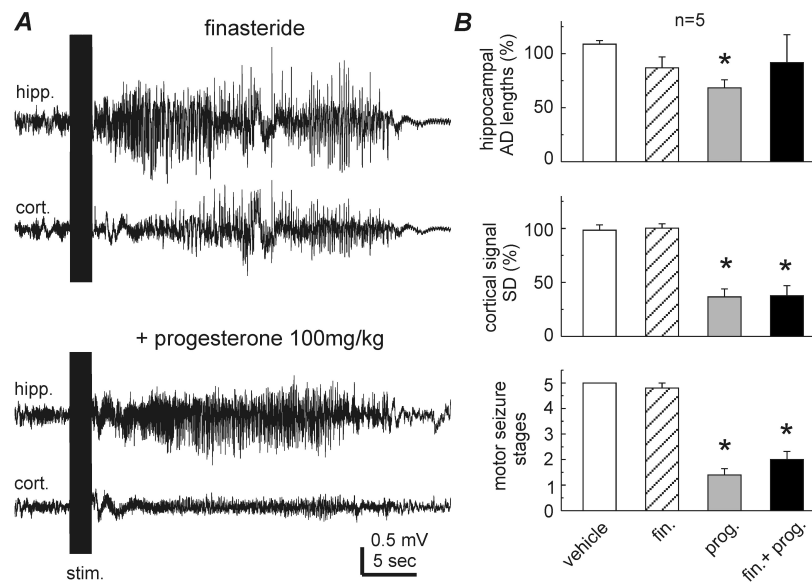


Figure 5

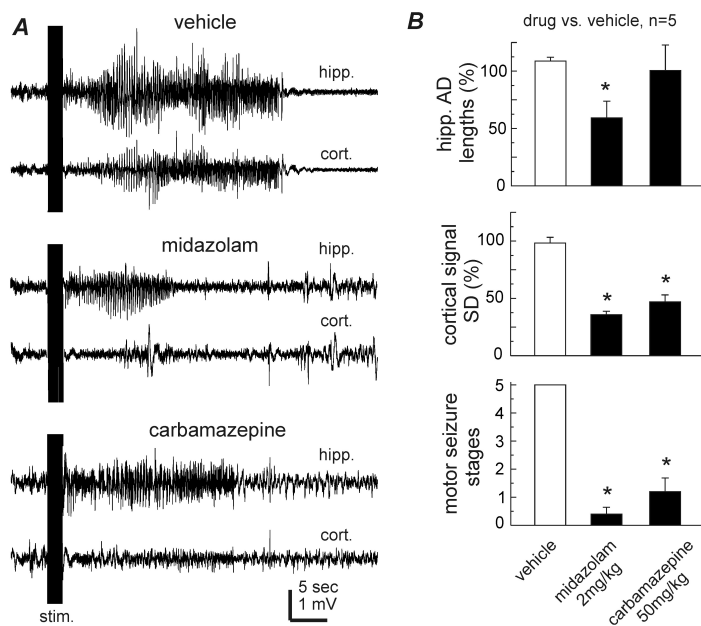


Figure 6

