MODULATION OF SEXUAL AND SLEEP FUNCTIONS BY ESTROGEN IN CASTRATED MALE RATS AS A MODEL FOR PROSTATE CANCER PATIENTS ON ANDROGEN DEPRIVATION THERAPY

by

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Submitted in partial fulfilment of the requirements
for the degree of Doctor of Philosophy

at

Dalhousie University
Halifax, Nova Scotia
August 2013

DEDICATION PAGE

I would like to dedicate my thesis to my late grandmother, Mrs. Kwee Kwa Lan Nio (1923 – 2013), who passed away as I was completing my final project. I truly cherish her hardwork and generous love. May God grant her soul eternal peace.

"I thank God… as night and day I constantly remember you in my prayers."
— 2 Timothy 1:3

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Advanced prostate cancer (PCa) patients are offered androgen deprivation therapy (ADT) to control their cancer's growth. ADT impairs sexual function and the sleep patterns of ADT patients. Since ADT deprives patients of estrogen, and supplemental estrogen reduces such problems in menopausal women, I studied whether administering estrogen reduces these problems for castrated male rats as a model for PCa patients on ADT.

First, I tested how early versus late estradiol treatment after castration influenced rats' sexual behaviour. Estradiol increases mounting behaviour to comparable levels regardless of when the treatment was started after castration, suggesting that estrogen's ability to restore male sexual interest is insensitive to a delay since castration.

Secondly, to understand the biological basis of these behavioural effects, I examined brain and muscle tissues from the same animals. Specifically, I compared changes in 1) estrogen receptors (ERs) and c-Fos protein (a neuronal activation marker) levels in brain areas controlling sex behaviour; 2) ERs levels in pelvic floor muscles, important for erection; and 3) ERs levels in the hippocampus and prefrontal cortex. Prolonged castration increases ERα levels in the preoptic area (POA), a key brain area that regulates mating behaviour, and estradiol treatment reduced these effects. In the POA, mating-induced c-Fos expression was not affected by estradiol regardless of when the treatment began post-castration. Estrogen may upregulate ERs in pelvic floor muscles, and downregulate ERs in the hippocampus and prefrontal cortex, depending on administration time after castration. These findings suggest that mating activates POA neurons, and this activation induces mounting only in the presence of estrogen. Additionally, the duration after castration influences ER autoregulation in the pelvic floor muscles, hippocampus, and prefrontal cortex in response to estradiol.

Lastly, I studied how estrogen modulates the sleep-wake behaviour of orchiectomized rats. Estradiol promotes baseline wakefulness during the dark period and prevents castration-induced impairment in sleep recovery after sleep deprivation. These findings suggest that estradiol may positively influence the sleep-wake behaviour of castrated males.

Collectively, I demonstrate that estrogen administered to castrated rats improves sexual and sleep functions. It may similarly improve the quality of life of PCa patients on ADT.

LIST OF ABBREVIATIONS USED

ADT	Androgen Deprivation Therapy
BC	Bulbocavernosus
BNST	Bed Nucleus of the Stria Terminalis
DES	Diethylstilbestrol
E	Estrogen
E2	Estradiol
EB	Estradiol Benzoate
EEG	Electroencephalography
EMG	Electromyography
ER	Estrogen Receptor
HPT	Hypothalamic-Pituitary-Testicular
i.p	intraperitoneal
LA	Levator Ani
LHRH	Luteinizing Hormone-Releasing Hormone
MeA	Medial Amygdala
NAc	Core Area of Nucleus Accumbens
NAs	Shell Area of Nucleus Accumbens
NREM	Non-Rapid Eye Movement
P	Progesterone
PCa	Prostate Cancer
PFC	Prefrontal Cortex
PFM	Pelvic Floor Muscle
POA	Preoptic Area
REM	Rapid Eye Movement
s.c	subcutaneous
SD	Sleep Deprivation
SEM	Standard Error of Mean
T	Testosterone

ACKNOWLEDGEMENTS

I would like to express much gratitude to all who have contributed to the completion of this thesis; you have continuously and tirelessly supported both this body of work as well as myself throughout this program. Thank you.

Thesis panel:

Dr. Richard Wassersug, your guidance, openness to ideas and your infectious ability to make me think outside the box are qualities I won't forget. Working with you has been a great pleasure. Your dedication to research has always inspired me and will continue to do so with the work I have yet to accomplish.

Dr. Kazue Semba, your encouragement and constructive criticism of my work is truly an art. I am privileged to have been a part of the Semba lab. Your ongoing commitment to sleep research is exemplary, and I have learned a great deal of knowledge from you that I will bring forward with me on my career path.

Dr. Richard Brown, Dr. Tara Perrot, Dr. Frank Smith, Dr. Lori Wood, for all the feedback during committee meetings, my comprehensive exam, and defence. **Dr. Brown,** for discussing and advising me on my sexual behaviour experiments. **Dr. Perrot** and **Amanda Green,** for consultation on micropunch techniques and western analyses.

Dr. Jessica Mong, for examining this body of work that is so close to me.

Those who have supported my projects:

Dr. William Currie, for advice on molecular research, providing a space to do western analysis, and giving me an opportunity to expand my anatomy knowledge and dissection skills. **Dr. William Baldridge,** for access to the cryostat and guiding me through my admission process. **Dr. Daniel Marsh,** for access to a plate reader and centrifuge. **Dr. Boris Kablar,** for a lab space to conduct the histology experiment.

Past Members of Wassersug lab. Thanks to all lab members mentioned here, who helped me with proof-reading my written works. Additional thanks to **Hannah Calich,** I am highly indebted to you for your help with the behaviour testing, western and histology analyses as well as for making the final push to the thesis submission. **Joanne Phillips,** for providing administrative support and ordering lab supplies. **Imhokhai Ogah,** for substantial editorial support, especially during the first few years of my program. **Isaac Siemens,** for rat handling. **Kristen Kukula, Emily MacLeod, Joshua Amiel**.

Current and Past Members of Semba Lab. Dr. Samuel Deurveilher, for all the support in the sleep study, discussion on new data, statistical advice. **Joan Burns,** for introducing me to animal handling and surgery, technical advice, and for making me a qualified castrator. **Chantalle Briggs, Inga Westermann, Katie Miller, Rebekah Stetson,** for helping me with sleep deprivation. **Jacquelyn Bush**, for daily rat handling.

Kay Murphy, for spending intense hours helping me with cryosectioning and western analyses, as well as tissue collection.

Dr. Mark Baguma-Nibasheka, for assistance in the H&E staining.

Members of the CACF for their support of my animal study. Special thanks to **Joanne Shewchuck, Amber Peck, and Sarah Whitehead**, for taking special care of my rats during a strict schedule every morning.

Thanks also to the members of the Department of Medical Neuroscience for making my graduate experience a memorable one. I am truly grateful for the opportunity to complete my PhD as well as for helping me appreciate the art of anatomy dissection. **Dr. Irena Rot,** for co-ordinating the gross anatomy course. **Rob Sandeski, Trevor Maclaren,** and **Kelly Miles** for organizing the anatomy lab. **Pauline Fraser, Brenda Armstrong, Catherine Currell, Suzanne Hayes, Heather-Ann Jennex,** and **Jennifer Wipp** for administrative support.

I thank God for continually blessing me; my family—my parents, my sister (Belinda), my brother-in-law (Arri) and Zoe—for the invaluable support throughout my time studying far from home; My partner, Shyronn, for the tremendous encouragement and for always trusting in me; my Godchild, Marcelo, for inspiring me to work hard, and to all my friends for all their support.

I would like to also acknowledge those that provided financial assistance: Faculty of Graduate Studies of Dalhousie University, Department of Anatomy and Neurobiology of Dalhousie University, Canadian Cancer Society through the Beatrice Hunter Cancer Research Institute, and the Nova Scotia Health Research Foundation.

CHAPTER 1: INTRODUCTION

1.1 BACKGROUND ON PROSTATE CANCER

Prostate cancer (PCa) is now the most common cancer in men. When first described just 160 years ago, Dr. John Adams (1853) listed its symptoms as: 1) frequent but difficult urination, 2) enlarged prostate gland, and 3) enlarged lymph nodes in lumbar and pelvic areas. Also, in the same paper, PCa was considered a "very rare disease". Today however, one in seven men in Canada can expect to be diagnosed at some time with PCa (Canadian Cancer Society's Steering Committee on Cancer Statistics, 2013). Interestingly, a metastasis pattern similar to what Adams described recently helped archeologists to identify the oldest (Schultz et al., 2007) and second oldest (Prates et al., 2011) known PCa cases, which date back over 2000 years ago. Thus, PCa is neither a 'modern' nor rare disease.

Although PCa is the most prevalent type of cancer in men, the mortality rate for PCa is lower than for lung and colorectal cancer (Canadian Cancer Society's Steering Committee on Cancer Statistics, 2013). This is partly because PCa is slow-growing and, therefore, men who are diagnosed with PCa often have a good prognosis. In most cases, men are diagnosed with PCa in their 60s or older. However, since the introduction of the prostate specific antigen test (Stamey et al., 1987), there have been increasing incidences of men being diagnosed with PCa at a younger age (Canadian Cancer Society's Steering Committee on Cancer Statistics, 2013).

If diagnosed early, when the cancer is localized within the prostate gland, PCa can be cured with a prostatectomy. However, for advanced (metastasized) PCa, patients are offered androgen deprivation therapy (ADT). ADT is used because androgens are the main endogenous factor that stimulates the cancer's growth. Unfortunately, for many men, ADT becomes a life-long treatment.

1.2 ANDROGEN DEPRIVATION THERAPY

ADT can be achieved by surgical or chemical castration. Castration was first described to reduce the size of prostate gland in 1786 by John Hunter (Hunter, 1786). In that report, Hunter described the prostate gland of a castrated animal as "*small, flabby, tough and ligamentous, and* [having] *little secretion*". More than a century later, castration was used by two surgeons to treat urinary blockade due to prostatic enlargement (White, 1895; Cabot, 1896). However, not until almost five decades later, was castration used as a specific treatment for PCa.

In 1941, Huggins and Hodges discovered that orchiectomy and/or administering high doses of diethylstilbestrol [DES, an oral estrogen (E)] to PCa patients reduced their serum acid phosphatase levels, a biochemical marker for PCa (Huggins and Hodges, 1941). Both orchiectomy and high doses of E reduce plasma androgen levels and subsequently suppress acid phosphatase production. For the next 40 years, DES and/or orchiectomy were common treatments for PCa (Denmeade and Isaacs, 2002).

The search for new androgen-depriving agents began following reports that DES increased lethal thromboembolic events (The Veterans Administration Co-operative Urological Research Group, 1967a, b). The risk of these events was elevated because oral E (i.e., DES) is absorbed in the digestive system and transported to the liver via the hepatic portal system, where it upregulates clotting factors (von Schoultz et al., 1989). However, when E is administer via parenteral routes (such as transdermal or intramuscular injection, thereby bypassing a portal surge to the liver), the hepatic clotting factors are not elevated (Henriksson et al., 1990; Ockrim et al., 2005). Recent evidence confirms that parenteral E does not elevate the cardiovascular morbidity risk any more than other androgen-depriving drugs (Abel et al., 2011; Langley et al., 2013).

In the early 1980s, luteinizing hormone-releasing hormone (LHRH) agonists became available to treat metastatic PCa patients (Tolis et al., 1982). Similar to DES, LHRH agonists suppress testosterone (T) production, resulting in decreased prostate size, lower

acid phosphatase levels, fewer osteoblastic lesions, and less bone pain. However, unlike DES, LHRH agonists do not increase the thromboembolic risk (The Leuprolide Study Group, 1984). Due to this benefit, LHRH agonists have replaced oral E as the primary systemic treatment for ADT. Other hormonal therapies for PCa include LHRH antagonists, anti-androgens, ketoconazole, and 5α-reductase inhibitors (Singer et al., 2008), but they are less commonly used than LHRH agonists as a treatment for PCa because of lower effectiveness, adverse side effects, or greater cost.

1.3 Mechanism of Androgen Deprivation Therapy

In males, the hypothalamic-pituitary-testicular (HPT) axis controls the production of androgens in the testes. Select neurons in the hypothalamus produce LHRH, which stimulates cells in the anterior pituitary to secrete luteinizing hormone (Miyamoto et al., 2004). Luteinizing hormone activates Leydig cells in the testes to produce androgens. Testosterone (T), the most abundant androgen, can be converted to E by the enzyme aromatase (Naftolin et al., 1975). An increase in the plasma T or E level triggers negative feedback inhibition at every level of the HPT axis, thus reducing the subsequent release of androgens.

How ADT suppresses androgen levels and androgen activity varies depending on the specific treatment modality. Obviously, by performing surgical castration, the main source of androgens in males, the testes, is physically removed. In contrast, high doses of E reduce the production of androgens by enhancing negative feedback inhibition to the HPT axis (Miyamoto et al., 2004; Singer et al., 2008).

In contrast, prolonged treatment with LHRH agonists shuts down androgen production by downregulating LHRH receptors in the pituitary gland (Singer et al., 2008). During initial LHRH agonists treatment, there will be a surge in T production, which can result in the ‘flare’ phenomenon (Bubley, 2001). The ‘flare’ phenomenon is a condition where elevated T levels worsen PCa clinically, exacerbating symptoms such as bone pain and nerve compression (Bubley, 2001). However, when the pituitary gland is continuously

stimulated by LHRH agonists, the pituitary cells eventually downregulate their LHRH receptors, decreasing the availability of receptors to bind to LHRH (Miyamoto et al., 2004). As a result, the production of lutenizing hormone from the pituitary (and subsequently the gonadal hormones) is reduced.

1.4 Side Effects of Androgen Deprivation Therapy

As early as 1941, Huggins *et al*. (1941) identified many adverse effects of androgen deprivation in his PCa patients. These included weight gain, sexual dysfunction, and hot flashes. Interestingly, many of the side effects of ADT are identical to those experienced by women during menopause. In fact, these side effects may be due to E deprivation, because E in males is derived from the aromatization of T (Naftolin et al., 1975). As such, ADT can lead to deprivation of both androgen and E in men.

The three most common side effects of LHRH agonists experienced by more than 70% of patients include hot flashes (Engstrom, 2008), loss of libido, and erectile dysfunction (Higano, 2012). All three may impact not only the patient's quality of life but also their partner's. Hot flashes can be severe enough to interfere with daily activities or wake up patients during the night (Engstrom, 2008; Jones et al., 2012). A patient's nocturnal awakening may accidentally disrupt his partner's sleep as well. As a result, both patients and their partners can experience sleep disturbances. Similarly, sexual problems (such as erectile dysfunction or diminished libido) can detrimentally affect intimacy between PCa patients and their partners (Higano, 2012).

In addition to sexual function, LHRH agonists have other serious health risks, such as osteoporosis, metabolic syndrome, and anemia (Higano, 2006). Metabolic syndrome makes patients vulnerable to cardiovascular morbidity or even mortality (Collins et al., 2012), and osteoporosis increases the risk of bone fractures and, potentially, immobility (Al-Shamsi et al., 2012). Anemia in conjunction with sleep disturbances can result in daytime fatigue.

Due to low androgen levels, PCa patients on ADT also experience physical changes including loss of body hair (Navon and Morag, 2003), reduced penile length (Park et al., 2011), reduced muscle mass and increased fat mass (leading to weight gain) (Collins et al., 2012). All of these changes collectively lead to body feminization and, as a consequence, the patients may lose self-esteem and avoid some social activities.

ADT can also influence cognition and affection (reviewed in Elliott et al., 2010; Casey et al., 2012). Declines in memory function have been reported in PCa patients on ADT (Jamadar et al., 2012). Furthermore, emotional changes, including increased tearfulness and irritability (Ng et al., 2006) as well as depressive symptoms (Pirl et al., 2002; Chipperfield et al., 2013), may occur and disrupt patients' daily functioning and social interaction.

In the next two subsections I describe in more detail the sexual dysfunctions and sleep problems experienced by PCa patients on ADT as these two areas are the main foci of my thesis.

1.4.1 Sexual Dysfunction in Prostate Cancer Patients

Before treatment, most patients are focused on staying alive and may not give much consideration to the sexual side effects of PCa treatments (including ADT). Yet, shortly after starting ADT, they realize that not only are their erections gone, but their sexual desire, erotic dreams, and the frequency of sexual fantasies are also diminished (Navon and Morag, 2003). This loss of sexual interest may not only affect the patients, but also their partners.

Treatments, such as penile implants or vacuum devices, are available to address erectile problems, but there is no established intervention for loss of libido. Due to their low levels of sexual desire, patients neglect sexual intimacy, which leaves the sexual needs of their partners unmet.

Factors that influence the extent to which sexual desire can be preserved after androgen-deprivation are not well-investigated. For some men, being androgen-deprived does not necessarily lead to cessation of sexual activity (Wibowo et al., 2012b). Historical records show that many eunuchs in different cultures remained sexually active after castration (Aucoin and Wassersug, 2006). Additional reports from voluntarily-castrated men in the western world (Brett et al., 2007) and the Hijra in India (Reddy, 2005) also indicate that castration may not abolish libido in all men. Independently, Davidson (1966) demonstrated that almost 50% of male rats lose ejaculation within the first few weeks after castration. However, some retained ejaculatory behaviour long after castration. Indeed, Davidson noted that one rat still displayed ejaculatory-like behaviour at 21 weeks after castration. These findings suggest that there is individual variation in how male sexual behaviour is affected by androgen deprivation.

Additionally, external factors including age, stress, other medications, or comorbidities may influence sexual function following ADT. Older age, depression and cardiovascular diseases have all been linked to lower frequencies of sexual activities in intact men (Corona et al., 2010). Pre-castration sexual behaviour also correlates with patients' sexual activity after treatment; i.e., men who were not sexually active before ADT are not likely to remain sexually active following ADT (Ellis and Grayhack, 1963; Choi et al., 1998). In contrast, in one case report, a hypersexual man still maintained coitus twice per week two years after castration (Ellis and Grayhack, 1963). Thus, how sexual a person is prior to ADT may influence sexual function after starting ADT.

1.4.2 Sleep Disturbance in Prostate Cancer Patients

Sleep problems are common in androgen-deprived PCa patients. Stephens *et al*. (2007), in a questionnaire-based prospective study, reported that PCa patients slept "*less well*" after beginning ADT. Similarly, Savard *et al*. (2012) studied insomnia in PCa patients undergoing radiotherapy with or without ADT. The severity of insomnia increased over time in patients on radiotherapy plus ADT, but not in those treated solely with radiotherapy. In addition, the patients in the first group had improved sleep quality when

the ADT was stopped. In a more quantitative study, Hanisch *et al*. (2011) used actigraphy to assess daily wake and sleep episodes in androgen-deprived PCa patients. On average, the patients in that study required more than 30 minutes to fall asleep and only slept for 6 hours per night with episodic awakening (mostly due to nocturia). The patients also took frequent daytime naps, indicative of daytime fatigue.

Sleep can be divided into non-rapid eye movement (NREM) sleep and REM sleep (Harrington and Lee-Chiong, 2012). Each stage is regulated by different neural circuits and is accompanied, respectively, by a distinct electroencephalogram (EEG) pattern. To date, no study has used the EEG to investigate how specific sleep stages in men are affected by ADT. However, some studies have investigated how sleep parameters change following castration in male rodents. In one such study, REM sleep during the dark phase increased following castration (Yamaoka, 1980), while other studies failed to find any difference in the amount of NREM or REM sleep (Peder, 1987; Paul et al., 2006). Collectively, there is some evidence that androgen depletion may influence sleep regulation in both men and male rodents (Yamaoka, 1980; Stephens et al., 2007; Hanisch et al., 2011; Savard et al., 2012).

1.5 Brain Sexual Differentiation

Since androgen depletion lowers both androgen and E levels in men, some of the side effects of ADT that PCa patients experience may be alleviated with E administration. In this thesis, I tested whether E treatment reduces some of the negative effects of castration in male rats, as a model for PCa patients on ADT. To date, the majority of studies on the physiological, behavioural and psychological effects of E have been with females, driven in part by concerns about the symptoms women experience with menopause. As such some of the rationale for my studies comes from findings on E treatment in females, because the effect of E on male functions has not been investigated. However, males and females may be affected by E differently, given the divergence that occurs during sexual differentiation in the brain during development (McCarthy, 2008).

In rats, sexual differentiation of the brain occurs during the perinatal period, and E is thought to be the key hormone that masculinizes the brain (McCarthy, 2008). Although fetuses of both sexes are exposed to high levels of maternal E, the endodermal cells of the embryo produce α-fetoprotein, a plasma protein that has a high affinity for E (Bakker et al., 2006). As a result, maternal E binds to α-fetoprotein, and no significant amounts of E can enter and masculinize the brains of female fetuses (Bakker et al., 2006). In contrast, male fetuses additionally produce T, which can be converted to E in the brain through neurons that express the aromatase (Lephart, 1996). The E produced through T aromatization is what masculinizes the brains of male fetuses [at least in rodents in which this phenomenon is best studied to date (McCarthy, 2008)].

Based on the organizational/activational hypothesis of brain sexual differentiation, steroid action during development permanently organizes the brain in a sex-specific manner that eventually determines how an individual will respond to gonadal hormones in adulthood (Wallen, 2009; McCarthy, 2010). In males, the action of E (derived from T) during the perinatal period organizes various processes to make male brains distinct from those of females. These processes include neurogenesis, apoptosis, synaptogenesis, steroid receptor expression, and formation of neurochemical pathways (Gillies and McArthur, 2010). In addition, during puberty, the rise of gonadal steroids further differentiates male- and female-specific neural circuits (Schulz et al., 2009). As a result of the actions of gonadal steroids during perinatal period and later in puberty, sexual dimorphism in the brain emerges. Consequently, administering gonadal hormone treatment to males and females gonadectomized in adulthood does not always produce identical responses between sexes (Gillies and McArthur, 2010). For these reasons, in my study, I would expect to see some differences in how castrated male rats respond to E treatment as compared to previously-reported results from female rats.

1.6 AUTOREGULATION OF ESTROGEN RECEPTORS

E influences many behaviours of castrated male animals by acting on estrogen receptors (ERs) (Rissman, 2008; Gillies and McArthur, 2010). The ERs that have been identified to

date include: 1) nuclear receptors, ERα and ERβ, and 2) the more recently discovered membrane receptors, GPR30 and ER-X (Pak and Handa, 2008). The effects of E via nuclear receptors involve the ligand's binding to the receptors, which results in the translocation of the E-ER complex to the E response element in the DNA to affect gene transcription. This process takes hours to days to occur. In contrast, the binding of E to membrane ERs leads to a rapid intracellular signaling cascade, the effects of which may occur within minutes (Simpkins et al., 2012).

The binding of E to nuclear ERs may lead to the autoregulation of ERs, which either enhances (through autoinduction) or homeostatically maintains (through autorepression) the cellular effects of E, depending on the tissue type (Bagamasbad and Denver, 2011). Autoinduction leads to an increase whereas autorepression leads to a decrease of ERs. A consequence of the autoregulation of ER is that the physiological response of a cell to E can vary greatly depending, not simply on the dose of E, but on the ER levels at a given time that can fluctuate.

Several mechanisms have been proposed for the autoregulation of ERs which vary depending the direction of change (i.e., autoinduction or autorepression) as well as on the tissue type. For autoinduction, E has been shown to directly enhance ER gene transcription in breast cancer cells (Saceda et al., 1988), stabilize ER mRNA in uterine cells (Ing et al., 2008), and reduce proteasome degradation of ER protein in the hippocampus (Zhang et al., 2011). In contrast, ER downregulation in mice mammary gland is due to a reduction in phosphorylation of a transcription factor that normally regulates ER gene (Hatsumi and Yamamuro, 2006). The interplay of additional factors like epigenetic mechanisms and the involvement of co-activators or co-repressors may also contribute to the regulation of ERs by E (Liang and Shang, 2012).

When cellular ERs are not autoregulated normally, the impact of E treatment may be attenuated. As an example, ERα in the hippocampus increases in female rats receiving E treatment immediately after ovariectomy, but not in those that receive E treatment 5 months later (Bohacek and Daniel, 2009). Similarly, administering E to recently

ovariectomized rats elevates choline acetyltransferase levels, a synthesizing enzyme for acetylcholine, in the hippocampus (Bohacek et al., 2008). However, the same E treatment cannot raise choline acetyltransferase levels in long-term ovariectomized rats (Bohacek et al., 2008). These findings suggest that when ER is not autoregulated (autoinduced) by E treatment, the physiological effects of E may be altered, for example E does not improve hippocampal-dependent tasks of long-term ovariectomized rats (Daniel, 2013).

1.7 THE "CRITICAL PERIOD HYPOTHESIS" FOR E TREATMENT

Clinically, some PCa patients on ADT elect to take supplemental E therapy to counteract hot flashes (Jones et al., 2012); however, the initiation time of E therapy relative to the onset of ADT varies among different patients. Though still unexplored, it is possible that those who begin E therapy long after the initiation of ADT will experience fewer benefits from the E treatment than those who start soon after beginning ADT. This idea is based on the "Critical Period Hypothesis" on how E affects female functions.

The "Critical Period Hypothesis" proposes that there is a critical period after the onset of steroid deprivation (i.e., menopause or surgical ovariectomy) in females when exogenous E treatment will be most beneficial (Daniel, 2013). Early, but not delayed, E treatment after steroid deprivation improves cognitive (Daniel, 2013), cardiovascular (Scott et al., 2012), and sexual functions (Damassa and Davidson, 1973; Czaja and Butera, 1985) in females. Why the duration of steroid deprivation influences the effects of E has not been widely investigated. It is possible that long-term steroid deprivation results in cellular changes that will eventually diminish the effects of E. To support this possibility, as mentioned in the previous section, E only increases ERα protein levels in the hippocampus of female rats if administered soon after ovariectomy, but not when E treatment is delayed (Bohacek and Daniel, 2009). This finding suggests that the autoregulatory (in this case autoinduction) capability of ER may be disrupted with prolonged steroid deprivation. As a consequence, there may be only basal levels of ERs, and the binding of E to these ERs may not be sufficient to induce the normal physiological effects of E.

Whether there is a time window after castration where E may have the most beneficial effects for males is not known. I hypothesize that early E treatment may bring more benefits to males when administered sooner than later after castration.

1.8 The Rationales and Hypotheses of the Three Studies

For this thesis, I conducted three main studies to investigate how E modulates sexual and sleep behaviours in castrated male rats as a model for PCa patients on ADT. The following sections describe the rationale and hypotheses for each study in this thesis. The overarching hypothesis is that E treatment will improve the sexual and sleep-wake behaviours of castrated male rats. In addition, I also tested the hypothesis that the timing of E treatment after castration influences the effectiveness of that E treatment.

1.8.1 Study 1: Testing the "Critical Period Hypothesis" of E Treatment on Male Sexual Behaviour in Castrated Male Rats (Chapter 2)

High dose E has previously been used as a primary ADT treatment for PCa patients (Appendix B). In two studies (Ellis and Grayhack, 1963; Bergman et al., 1984) of the patients that were sexually active prior to treatment, more patients on high dose E therapy remained sexually active than those who were orchiectomized. Currently, the factors that determine the effectiveness of E treatment in preserving libido in androgen-deprived men have not been investigated.

It is well-established that administering E to castrated male rats increases mounting behaviour; however, the extent to which E changes libido varies among studies (Appendix B). Many factors influence the effectiveness of E in restoring sexual interest of castrated males, such as the age at which castration is performed, the dose, type of estrogen, and the method of E administration (see Appendix B for more details). In this study, I tested if the interval from castration to the onset of E treatment affects the extent to which sexual interest is elevated by E. Based on the "Critical Period Hypothesis" proposed for females, I hypothesized that exogenous E would raise sexual interest more

effectively when administered sooner rather than later after castration. I compared the sexual behaviour of male rats that received E treatment immediately, one month, or three months after castration.

[More detailed information on how various factors may affect E's influence on the sexual interest of castrated males has been published in my review article (see Appendix B)].

1.8.2 Study 2: Alterations in ERs and c-Fos Levels in the Brain and Periphery after E Treatment Beginning at Different Time Intervals Post-Castration in Male Rats

To study the neurobiological basis of the behavioural effects of E, at the end of the behavioural experiment described in Chapter 2, I collected the brains and pelvic floor muscles (PFM) of the rats immediately after a sexual encounter, and used the tissues for the second study described in Chapter 3-5.

1.8.2.1 Changes in ERs and c-Fos Levels in Brain Areas that Control Male Sexual Behaviour (Chapter 3)

To follow up on the behavioural findings described in Chapter 2, I studied the neuroendocrine mechanisms responsible for the behavioural observations, i.e., similar effects of E on sexual behaviour when E was administered at different times after castration. To achieve this goal, I used western blot analysis to quantify the levels of neuronal activation (by quantifying c-Fos levels) and ERs in brain areas that control sexual behaviour and are known to express c-Fos in response to sexual encounters. The presence of mating-induced c-Fos in a given brain area would suggest that the neurons in that area are involved in sexual behaviour and/or are responding to sexual stimuli. Furthermore, changes in ER levels in these brain regions, as a result of E treatment, could indicate alteration in the autoregulation of ERs. These, in turn, might be responsible for the effects on the sexual behaviours induced by E treatment at different time intervals after castration.

1.8.2.2 Changes in Morphology and ERs in the Pelvic Floor Muscles (Chapter 4)

In addition to brain ERs, I studied whether the duration of time between castration and E administration influences the morphology and levels of ERs in the pelvic floor muscles (PFM), which are important for sexual functions such as erection, orgasm and continence (Chapter 5). Intromission and ejaculatory behaviours in male rats require erectile function, and high-dose E treatment has been shown to restore both behaviours in castrated male rats (Davidson, 1969; Södersten, 1973). Therefore, if E can increase either behaviour, E may also affect the morphology and ER levels of the PFM, which are also known to express ERs (Dube et al., 1976; Rudolph and Sengelaub, 2013). To my knowledge, no study has investigated how E influences ER levels in the PFM in males. Furthermore, this study was the first to test the "Critical Period Hypothesis" of E treatment on non-neuronal tissue.

1.8.2.3 Changes in ERs in the Hippocampus and Prefrontal Cortex (Chapter 5)

Additionally, I tested whether the "Critical Period Hypothesis" for E treatment applies to the ERs in the hippocampus and prefrontal cortex (PFC) (Chapter 5). Both areas are involved in cognitive function; the hippocampus is important for spatial memory and the PFC is essential for working memory (Gillies and McArthur, 2010). To date, studies testing the "Critical Period Hypothesis" have shown that the positive effects of E are found mostly on cognitive function in females (Daniel, 2013). One of the mechanisms responsible for the beneficial effects of E on cognition is thought to depend on the action of E on ERα in the hippocampus and the PFC (Bohacek and Daniel, 2009). As mentioned earlier, long-term deprivation of ovarian hormones alters the autoregulatory mechanism of ERα in both brain areas in female rats (Bohacek and Daniel, 2009). However, it was not known whether the regulation of ERs by E in both brain areas in males is also sensitive to the timing of E treatment after castration. Given that background, I

investigated whether sex difference exists in how E modulates ERs in the hippocampus and PFC when the E is administered at different times after castration.

1.8.3 Study 3: Effects of E Administration on Sleep Regulation in Castrated Male Rats (Chapter 6)

My third main study (Chapter 6) was devoted to investigating the effects of E on the sleep behaviour of castrated male rats. I examined the effects of E treatment on the sleep-wake behaviour of castrated male rats during baseline, sleep deprivation and recovery periods.

As reviewed above, some postmenopausal women report better sleep quality after starting hormone replacement therapy. This is likely due to fewer nocturnal awakenings and increased REM sleep duration (Dzaja et al., 2005; Parry et al., 2006). In contrast, studies in ovariectomized rodents show that exogenous E, either alone or with progesterone (P), reduces REM sleep during the dark phase when the animals are mostly active, thus increasing the light:dark ratio (Colvin et al., 1969; Branchey et al., 1971; Matsushima and Takeichi, 1990; Pawlyk et al., 2008a; Pawlyk et al., 2008b; Deurveilher et al., 2009; Paul et al., 2009). Furthermore, E-treated ovariectomized rats show better REM rebound than those without E (Deurveilher et al., 2009, 2011; Schwartz and Mong, 2011, 2013).

In contrast to female studies, the effects of E on male sleep function are not well understood. To date, there has only been one study on the effects of E on sleep behaviour in the genetic human male population (Kunzel et al., 2011). In that study, E was administered to male-to-female transsexuals together with anti-androgens. This hormonal regimen had only minor effects on sleep parameters; i.e., reducing stage 1 sleep and increasing EEG beta activity.

Similarly, only two studies (Branchey et al., 1973; Yamaoka, 1980) have investigated the effects of E on the sleep parameters of castrated male rats. Those studies showed that low dose E alone did not affect sleep parameters. However, when E combined with P was

administered to neonatal castrates (but not to adult castrates), both NREM and REM sleeps were reduced in the dark period. Interestingly, administering low-dose E treatment to female rats that are ovariectomized in adulthood also reduced REM sleep only when administered with P (Deurveilher et al., 2009). This could be because when male rats are orchiectomized neonatally, their brains may not be fully masculinized, and as such may resemble female brains (McCarthy, 2008).

In sum, I investigated the potential benefits of administering E treatment to castrated male rats, specifically for improving their sexual and sleep behaviours. Additionally, I examined whether the interval from castration to the onset of treatment influences the effectiveness of E administration and the autoregulation of ERs in neuronal and non-neuronal tissues.

Chapter 2: DOES THE TIMING OF ESTROGEN ADMINISTRATION AFTER CASTRATION AFFECT ITS ABILITY TO PRESERVE SEXUAL MOTIVATION IN MALE RATS? – EXPLORING THE CRITICAL PERIOD HYPOTHESIS

Abstract

Loss of libido is a major side effect that reduces the quality of life of prostate cancer patients on androgen-deprivation therapy. Estrogen restores sexual motivation to some extent in castrated male mammals; however, the beneficial effects of estrogen vary greatly among different studies. We investigated whether the timing of estrogen treatment after castration affected its ability to restore sexual motivation in male rats.

For each rat, sexual behaviour was tested with receptive female rats before castration, and after two weeks of either oil alone (as a control) or oil plus estradiol (E2) treatment administered via Silastic tubes implanted immediately, at 1 month (Short-Term), or at 3 months (Long-Term) after castration.

Intromission frequency decreased and genital sniffing frequency increased significantly after castration compared to pre-castration levels, regardless of the testing time post-castration. E2 treatment at any time point did not reverse these changes. However, more E2-treated than control rats exhibited mounting behaviour, with a significant difference between the Long-Term groups. Mounting frequency did not differ from pre-castration levels for either E2 or control rats under the Immediate condition, but declined significantly in rats treated with oil only under both the Short- and Long-Term conditions. In contrast, E2 treatment elevated mounting frequency above the castrate levels to a similar extent in both the Short and Long-Term groups.

In conclusion, E2 administration partially restores sexual motivation in castrated male rats, and the length of post-castration delay in E2 administration does not affect the ability of E2 to restore mounting behaviour.

Publication Information

This chapter has previously been published as: Wibowo E, Wassersug RJ (2013) Does the timing of estrogen administration after castration affect its ability to preserve sexual interest in male rats? - Exploring the critical period hypothesis. Physiol Behav 110-111C:63-72. EW was involved in study design, running the experiments, data analysis and prepared the manuscript.

2.1 INTRODUCTION

Androgen deprivation therapy (ADT) is a standard treatment for advanced prostate cancer (Smith, 2007). ADT can be achieved by either bilateral orchiectomy or chemical castration with various anti-androgens, luteinizing hormone-releasing hormone (LHRH) antagonists, or more commonly with LHRH agonists. Sexual dysfunctions, such as loss of libido and erectile dysfunction, are two of the most common side effects of ADT (Higano, 2006), which reduce the quality of life of the patients and their partners.

Wibowo *et al*. (2011; Wibowo et al., 2012b; Wibowo and Wassersug, 2013a) reviewed reports on the ability of E to elevate libido for a wide variety of castrated animals and humans treated with exogenous estrogen (E) and found that the degree to which E could restore sexual motivation varied greatly among the different studies. Factors that could contribute to the variation include the timing of E administration, the type and dose of E used, age of castration, and species difference. In this study, we hypothesized that early rather than late E administration after androgen deprivation (castration) will preserve libido better in male rats.

Our study is based on the "Critical Period Hypothesis" that concerns the effect of the timing of E treatment on the sexual behaviour (Damassa and Davidson, 1973; Clark et al., 1981; Czaja and Butera, 1985), cognitive function (Sherwin, 2009; Daniel and Bohacek, 2010; Rocca et al., 2010), and risks for cardiovascular diseases (Scott et al., 2012) in hypogonadal females. According to this hypothesis, E administration must be initiated within a critical time period following the loss of ovarian function in order to maximize its positive physiological effects. Currently some prostate cancer patients receive supplementary E to alleviate the hot flashes they experience when on ADT (Guise et al., 2007). However, patients start E therapy at various times following the initiation of ADT; i.e., some start early, while others may start after years of ADT.

Here we compared the effectiveness of initiating estradiol (E2) treatment at different delays post-castration in restoring sexual motivation of castrated male rats. Specifically,

we treated castrated male rats with either oil vehicle (as a control) or E2 dissolved in oil at one of the three intervals after castration: immediately, one month later and three months later. In each treatment group the rats' sexual behaviour was assessed two weeks after E2 or oil treatment.

2.2 Materials and Methods

2.2.1 Animals

Sexually-naïve adult male Long-Evans rats (Charles River Canada, St. Constant, QC, Canada), weighing 275-300 g at the time of arrival, were singly housed under a reversed 14/10 h light/dark cycle (lights on at 7:30 PM) at 23 ± 1ºC ambient temperature, with rat chow and water available *ad libitum*. Animal handling protocols followed the guidelines of the Canadian Council on Animal Care and were approved by the Dalhousie University Committee on Laboratory Animals.

Each rat was screened for sexual behaviour with an estrous female rat once a week for 4 weeks beginning one week after arrival. Those that ejaculated ≤1 or ≥4 times in a 30-minute testing period were considered hypo- or hypersexual (Olivier et al., 2006). In order for us to detect a change in sexual behaviour from baseline levels after E treatment, only rats that showed 2 or 3 ejaculations during their fourth screening test were included in this study. After the fourth sexual experience, these rats were surgically castrated as described below.

2.2.2 Surgery and Oil/Estradiol Administration

Orchiectomy was conducted through bilateral scrotal incisions under anesthesia with a combination of 104 mg/kg ketamine, 4.8 mg/kg xylazine, and 0.9 mg/kg acepromazine (i.p.) as previously described in Wibowo et al. (2012a). After surgery, rats were given the analgesic Ketoprofen (5 mg/kg) and the antibiotic Baytril (5 mg/kg) through subcutaneous injections. All rats were monitored as they emerged from anesthesia. They

were then returned to the colony for further recovery. After castration, the rats had no further access to female rats until behavioural testing at two weeks after Silastic tube implantation.

Rats were randomly assigned to either the oil or the E2 (dissolved in oil) treatment group (n = 24 per group). In each treatment group, animals were further subdivided into 3 groups (n = 8 per group) according to when the treatment was started after castration (Figure 2.1): immediately (Immediate), after one month lag (Short-Term), or after 3 months lag (Long-Term).

In the Immediate groups, immediately following gonadectomy and during the same surgery, a Silastic implant (1.6 mm inner diameter, 3.2 mm outer diameter; 35 mm in length; Dow Corning Corporation, Midland, MI) was inserted subcutaneously on the dorsum of the rats. The implant contained either sesame oil (60 μL; Catalog No. S3547, Sigma-Aldrich, St Louis, MO) for oil groups, or 230 μg of 17β-E2 (Catalog No. E8875; Sigma-Aldrich) in 60 μL sesame oil for the E2 groups. In our previous study (Wibowo et al., 2012a), this E2 dose increased the plasma E2 to levels that are similar to those observed in female rats during proestrus.

For the Short-Term and Long-Term delayed E2 administration groups, the Silastic tube was implanted 4 weeks and 12 weeks after castration, respectively. This procedure was done under isoflurane anesthesia [4% for induction, 2% for maintenance; 1L/min] mixed with oxygen. The size of the Silastic tube and the dose of E2 were the same as the ones used for the Immediate group.

For the final behaviour test, each male rat was paired with an ovariectomized female rat made sexually receptive by subcutaneous injections of estradiol benzoate (EB, 20 μg) and progesterone (500 μg) at 48 hours and 4 hours respectively before each testing.

2.2.3 Sexual Behaviour Testing

All sexual behaviour testing was done under dim red light at 4-7 hours after lights off. Five minutes before each test, a male rat was placed inside the testing cage [a terrarium (50 cm X 26 cm X 30 cm)] for habituation. An estrous-induced female rat was introduced at the end of the habituation period. Sexual receptivity of the female was checked by introducing a stud male into her home cage prior to the test. Only females that showed lordosis when mounted by the stud male were used for the test. Each copulatory behaviour test lasted for 30 minutes and the testing was recorded with a digital camera (Flip Video Ultra, Cisco).

2.2.4 Blood Collection and Radioimmunoassay for Estradiol

After the final behaviour test (at two weeks after Silastic tube implantation), each rat was euthanized 1 hour after the end of testing by anesthetic overdose (208 mg/kg ketamine, 9.6 mg/kg xylazine, and 1.8 mg/kg acepromazine, i.p.). A blood sample was collected through cardiac puncture with a heparinized syringe and was then centrifuged at 3000 rpm for 10 minutes. The plasma was collected and stored at -80°C for later radioimmunoassay.

The plasma E2 levels were determined by using commercial kits (DSL 4800 Ultra-Sensitive Estradiol RIA kit; Immunotech, Prague, Czech Republic) with a detection limit of 3.5 pg/mL. The sample from each animal was assayed in duplicate (intra-assay coefficient of variation = 6.0%) and all assays were conducted in a single session.

2.2.5 Data Analyses

To analyze sexual behaviour, each video recording was played using Windows Media Player, and the sexual behaviour of the male rats was scored with the aid of Etholog freeware version 2.2.5 (Ottoni, 2000) run on Windows XP. We analyzed the frequencies of genital sniffing, mounting, intromission, and ejaculation as described in (Agmo, 1997).

[We also measured the latency for each behaviour but there was no significant difference between treatment groups (Table 2.2).]

For statistical analysis, Prism 4.03 (GraphPad Software, San Diego, CA), Statview 5.0 (SAS Institute Inc., Cary, NC), and SPSS 17.0 (SPSS Inc, Chicago, IL) were used. All sexual behaviour parameters were analyzed with the Mann-Whitney and Kruskal-Wallis tests, unless specified, followed by Dunn post hoc test when statistical significance was reached. The percentages of rats showing each sexual behaviour were compared using Fisher's exact test. Paired comparisons in sexual behaviour parameters between pre-castration and at 2 weeks after treatment, as well as the behaviour frequency between the first and second half of the 30-minute testing period, were evaluated using Wilcoxon signed-rank test. The relationship between body weight change and total mounting frequency was analyzed using Spearman's correlation. Probabilities of < 0.05 were considered statistically significant.

2.3 RESULTS

2.3.1 PLASMA ESTRADIOL

The plasma E2 levels in the blood samples drawn after the final behavioural test were not significantly different from each other in either the three oil (control) groups or the three E2 groups (Table 2.1). However, all three E2 groups had higher plasma E2 levels than their corresponding oil groups (Table 2.1, Mann-Whitney test, all $P < 0.01$).

2.3.2 BODY WEIGHT CHANGE

All rats had similar body weights at the time of castration and, if they did not receive E2, they subsequently gained weight, mostly in the following 2 weeks. Despite these changes in weight, both the oil and E2 implanted rats had similar body weights at the time of Silastic tube implantation regardless whether they were in the Short-Term or Long-Term group (Table 2.1).

We analyzed the change in body weight from the day of Silastic tube implantation to the day of euthanasia after the final behavioural test (2 weeks later). Among the oil-treated groups, weight gain was greater in the Immediate oil group than in the Short-Term and Long-Term groups (Kruskal-Wallis test, $H = 14.9$, $P < 0.001$; Immediate > Short-Term, Long-Term, both $P < 0.01$, Figure 2.2). In contrast, all E2-treated groups lost weight, and the Long-Term group lost more body weight than the Short-Term group (Kruskal-Wallis test, $H = 9.3$, $P < 0.01$; Short-Term > Long-Term, $P < 0.01$).

2.3.3 Effects of the Timing of E2 Treatments on the Percentage of Rats Showing Specific Sexual Behaviours

We calculated the percentage of rats that showed each mating related behaviour at least once during the final test. All of these rats showed each of these behaviours before castration.

Among the oil groups, the number of rats showing mounting decreased over time, i.e., 7 (87.5%) in the Immediate, 5 (62.5%) in the Short-Term and 3 (37.5%) in the Long-Term groups (Table 2.1). In contrast, 7-8 (87.5-100%) of the E2-treated rats displayed mounting at any time post-castration. More E2-treated rats showed mounting than the oil-treated rats regardless of the delay between castration and E2 administration; with significant differences between the Long-Term groups (Fisher's exact test, $P < 0.05$, Table 2.1).

Among the oil groups, 7 (87.5%) of the Immediate group rats showed at least one intromission in their encounter with the female but this percentage gradually declined with the increasing time since castration, as observed in the other two groups [3 (37.5%) for Short-Term and 1 (12.5%) for Long-Term oil groups], with a significant difference between the Immediate and Long-Term groups (Fisher's exact test, $P < 0.05$, Table 2.1). In each of the E2 groups, 4-6 (50-75%) of the rats displayed intromission at least once during the final behavioural testing session. Though not statistically significant, fewer rats intromitted in the Immediate E2 group than the Immediate oil group, whereas more

rats intromitted in Short-Term and Long-Term E2 groups than the corresponding oil groups.

The number of rats that showed at least one ejaculation during the final test declined in all groups. Two rats (25%) ejaculated in the Immediate oil group but none in the Short or Long-Term groups (Table 2.1). In contrast, three rats (37.5%) ejaculated in Immediate E2 group, two (25%) in Short-Term group and none in Long-Term group.

2.3.4 Comparison of Sexual Behaviour Frequencies before Castration vs. after E2 Treatment

During the final test all of the rats in both the E2 and oil (control) groups (regardless of the delay between castration and treatment) showed an increased frequency of genital sniffing (average increase = 781%) and decreased intromission frequency (average decrease = 80%), (Wilcoxon signed-rank test, all $P < 0.05$, Figure 2.3A-C; G-I).

The mounting frequencies of the rats in both the Immediate oil and E2 groups did not differ from pre-castration levels (Figure 2.3D). There was no significant difference between the total mounting frequency of the oil and E2 groups. However, mounting frequencies of the Short-Term and Long-Term oil groups were significantly lower at two weeks following treatment compared to before castration (Figure 2.3E,F, Wilcoxon signed-rank test, both $P < 0.05$). The final mounting frequencies of both the Short-Term and Long-Term groups were lower than the Immediate oil group (Kruskal-Wallis test, $H = 11.1$, $P < 0.01$; Immediate > Short-Term and Long-Term, $P < 0.05$ and $P < 0.01$ respectively); suggesting that without the add-back gonadal steroid, mounting frequency progressively declined over time for male rats after castration.

Two weeks following post-castration treatments, unlike the oil groups, the total mounting frequencies of the Short-Term and Long-Term E2 groups were comparable to those of the intact rats (Figure 2.3E,F). The mounting frequencies of both the Short and Long-Term E2 groups were significantly higher than those of their corresponding oil groups

[Mann-Whitney test, both $P < 0.05$]; suggesting that E2 helped restore mounting behaviour for castrated male rats, even when the treatment is delayed for as much as three months.

2.3.5 Percentage Changes in Sexual Behaviour Parameters

We analyzed the percentage change in each sexual behaviour from intact levels to the levels at two weeks after treatment (i.e., during the final testing session). The percentage changes in genital sniffing and intromission frequencies were not significantly different between treatment groups or the post-castration intervals to the onset of treatment (data not shown).

During the final test the mounting frequencies declined significantly from intact levels in the Short-Term and Long-Term oil groups, compared to the Immediate oil group (Kruskal-Wallis test, $H = 11.0$, $P < 0.01$; Immediate > Short-Term and Long-Term, $P < 0.05$ and $P < 0.01$ respectively, Figure 2.4). Mounting frequencies still decreased in Short-Term and Long-Term E2 groups but the decline was less than in the corresponding oil groups (Mann-Whitney test, $P < 0.05$ and $P < 0.01$ respectively), further confirming that E2 helps dampen the castration-induced decline in mounting frequency.

2.3.6 Changes in Sexual Behaviour Frequencies during Behavioural Testing

Here, we analyzed the changes in behaviour during the first and last 15 minutes of the half hour long final test between each castrated male and a receptive female. Our baseline data on intact individuals included all males tested prior to castration. Intact rats normally show low genital sniffing frequency but high rates of mounting and intromission. As shown in Figure 2.5A,D,G, all of these sexual behaviours for intact rats were higher in the first than in the second half of the testing period (Wilcoxon signed-rank test, $P < 0.001$ for genital sniffing and mounting; $P < 0.0001$ for intromission).

As previously mentioned, total genital sniffing frequency increased after castration (Figure 2.4A-C). In both the Immediate groups, this elevation of genital sniffing frequency was prominent in the first 15-min but declined significantly over the half hour, with a mean decrease of 49% for oil and 73% for the E2 groups (Wilcoxon signed-rank test, $P < 0.05$ for oil and $P < 0.01$ for E2 group, Figure 2.5A). Similar pattern was observed in the Short-Term groups with declines of 60% for oil- and 53% for the E2-treated rats between the first and second half of the final test (Wilcoxon signed-rank test, $P < 0.05$ for oil and $P < 0.01$ for E2 group, Figure 2.5B). However, among the Long-Term groups, the elevated genital sniffing frequency only decreased significantly (by 74%) across the testing period in the oil group (Wilcoxon signed-rank test, $P < 0.01$) but not in the E2 group (Figure 2.5C).

Between treatments, the genital sniffing frequency was higher during the first half of the testing period in the Long-Term oil group compared to the Long-Term E2 group (Mann-Whitney, $P < 0.05$) (Figure 2.5C). No other difference between treatments was observed among the Immediate or Short-Term groups.

When analyzed across the testing period, the mounting frequency of the Immediate E2 group declined significantly (by 57%) from the first to the second half of the testing period (Wilcoxon signed-rank test, $P < 0.01$, Figure 2.5D) but not in the Immediate oil group. Mounting frequencies of the Immediate groups were not significantly different between treatments in either the first or second 15-min period. In contrast, in both Short-Term groups, mounting frequency declined significantly (by 98% for oil; by 55% for E2 groups) from the first to the second half of the testing period (Wilcoxon signed-rank test, both $P < 0.05$, Figure 2.5E). In addition, in both the first and second 15 min periods, mounting frequency was higher in the Short-Term E2 group than the Short-Term oil group (Mann-Whitney test, both $P < 0.05$). Like the Immediate E2 group, mounting frequency only declined significantly (by 67%) across the testing period in the Long-Term E2 group (Wilcoxon signed-rank test, $P < 0.05$, Figure 2.5F) but not in the Long-Term oil group. As in the Short-Term groups, in both the first and second 15-min of the testing period, mounting frequency was higher in the E2 group than the oil group (Mann-

Whitney test, $P < 0.01$ in the first half and $P < 0.05$ in the second half). In sum, E2 treatment helps maintain mounting behaviour for castrated male rats during staged-mating encounters, even when the E2 was administered three months after castration.

In the first 15-min of the testing, mounting frequencies were significantly lower in the Long-Term oil group than the Immediate oil group (Kruskal Wallis, $H = 6.6$, $P < 0.05$; Immediate > Long-Term, $P < 0.05$, Figure 2.5D,F). In contrast, in the second 15-min of the testing, mounting frequencies were significantly lower in the Short-Term and Long-Term oil groups than in the Immediate oil group (Kruskal Wallis, $H = 16.5$, $P < 0.001$; Immediate > Short-Term and Immediate > Long-Term, $P < 0.01$ and $P < 0.001$ respectively, Figure 2.5D-F). This suggests that not only the ability to start, but also to maintain, mounting behaviour diminishes after prolonged castration.

Among the oil-treated groups, intromission frequency was similar between the first and second 15-min. However, intromission frequency declined significantly across the testing period in the Short- and Long-Term E2 groups (Wilcoxon signed-rank test, $P < 0.05$ for both groups), but not in the Immediate E2 group. In addition, in the second 15-min, intromission frequency was lower in Short- and Long-Term oil groups than the Immediate oil group (Kruskal-Wallis, $H = 9.1$, $P < 0.05$; Immediate > Short-Term and Immediate > Long-Term, $P < 0.05$ for both, Figure 2.5G-I).

2.3.7 Correlation between Body Weight Change and Mounting Frequency

In the Short-Term E2 group, body weight loss from Silastic tube implantation to the day of sacrifice (2 weeks later) was positively correlated with mounting frequency ($\rho = -0.762$, $P < 0.05$, Figure 2.6B). There was no significant correlation between body weight change and mounting frequency in the other groups, although it approached significance in the Immediate oil group, in which lower body weight gain showed a trend toward a positive correlation with mounting frequency ($\rho = -0.667$, $P < 0.08$, Figure 2.6A).

Figure 2.1. Experimental protocol for implantation of E capsules and sexual behaviour testing in castrated male rats. “Silastic Implant” refers to the implantation in the castrated rats of a silastic tube filled with either oil alone as a control or estradiol dissolved in oil. See Methods for additional details.

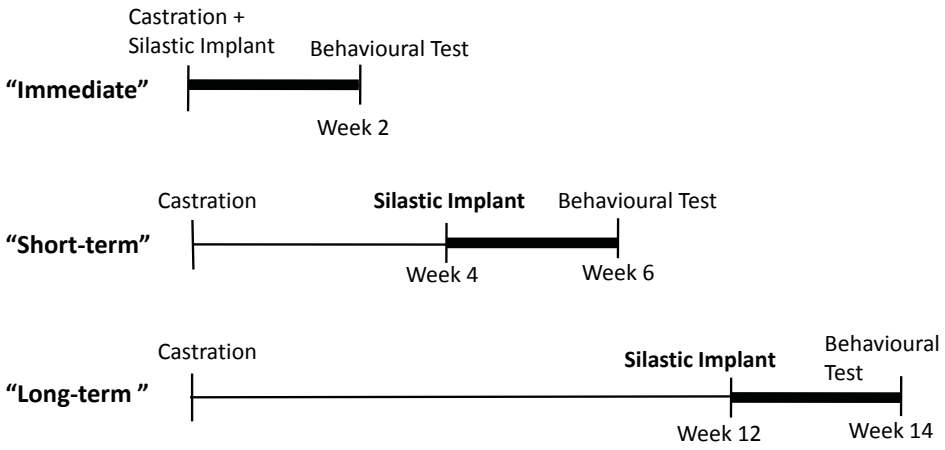

Castration +
Silastic Implant
Behavioural Test
"Immediate"
Week 2
Castration
Silastic Implant
Behavioural Test
"Short-term"
Week 4
Week 6
Castration
Silastic Implant
Behavioural
Test
"Long-term "
Week 12
Week 14

Figure 2.2. Changes in body weight (in grams) of castrated male rats treated for two weeks with oil (grey) or estradiol dissolved in oil (E2, white) immediately (Immediate, left), one month (Short-Term, middle), and 3 months (Long-Term, right) after castration (n = 8 per group). One outlier is indicated as a black dot, but it is included in the statistical analysis. The Short-Term and Long-Term oil-treated rats had smaller gain in body weight than the Immediate oil group. Among the E2 groups, the Long-term group lost significantly more body weight than the Short-Term group. (Kruskal Wallis test followed by Dunn post hoc comparisons for differences between groups of the same treatment; Mann-Whitney test for comparisons between treatment groups). * indicates significant difference from each other. In this and subsequent figures, * or # = $P < 0.05$, ** or ## = $P < 0.01$, *** or ### = $P < 0.001$, and **** = $P < 0.0001$.

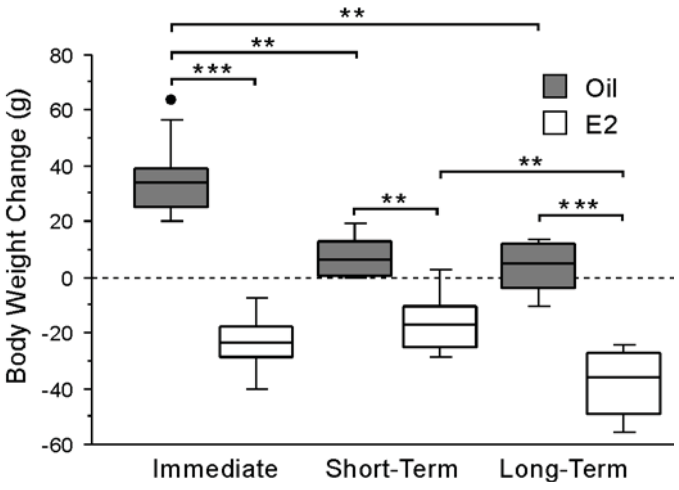

**
**
***
Oil
E2
**
**
***
80
60
40
20
0
-20
-40
-60
Body Weight Change (g)
Immediate
Short-Term
Long-Term

Figure 2.3. The total frequency of genital sniffing (A-C), mounting (D-F), and intromission (G-I) of male rats in a 30-minute testing period before castration (hatched) and after castration followed by two weeks of oil or E2 treatment (dotted) given immediately (Immediate, A,D,G), a month (Short-Term, B,E,H) and three months (Long-Term, C,F,I) following castration (n = 8 per group). For each graph, the two left boxplots represent the oil groups and the two on the right represent the E2 groups. Outliers are indicated with black dots, but were included in the statistical analyses. All rats had significantly more genital sniffing (A-C) and fewer intromissions (G-I) at two weeks after treatments post-castration than before castration regardless of treatment or the onset of the treatment. In both Short-Term and Long-Term oil groups, mounting frequency declined after castration (E,F). In contrast, all E2 groups displayed significantly more mounting than Short-Term and Long-Term oil-treated groups. (G-I) The Long-Term oil groups had significantly fewer intromissions than the Immediate oil group. See Fig. 3.2 caption for interpretation of * and # symbols. (Wilcoxon signed-rank test for comparisons before castration and after post-castration treatment; Mann-Whitney test for comparisons between treatments; Kruskal-Wallis test followed by Dunn post hoc comparisons for differences between groups of the same treatment). * indicates significant difference from each other and # indicates significant difference from Immediate control group.

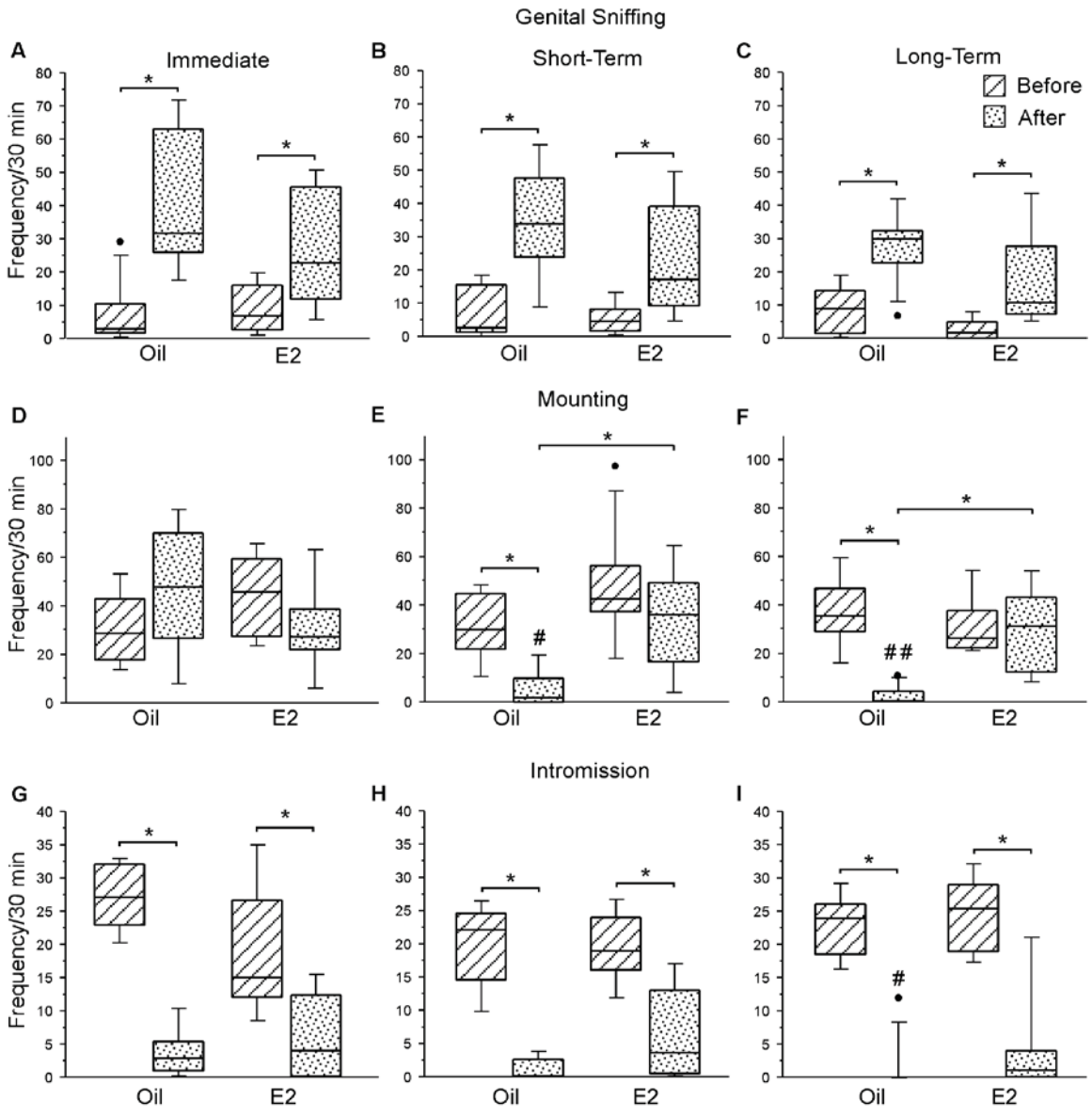

Genital Sniffing
A
Immediate
B
Short-Term
C
Long-Term
Before
After
Frequency/30 min
Oil
E2
D
Mounting
E
F
G
Intromission
H
I
*
#
##

Figure 2.4. Percentage change in mounting frequency of castrated male rats from pre-castration period to the testing time at two weeks after oil (grey) or E2 (white) treatment immediately (Immediate, left), one month (Short-Term, middle) and three months (Long-Term, right) (n = 8 per group). Outliers are indicated as the black dots; but were not excluded from the statistical analysis. Mounting frequency was significantly reduced to minimum in Short-Term and Long-Term oil groups. E2-treated rats showed less decrease in mounting frequency than the oil rats in both Short-Term and Long-Term groups. See Fig. 3.2 caption for interpretation of * symbol. (Kruskal Wallis test followed by Dunn post hoc comparisons for differences between groups of the same treatment; Mann-Whitney test for comparisons between treatment groups). * indicates significant difference from each other.

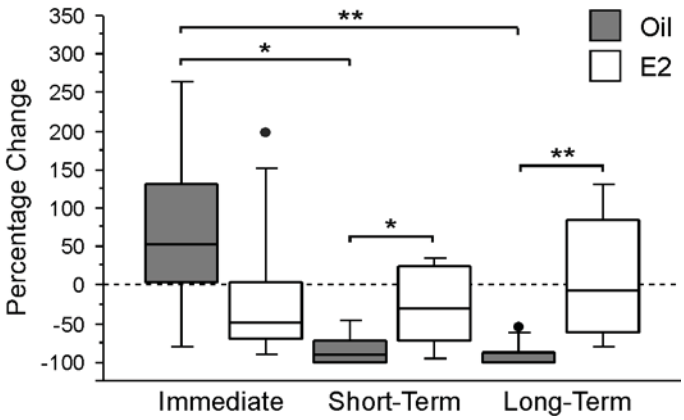

Oil
E2
Percentage Change
350
300
250
200
150
100
50
0
-50
-100
**
*
*
**
Immediate
Short-Term
Long-Term

Figure 2.5. Time course of genital sniffing (top row), mounting (middle row), and intromission (bottom row) frequencies of castrated male rats in 15-min bins during 30-min testing period before castration (Intact, dotted) and at two weeks after oil (grey) or E2 (white) treatment immediately (Immediate, A,D,G), one month (Short-Term, B,E,H), and three months (Long-Term, C,F,I) after castration (n = 8 per group). Outliers are indicated as black dots. Prior to castration, all sexual behaviour declined in the second half of the testing period (A,D,G). Similarly, after castration, in all cases except for Long-Term E2 group, genital sniffing frequency declined significantly in the second half of the testing period (A-C). (C) Long-Term E2 group had less genital sniffing frequency than the Long-Term oil group in the first 15-min. All E2 groups had significantly less mounting in the second half of the testing period than the first half whereas among the oil groups, this was only the case in the Short-Term oil group (D-F). E2 groups displayed more mounting than oil groups at all time-points in both Short-Term and Long-Term groups. Intromission frequency was significantly lower in the second half of the staged-mating encounter only in Short- and Long-Term E2 groups (H,I). See Fig. 3.2 caption for interpretation of * and # symbols. (Wilcoxon signed-rank test for differences between the first and second half of the testing period; Kruskal Wallis test followed by Dunn post hoc comparisons for differences between groups of the same treatment; Mann-Whitney test for comparisons between treatment groups). * indicates significant difference from each other and # indicates significant difference from Immediate control group.

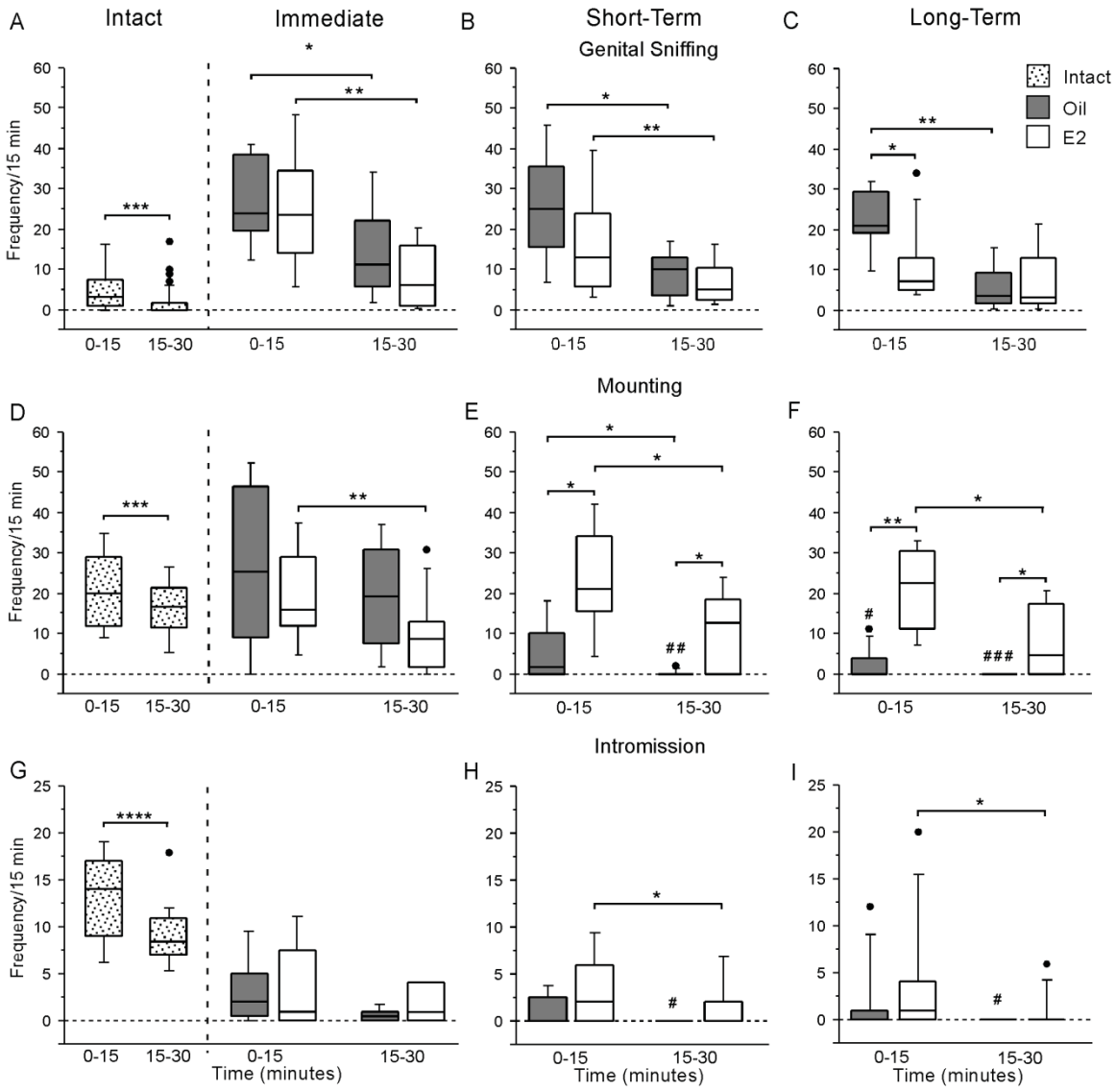

A
Intact
Immediate
B
Short-Term
C
Long-Term
Genital Sniffing
Intact
Oil
E2
Frequency/15 min
0-15
15-30
D
E
F
Mounting
G
H
I
Intromission
Time (minutes)

Figure 2.6. Scatterplots of body weight change (%) and total mounting frequency of castrated male rats at two weeks after oil (grey) or E2 (white) treatment immediately (Immediate), one month (Short-Term), and three months (Long-Term) after castration (n = 8 per group). (B) The percent change in body weight was negatively correlated with mounting frequency only in the Short-Term E2 group. NS = no significant correlation between body weight and mounting frequency.

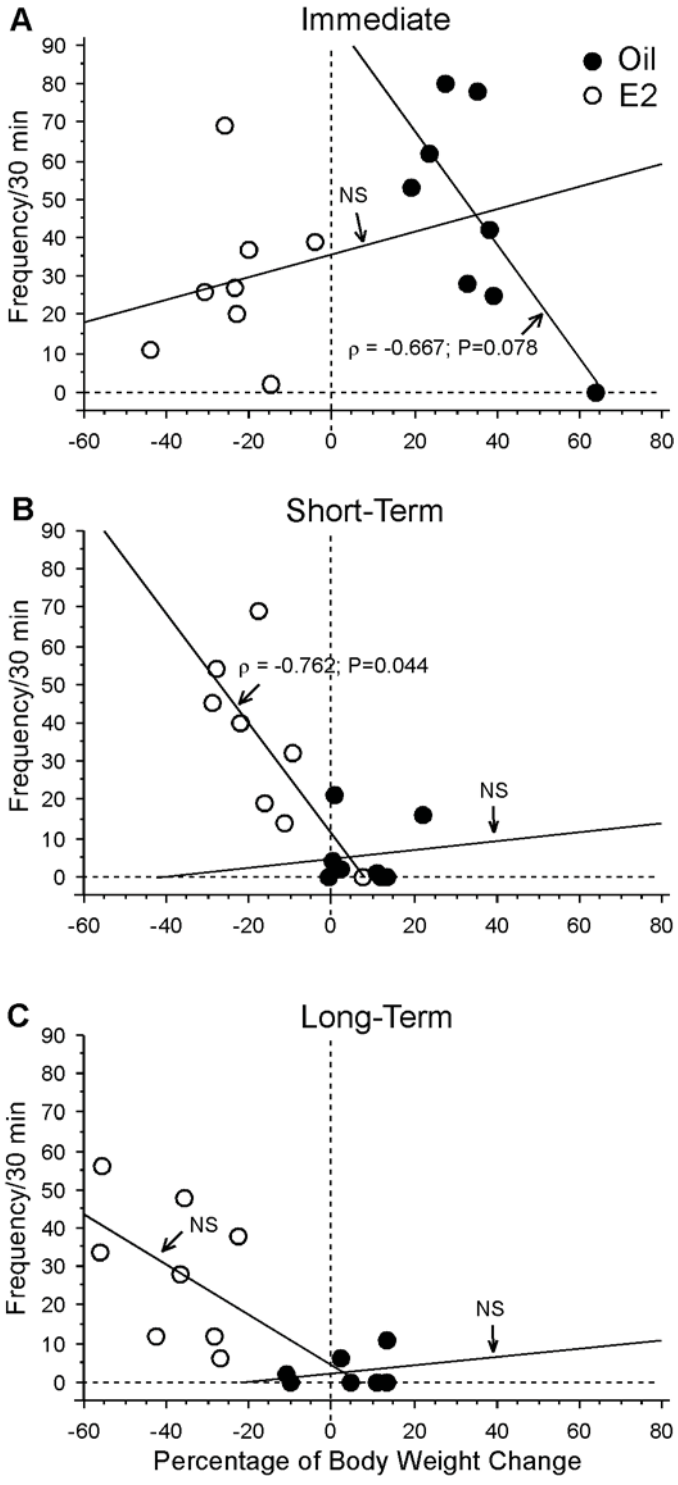

A
Immediate
Oil
E2
Frequency/30 min
NS
ρ = -0.667; P=0.078
B
Short-Term
ρ = -0.762; P=0.044
NS
C
Long-Term
NS
NS
Percentage of Body Weight Change

Table 2.1. Average body weights, plasma estradiol (E2), and the number of rats displaying at least one behaviour in the final test (n = 8 per group). Oil is the control in this and the next table.

	Immediate		Short Term		Long Term	
	Oil	E2	Oil	E2	Oil	E2
Plasma E2 levels (pg/mL)	7.2 (2.7)[a]	40.8 (42.1)[a]	4.7 (6.5)[b]	23.2 (23.1)[b]	7.7 (4.7)[c]	46.7 (23.6)[c]
Body Weight						
At Implantation	443.44	427.78	533.99	534.30	612.75	586.85
At Sacrifice	478.40	407.41	541.68	518.49	616.33	548.81
Behaviour						
Genital sniffing	8	8	8	8	8	8
Mounting	7	8	5	7	3[d]	8[d]
Intromission	7[e]	4	3	6	1[e]	5
Ejaculation	2	3	0	2	0	0

The median and interquartile range are given for the plasma E2 data. Same letters are significantly different from each other (Mann-Whitney test for plasma E2 levels and Fisher's exact test for mounting and intromission; $P < 0.05$ for all).

Table 2.2. Latencies (s) to the first mounting, intromission, and ejaculation behaviours (means ± SD) of male rats measured before castration (intact), as well as at two weeks after receiving either Oil (as a control) or E2 in oil immediately, one month (Short-Term), and three months (Long-Term) post-castration. Although there were 8 rats per group, not all animals showed all four behaviours; therefore, the data presented in each group are the averages from rats that showed each specific behaviour. The number of animals that displayed each behaviour per group are shown on Table 2.1.

	Intact	Immediate		Short-Term		Long-Term	
		Oil	E2	Oil	E2	Oil	E2
Genital Sniffing	144.7 ± 372.4	6.0 ± 6.2	4.8 ± 5.3	11.2 ± 8.4	8.4 ± 8.4	11.9 ± 12.1	11.4 ± 6.6
Mounting	29.0 ± 34.6	147.3 ± 346.9	108.5 ± 165.4	198.4 ± 337.2	37.4 ± 31.2	144.5 ± 140.3	37.2 ± 35.8
Intromission	33.1 ± 48.7	170.7 ± 353.9	53.3 ± 28.5	172.4 ± 239.9	100.5 ± 61.4	96.4	23.4 ± 13.1
Ejaculation	393.5 ± 148.5	510.8 ± 150.7	423.7 ± 144.2	-	708.8 ± 41.4	-	-

Table 2.3. Summary table of changes in sexual behaviours of castrated male rats in relation to intact levels measured 2 weeks after either oil or E2 treatment. Arrows indicate direction of change in behaviour. No change is indicated with an equal sign.

Behaviour	Immediate		Short-Term		Long-Term	
	Oil	E2	Oil	E2	Oil	E2
Ejaculation	↓	↓	↓	↓	↓	↓
Intromission	↓	↓	↓	↓	↓	↓
Mounting	=	=	↓	=	↓	=
Genital Sniffing	↑	↑	↑	↑	↑	↑

2.4 Discussion

As expected, castration altered male sexual behaviour (Table 2.3). Regardless of whether the rats were treated immediately, 1 month, or 3 months after castration, intromission frequency was markedly reduced while genital sniffing frequency increased. E2 treatment did not reverse these changes. Mounting frequency, another measure of sexual behaviour, was not significantly altered 2 weeks after castration but showed a decrease 1 and 3 months later. These declines in mounting frequency, however, were responsive to exogenous E2 administration, and E2 restored mounting frequency to similar levels when administered one or three months after castration. This finding shows that the restoration of mounting behaviour by E2 does not comply with the Critical Period Hypothesis; i.e., under the experimental conditions used in the present study, the time lag from castration to the initiation of E2 treatment does not affect how much sexual motivation can be restored in rats.

2.4.1 Comparison to Previous Studies

Several studies have reported that E can increase mounting in castrated males of different vertebrate species, including rats (Wibowo et al., 2012b; Wibowo and Wassersug, 2013a). Various intervals between castration and the initiation of E administration, however, were used in those studies. The percentages of our Immediate and Short-Term rats showing ejaculation (25-37.5 %) after two weeks of E2 treatment are similar to previous findings for E-treated immediate (Davidson, 1969) and 6-7 weeks castrates (Södersten, 1973; Södersten and Larsson, 1975). Similarly, the percentage of oil-treated rats ejaculating is similar to a previous study (Davidson, 1966). To the best of our knowledge, our study is the first with rodents to explore intervals as long as 3 months between castration and E2 administration. We acknowledge, though, that a 3-month interval is still a relatively short in light of the life span of the rat, which may be up to approximately three years.

2.4.2 Changes in Sexual Behaviour Parameters

In our study, intromission frequency decreased following castration and E2 did not reverse the effect. This may in part be explained by the fact that androgen-deprivation causes atrophy of the pelvic floor muscles that are important for erectile function; E2 cannot reverse this atrophy (Sengelaub and Forger, 2008; Wibowo and Wassersug, 2013a). However, in contrast to our findings, daily injection of a very high dose of estradiol benzoate (EB, $\geq$ 100 μg) reportedly restores intromission (Södersten, 1973; Holmes and Sachs, 1992), despite any atrophy of the pelvic floor. A possible mechanism to account for this restoration is E2's ability to preserve the electrophysiological property of the muscle despite atrophy (Holmes and Sachs, 1992; Fargo et al., 2003; Foster and Sengelaub, 2004). Differences in the route of administration, duration of E treatment, type and dose of E may also explain in part the different effects of E on intromission behaviour observed in the different studies.

The total mounting frequency of the Immediate groups was similar to the pre-castration level regardless of the hormonal treatment. As reviewed by Hull *et al.*(2006), in male mammals ejaculation diminishes first after castration, followed by a decline in intromission and lastly in mounting. Thus, as expected, at two weeks after castration (i.e., in our Immediate group), mounting behaviour had not been substantially affected by androgen deprivation, nor by E2 treatment. However, mounting frequency for the oil-treated rats was greatly reduced at 6 and 14 weeks after castration. These effects were even more conspicuous when we analyzed the behaviour in two 15-min periods. Short- and long-term androgen deprivation led to minimal mounting in the first 15-min of the testing period, and mounting almost completely disappeared in the second 15 min. This suggests that castration not only reduces sexual desire to initiate copulatory activity but also reduces the maintenance of sexual motivation during sexual encounters.

As with most sexual behaviour tested in laboratory setting, mounting frequency for the rats diminished over the time period during the staged encounter between the male and female rats. While short- and long-term oil-treated castrates showed minimal mounting in

the second 15-min period, E2 treatment restored mounting not just in the first but also in the second half of the testing period. This suggests that E2 does not only reinstate sexual motivation to a degree that is sufficient to initiate copulation, but also helps sustain the sex drive throughout a 30 min mating session.

As previously described (Shishkina et al., 2001), genital sniffing frequency increased after castration and may be viewed as a displacement activity or a minimal socio-sexual activity in the absence of any more explicit mating behaviours. Due to the decline in intromission, in the presence of an estrous female, castrated rats only either mount (which also diminishes over time after castration) and/or sniff her anogenital area. Like mounting frequency, genital sniffing frequency is higher in the first than in the second half of the testing period. In contrast to previous studies (Cross and Roselli, 1999; Roselli and Chambers, 1999), we found that E2 administered to castrated male rats did not elevate genital sniffing frequency. Instead, E2 decreased genital sniffing frequency in long-term castrates, particularly in the first half of the testing period. Differences in strains, treatment regimens, housing condition or testing protocol could be factors accounting for the differences among these studies.

2.4.3 The Timing of E2 Treatment does not Alter its Protective Effects on Sexual Behaviour

Contrary to the Critical Period Hypothesis, the lag between castration and the initiation of E2 treatment did not affect mounting frequency. Only one study to date has investigated the effect of timing of E administration on castrated males. Antliff and Young (1956) compared the sexual behaviour of castrated male guinea pigs that were treated with estrone at either 1 or 10 weeks post-castration and stated that "*the results* [from the 10-weeks castrates] *are so similar to those obtained from the* [1-week castrates] *that the graph and table… are not reproduced*." Although imprecise, this is concordant with our results. It should be noted, however, that Antliff and Young also tested α-estradiol benzoate, which failed to increase sexual motivation in the castrated male guinea pigs.

This finding suggests that the effectiveness of one estrogenic compound versus another in restoring sexual motivation may vary among different animal models.

Our findings show that early and late E treatment after gonadectomy produces similar effects on male sexual behaviour; however, this is in contrast to E's effect on female sexual behaviour. Delayed E administration after ovariectomy resulted in blunted responses in female sexual behaviour (as indicated by lordosis), compared to when E was started earlier after ovariectomy (Damassa and Davidson, 1973; Clark et al., 1981; Czaja and Butera, 1985). A similar result in lordosis behaviour has been observed in castrated male rats—intact male rats do not normally display lordosis, but after castration some males exhibit lordosis when treated with E (Sodersten and Larsson, 1974; Södersten and Larsson, 1975). In one study (Davidson, 1969), early EB treatment after castration in male rats resulted in high lordosis response, but the response was reduced when the same rats were re-treated with EB five weeks later. The differences in E's effect on mounting (typical male sex behaviour) and lordosis (typical female sex behaviour) may reflect the fact that masculinization and defeminization are regulated by different hypothalamic areas; i.e., mounting by the preoptic area (POA (Hull et al., 2006) and lordosis by the ventromedial nucleus (VMN, Flanagan-Cato, 2011).

A change in expression levels of estrogen receptors (ERs) in the hypothalamus after castration may explain why we failed to see a timing effect despite what would be expected from studies on E replacement for steroid-deprived females (Sherwin, 2009; Daniel and Bohacek, 2010; Rocca et al., 2010). Following gonadectomy in male rats, ER mRNA levels increase in the POA, and these increases are maintained for 2-4 months (Handa et al., 1996). In the same study, E2 administered to castrated male rats was found to down-regulate the ERs mRNA levels back to the gonad-intact level. Thus, this may explain the similar level of restoration of mounting behaviour that we observed achieved by E2 treatment regardless of the lag between castration and E2 administration. Currently, whether early versus delayed E2 treatment in castrated male rats modulates ERs' expressions differently in the VMN is not known. We note that varied timing of E administration in ovariectomized rats results in inconsistent changes in ER levels in

different brain areas; i.e., ERα levels in hippocampus increased only after immediate E2 treatment whereas in prefrontal cortex ERα is elevated only following delayed E2 treatment (Bohacek and Daniel, 2009). By extension, E2 treatment in castrated male rats may exert different modulatory effects in different hypothalamic areas.

In sum, the timing of the onset of E2 treatment after castration does not affect E2's ability to preserve or restore male mounting behaviour, but early rather than late E2 treatment post-castration may be superior in eliciting female-typical sexual behaviours. Future studies need to investigate the modulatory effects of E2 on the POA versus the VMN at various time points after gonadectomy in order to understand why the timing of E2 treatment is crucial for female-typical, but not for male-typical sexual behaviour.

2.4.4 Change in Plasma E2 Levels and Body Weights

Within two weeks of Silastic tube implantation, E2 dosing raised the plasma E2 levels in the castrated male rats to levels similar to those of female rats in a proestrous state. The plasma E2 levels remained low, however, in the castrated rats that only received the oil vehicle. All male rats treated with oil gained body weight within this two-week period. One or 3 months later, oil-treated castrates in contrast had lower body weight gain than immediately after castration and implantation. This is consistent with the natural plateau in body weight gain over time as observed for male rats (Gentry and Wade, 1976b). In contrast, as described in other studies in male (Gentry and Wade, 1976a; Kritzer et al., 2001; Turvin et al., 2007; Wibowo et al., 2012a) and female (Ke et al., 1997; Westerlind et al., 1998; Gogos and Van den Buuse, 2004) rats , E2 treatment reduces body weight of castrated male rats. The long-term castrates lost more body weight than short-term castrates following E2 administration. In addition, we found that in Short-Term E2-treated rats, there was a moderate correlation between body weight loss and mounting frequency; i.e., the greater the body weight loss, the higher the mounting frequency (Figure 2.6).

These weight changes may have implications to the long-term health of androgen-deprived males. Androgen-deprivation has been known to cause the metabolic syndrome in humans, consisting of poor lipid profiles, decreased lean body mass, elevated fat mass, and eventually increased body mass index (Braga-Basaria et al., 2006). In contrast, parenteral E2 therapy can reduce LDL cholesterol and inflammatory marker in prostate cancer patients on ADT (Purnell et al., 2006). Furthermore, Purnell et al. noted that there was no change in the lean body mass or fat mass before and after E2 treatment, suggesting that E2 may help dampen the increase in the body fat content in androgen-deprived males. These data from humans, though not identical to our rodent data (where E2 decreased body weight in the castrated rats), suggest that clinically, E2 may help prostate cancer patients maintain their weight and avoid the increase in body fat mass that typically occurs when they are on ADT (Braga-Basaria et al., 2006).

An unanticipated finding from our study is that the more weight that is lost the greater sexual motivation in short-term E2-treated castrated rats. Possibly, this correlation is due to the sensitivity of the rats to E2 treatment; i.e., rats that are more sensitive to E2 lost more body weight and showed more mounting than those that have less sensitivity to E2.To the best of our knowledge, this association has not been investigated in humans. Our data suggest that future studies with patients starting on ADT should explore whether E2 helps them concurrently maintain both a good body mass index as well as their libido.

2.4.5 Clinical Implications

At any time in North America, over a half million men are on androgen depriving drugs as part of their treatment for prostate cancer (Smith, 2007). Loss of libido in patients on ADT not only burdens the patients, but also their partners (Elliott et al., 2010). Even in the absence of coital sex, the loss of libido by the male can lead to a feeling of abandonment by their partners (Navon and Morag, 2003; Soloway et al., 2005; Kim et al., 2008; Walker and Robinson, 2011). Our study is relevant to helping these patients and their partners adapted to the side effects of ADT.

We confirmed that supplemental E2 increased mounting behaviour in rats—a proxy for libido in adult human males. Although the potential for E to raise libido above castrate levels was previously suggested for various mammalian species (Wibowo et al., 2011), our results document this rigorously with concomitant data on plasma E2 concentrations. Our results confirm that E2 not only may help preserve some libido, but that this can be achieved with the primary form of E in human males, and at physiologically normal levels. Although some studies with androgen-deprived men have independently suggested that E may help preserve sexual interest in humans ((Ellis and Grayhack, 1963; Davidson et al., 1983; Bergman et al., 1984; Brett et al., 2007; Wassersug and Gray, 2011) reviewed in (Wibowo et al., 2011; Wibowo et al., 2012b; Wibowo and Wassersug, 2013a)), there is only a single case study that explored this in a blinded fashion to control for placebo effects (Davidson et al., 1983).

Supplemental E2 treatment is not commonly offered to androgen-deprived prostate cancer patients. In part, this is due to the high thromboembolic risk with oral E, but this can be circumvented by parenteral administration (Ockrim et al., 2005; Hedlund et al., 2008; Schellhammer, 2012). Currently, when E2 is offered to patients on ADT, it is primarily in a transdermal form and used to alleviate severe hot flashes. Due to the limited use of E2 in men, the Critical Period Hypothesis on the effect of E2 on sexual interest after androgen deprivation has not been assessed. Thus, we do not know if the timing of E2 in androgen-deprived males would influence E2's ability to elevate libido in men. If humans respond as our male rats do, then supplemental E2 may sexually benefit them regardless of whether it is started simultaneously with ADT or some weeks to months later. However, early E2 supplementation may still benefit patients more than late E2 for traits not explored in our study. As mentioned earlier, E2 treatment to steroid-deprived females may preserve cognitive function better when administered in the perimenopausal period rather than years later (Sherwin, 2009; Daniel and Bohacek, 2010; Rocca et al., 2011). In addition, E2 also has other benefits for androgen-deprived men, such as in maintaining bone mineral density (Ockrim et al., 2004; Schellhammer, 2012).

E2's potential to restore libido may help improve the quality of life of androgen-deprived men, and that of their partners. Erectile capability may not be restored by E2 administration, but preserving sexual drive could still be vital in maintaining patient-partner intimacy of a non-coital nature.

2.5 CONCLUSIONS

Castration has detrimental effects on all parameters of male rat sexual behaviour. We show here that E2, whether administered one or three months after castration, can increase mounting behaviour equally well and significantly above the level shown by castrated rats without E2 treatment. Though this does not support the Critical Period Hypothesis, clinically, this suggests that E2 may potentially benefit not just patients starting on androgen deprivation but those who have been on ADT for some length of time. Given the detrimental impact that loss of libido has to not only prostate cancer patients but also their partners (Elliott et al., 2010), our study bolsters the rationale for clinical trials to determine if supplemental E2 can improve the quality of life for prostate cancer patients on ADT.

Chapter 3: REGULATION OF ESTROGEN RECEPTORS AND C-FOS BY ESTRADIOL IN BRAIN AREAS RELATED TO MALE SEXUAL BEHAVIOUR

Abstract

Male copulatory behaviour declines after castration. In our previous study, we treated castrated male rats with either oil as a control or E2 in oil, and varied the interval between castration and treatment, and found that estradiol (E2) can restore mounting behaviour in castrated male rats regardless of when the treatment is started after castration. The aim of the present study was to investigate the mechanisms by which E2 elevates mounting behaviour equally well in the short- and long-term castrates. We used Western blot analysis to examine estrogen receptor (ER) α, ERβ, and c-Fos protein levels in brain areas that control or modulate male sex behaviour, in those rats after their final sexual encounter. ERα levels in the preoptic area (POA) increased at three months after castration but decreased when castrated rats were treated with E2. Mating-induced c-Fos induction in the POA was similar regardless of the treatment or the timing of the onset of treatment after castration. The mounting frequency of rats treated with E2 one month after castration was negatively correlated with ERβ levels in the POA, but positively correlated with c-Fos levels in the core area of nucleus accumbens and the POA. Among rats that started E2 treatment 3 months post-castration, mounting frequency was positively correlated with ERβ levels in the medial amygdala. Collectively, E2 is likely to increase mounting by acting on the POA. Mounting is elevated to a similar level in short- and long-term castrates possibly because mating-induced c-Fos in the POA is not affected by E2 treatment or the duration of androgen deprivation.

Publication Information

This chapter is under review for publication at the journal "*Hormones and Behavior*" at the time of submission of this thesis. EW, RWC and RJW was involved in study design. EW and HJC ran the experiments and analyzed the data. RWC provided critical feedback on the experimental methods. EW made the first draft of the manuscript and all authors provide editorial inputs in the manuscript.

3.1 INTRODUCTION

Of various male sex hormones, testosterone is often considered as the main sex steroid that regulates sexual interest. Depriving men of androgens reduces libido (Corona et al., 2012), and testosterone supplementation to hypogonadal men can improve their sexual interest (Jacob, 2011). However, there are cases when testosterone replacement is not ideal for androgen-deprived castrated men, such as prostate cancer patients or male-to-female transsexuals. In those cases, their libido plummets in the absence of gonadal steroids (Higano, 2012).

Estrogen (E) has beneficial effects on maintaining sexual interest among castrated (i.e., androgen-deprived) male animals, including humans (Wibowo et al., 2012b; Wibowo and Wassersug, 2013a). In male rats, this is indicated by an increase in mounting behaviour (Pfaus et al., 2003). E exerts this behavioural effect by binding to estrogen receptors (ERs) in brain areas that control male sexual behaviour (Rissman, 2008). Several types of ERs have been identified, including the nuclear membrane receptors ERα and ERβ, and the membrane receptor GPR30 (Pak and Handa, 2008). ERα is thought to mediate male copulatory behaviour, but the role of ERβ in male sex behaviour remains unclear (Rissman, 2008). In response to E treatment, ERs autoregulate their expression to maintain normal physiological function (Bagamasbad and Denver, 2011).

Different brain regions regulate mounting behaviour as suggested by elevated expression, after sexual encounter, of c-Fos protein, the product of the immediate-early gene *c-fos*, which indicates neuronal activation (Robertson et al., 1991; Baum and Everitt, 1992). ERs are present in these same brain areas (Simerly et al., 1990; Shughrue and Merchenthaler, 2001). The preoptic area (POA) is one of these centers and is the main area that integrates inputs from other brain areas to activate male sex behaviour (Hull and Rodrigues-Manzo, 2010). The medial amygdala (MeA) also expresses ERs and processes chemosensory as well as somatosensory signals and transmits them, either through the bed nucleus of the stria terminalis (BNST) or directly, to the POA (Hull and Rodrigues-Manzo, 2010). Furthermore, sexual behaviour activates the dopaminergic mesolimbic

pathways that are involved in reward and motivational functions; increased activity in the mesolimbic system, in turn, activates the nucleus accumbens (NA), where ERs are present (Hull and Rodrigues-Manzo, 2010).

To investigate the issue of the timing of estrogen therapy after castration, in a previous study, we compared how early versus late estradiol (E2) administration after castration affects male rat sexual behaviour (Wibowo and Wassersug, 2013b). We found that E2 increased mounting behaviour equally well regardless of whether it was administered soon (i.e, 1 month) or long (i.e., 3 months) after castration. Thus, within that time frame the effect of E2 on mounting behaviour (as a measure of sexual motivation) was insensitive to when the treatment was started.

To follow up on our previous behavioural study (Wibowo and Wassersug, 2013b), we studied here how E2 treatment, and the interval from castration to E2 administration influence neuronal activation in brain areas that are involved in male sexual behaviour. We also examined how E2 affects the autoregulation of estrogen receptors (ERs) in brain areas associated with the control of male copulatory behaviour.

3.2 MATERIALS AND METHODS

3.2.1 ANIMALS

The tissues used in this study were collected from the rats used in our previous behavioural experiment, and the experimental design, surgery and treatment protocols were described previously (Wibowo and Wassersug, 2013b). In brief, adult male sexually-naïve Long-Evans rats (Charles River Canada, St. Constant, QC, Canada, 275-300 g at the time of arrival) were housed singly under a reversed 14:10 light:dark cycle (lights on at 7:30 PM) at 23 ± 1ºC ambient temperature, with food and water available *ad libitum*. Animal handling protocols followed the guidelines of the Canadian Council on Animal Care and were approved by the Dalhousie University Committee on Laboratory Animals.

Prior to castration, each rat received four weekly staged sexual encounters with an estrus female rat starting one week after arrival. Only male rats that ejaculated 2 or 3 times in their fourth sexual encounter were used to ensure that no hyper- or hypo-sexual rats were included. A total of 48 male rats met that inclusion criterion.

3.2.2 Surgery and Oil/Estradiol Administration

Male rats were subjected to bilateral scrotal orchiectomy under anesthesia (104 mg/kg ketamine, 4.8 mg/kg xylazine, and 0.9 mg/kg acepromazine, i.p) as previously described in Wibowo *et al*. (2012a). After surgery the rats were given an analgesic (Ketoprofen, 5 mg/kg, s.c) and an antibiotic (Baytril, 5 mg/kg, s.c), and returned to the animal care facility for recovery.

The castrated rats were randomly divided to receive either sesame oil (as control) or E2 (dissolved in sesame oil) treatment (n = 24 per treatment group). Animals in the same treatment group were further assigned randomly into 3 groups (n = 8 per group) according to the timing at which a Silastic tube (1.6 mm inner diameter, 3.2 mm outer diameter, 35 mm in length; Dow Corning Corporation, Midland, MI) was implanted: immediately (Immediate), one month (Short-Term), or 3 months (Long-Term) after castration. Each implant was filled with either sesame oil (60 μL; Catalog No. S3547, Sigma-Aldrich) for the oil groups, or 230 μg of 17β-E2 (Catalog No. E8875; Sigma-Aldrich) in 60 μL of sesame oil for the E2 groups. This E2 dose raised the plasma E2 level of the castrated male rats to levels similar to those of proestrus female rats (Wibowo et al., 2012a).

Rats in the Short-Term and Long-Term groups received their Silastic implants under isoflurane anesthesia (4% for induction, 2% for maintenance; 1 L/min) mixed with oxygen. The Silastic implant size, oil volume, and E2 dose were the same as those used with the Immediate groups. After castration, the males and females did not have further sexual encounters until the final behavioural test, which occurred two weeks after Silastic tube implantation.

3.2.3 Tissue Collection

Two weeks after Silastic tube implantation, each male rat was given an opportunity to mate with an estrus female rat, and their sexual behaviour was monitored (Wibowo and Wassersug, 2013b). We recorded the frequencies of mounting, intromission, and ejaculatory behaviours in each male (Wibowo and Wassersug, 2013b). One hour after testing, the male rat was killed by anesthetic overdose (208 mg/kg ketamine, 9.6 mg/kg xylazine, and 1.8 mg/kg acepromazine, i.p). The brain was quickly removed and frozen at -80°C.

Brains were cut into 300-µm thick sections using a cryostat (Leica CM1850) at temperatures between -7 and -10°C. Brain areas of interest were micropunched according to the techniques described by Palkovitz and Brownstein (1988). The bed nucleus of the stria terminalis (BNST), plus the shell (NAs) and core (NAc) regions of nucleus accumbens were sampled using a 1.0 mm Harris MicroPunch^{TM} (Catalog No. 69035-10, Electron Microscopy Sciences). The POA and MeA were sampled using a 0.5 mm Harris MicroPunch^{TM} (Catalog No. 69035-05, Electron Microscopy Sciences). Each brain tissue sample was immediately placed into either 25 µL (POA, MeA), or 40 µL (BNST, NAs, NAc) of homogenization buffer (0.32 M sucrose in 0.1 M phosphate-buffered saline with one complete mini protease inhibitor tablet (Catalog No. 04 693 124 001, Roche) per 10 mL. The locations for the micropunches are shown in Appendix C.

All tissues were homogenized for 2 minutes using a pellet pestle (Catalog No. Z359947, Sigma-Aldrich) and its motor (Catalog No. Z359971, Sigma-Aldrich), then centrifuged for 10 minutes at 13,000 X g in an IEC multi-RF centrifuge (Thermo Fisher Scientific). The supernatant was collected and protein determination was conducted using the Bio-Rad protein assay.

3.2.4 Western Blot

Aliquots of 15 µg (POA), or 20 µg (BNST, NAs, NAc, MeA) were mixed with Laemmli sample buffer (Catalog No. 161-0737, Bio-Rad) in a 1:1 ratio, and samples were heated at 95°C for 10 minutes. Protein samples and molecular ladder (LC5800, Novex® Sharp Pre-Stained Protein Standard, Invitrogen Life Technologies) were separated in 7.5% SDS-polyacrylamide gels for 20 minutes at 75 V, followed by 1 hour at 175 V. The gels were transferred using a multi-strip western blot technique as described by Aksamitiene et al. (2007). This technique allowed us to quantitatively compare samples from all animals on one membrane. Briefly, we cut each gel into two strips with the following protein ranges: one between 40-60 kDa and another between 60-80 kDa. The strips containing protein of the same molecular weight range were then assembled on a single filter paper and transferred to a PVDF membrane (IPVH00010, Millipore) for 4 hours at 100 V.

3.2.5 Immunolabeling

The membranes were blocked for 1 hour at room temperature in a 5% skim milk in Tris-buffered saline solution with 0.1% TWEEN® 20 (TBST). The membranes were then incubated in primary antibodies for two days at 4°C. Primary and secondary antibody incubation was done in the same TBST solution as described above. The information on the primary antibodies used and their concentrations is provided in Table 3.1. All our antibodies detected respective antigens within the expected molecular weight ranges. After washing in TBST (2 X 1 min, 2 X 15 min, 3 X 5 min), the membranes were incubated for 1 hour at room temperature in 1:10000 chicken anti-rabbit IgG-HRP (sc-2963, Santa Cruz Biotechnology). After secondary antibody incubation, the membranes were washed in TBST again (2 X 1 min, 2 X 15 min, 3 X 5 min). We used an ECL 2 kit (Product No. 80196, Pierce) and Typhoon 9410 scanner (Amersham Biosciences) to visualize the bands on all membranes.

3.2.6 Densitometry and Statistical Analyses

The density (RawIntDen value) of each protein band was measured using NIH ImageJ 1.46r. First, the density of each band (ERα, ERβ, c-Fos, and actin) was subtracted from the background density. To obtain a normalized band density, the ERα and ERβ bands were divided by the density of the loading control (actin).

Statistical analyses were conducted using Statview 5.0 (SAS Institute Inc., Cary, NC). Normalized density values were analyzed using Mann-Whitney and Kruskal-Wallis tests. We also performed Spearman's correlation tests to analyze how mounting in the final behavioural test correlated with the abundance of ERα, ERβ, and c-Fos protein in each of the five brain areas studied. Probabilities less than 0.05 were considered statistically significant.

3.3 Results

3.3.1 Changes in ERα, ERβ, and c-Fos Levels

Although levels of ERα, ERβ, and c-Fos were assessed in select brain areas, significant effects of E2 treatments were found only in several brain areas for certain molecules (Figure 3.1).

Among the Oil groups, ERα levels in the POA increased with prolonged androgen-deprivation, with the Immediate group having significantly lower levels than the Long-Term group (Kruskal-Wallis test, $H = 6.72$, $P < 0.05$; Immediate < Long-Term, $P < 0.05$: Figure 3.1A). In contrast, the ERα levels in the POA of the E2 treated rats decreased as the interval between castration and treatment onset increased, with the Immediate group having significantly higher levels than the Long-Term group (Kruskal-Wallis test, $H = 10.48$, $P < 0.01$; Immediate < Long-Term, $P < 0.01$). In addition, treating long-term castrates with E2 significantly lowered the ERα levels in their POA compared with the untreated long-term castrates (Mann-Whitney test, $P < 0.001$, Oil > E2).

In the absence of E2 treatment, ERβ levels in the NAc decreased at one month after castration and remained at similar levels thereafter (Kruskal-Wallis test, $H = 10.75$, $P < 0.01$; Immediate > Short-Term, $P < 0.01$, and Immediate > Long-Term, $P < 0.05$; Figure 3.1B). Starting E2 treatment immediately after castration, but not later, tended to reduce ERβ in the NAc (Figure 3.1B).

The timing of the onset of treatment after castration did not affect the abundance of mating-induced c-Fos protein in the BNST (Figure 3.1C). E2 treatment, however, reduced c-Fos levels in the BNST of the Short-Term castrates, but not in the Immediate or Long-Term castrates (Mann-Whitney test, $P < 0.05$: Figure 3.1C). In other brain areas studied, c-Fos levels were neither significantly different between treatments nor influenced significantly by the duration of the interval between castration and E2 administration.

3.3.2 Correlation between Mounting Frequency and ERα, ERβ, or c-Fos Levels

We analyzed how the expression of ERα, ERβ, and c-Fos proteins correlated with mounting behaviour observed at the final sexual encounter before sacrifice in the same rats. We correlated the level of each of the three proteins in the POA, BNST, MeA, NAc, and NAs with the frequency of mounting behaviour. Significant correlations were observed for several brain areas (Figures 3.2-4).

In the Short-Term Oil group, mounting frequency was positively correlated with ERα levels in the NAc (Figure 3.2A, middle panel, $P < 0.05$) and with c-Fos levels in NAs (Figure 3.4B, middle panel, $P < 0.05$). In contrast, in the Short-Term E2 group, mounting frequency was negatively correlated with ERβ levels in the POA (Figure 3.3C, middle panel, $P < 0.05$), and positively correlated with c-Fos levels in the NAc and POA (Figure 3.4A, middle panel, $P < 0.05$, and Figure 3.4C, middle panel, $P < 0.05$, respectively).

In the Long-Term groups, mounting frequency was positively correlated with ERβ levels in the NAc of the Oil group and in the MeA of the E2 group (Figure 3.3B, right panel, $P < 0.05$).

In the Immediate Oil group, mounting frequency was negatively correlated with c-Fos levels in the NAs (Figure 3.4B, left panel, $P < 0.05$) and positively correlated with c-Fos levels in BNST (Figure 3.4D, left panel, $P < 0.05$).

Figure 3.1. The abundance (mean + SEM normalized optical density) of ERα in the POA (A), ERβ in the NAc (B), and c-Fos protein in the BNST (C) of male rats euthanized one hour after a sexual encounter. Each sexual encounter occurred with an estrus female rat two weeks after treatment with oil (white) or E2 dissolved in oil (grey) beginning immediately (Immediate), one month (Short-Term), or three months (Long-Term) after castration. The number of rats in each group is indicated at the bottom of each bar. Representative images from the Western analysis (including the loading control) are shown below each graph with each band corresponding to the bar directly above it. ERα in the POA (A) and c-Fos in the BNST (C) were expressed at lower levels in E2-treated rats from the Long-Term and Short-Term groups, respectively, compared to the corresponding oil-treated rats. * indicates that two groups connected with a bar are significantly different from each other, $P < 0.05$. *** $P < 0.001$. $^{@}$ indicates that a group is significantly different from the Immediate group of the same treatment, $P < 0.05$. $^{\#}$ indicates that the difference between the groups approaches significance, $P = 0.09$.

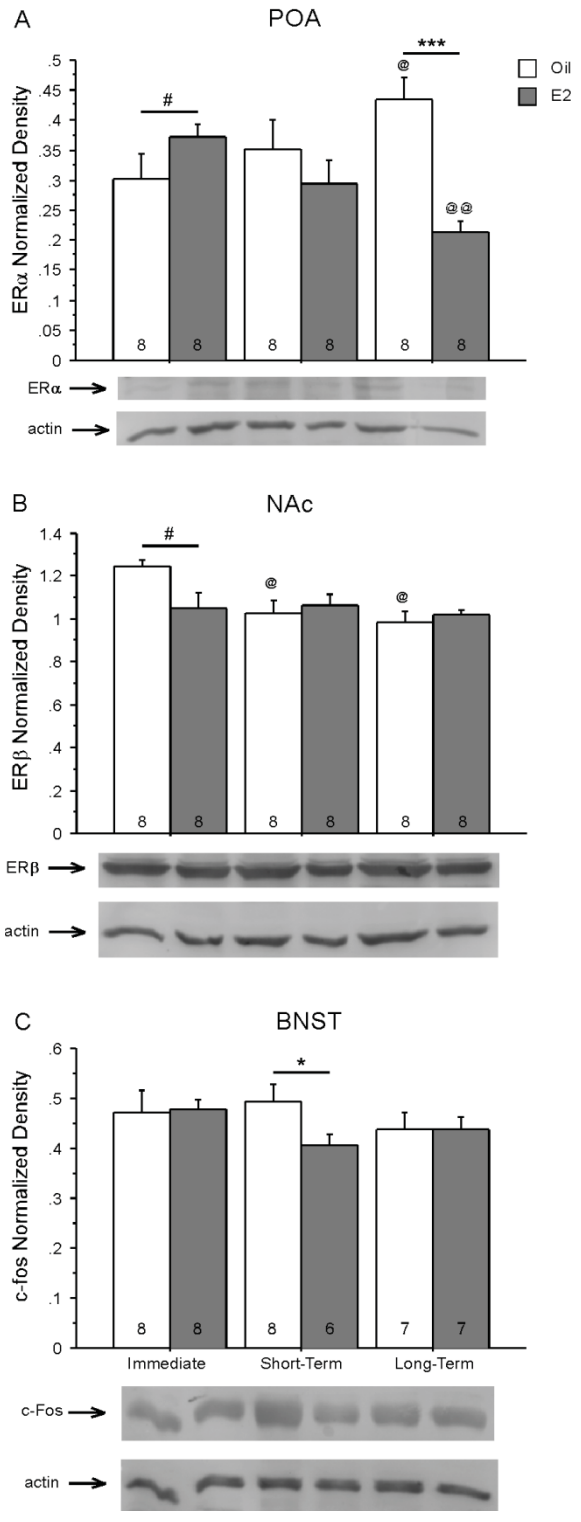

A
POA
Oil
E2
ERα Normalized Density
.5
.45
.4
.35
.3
.25
.2
.15
.1
.05
0
#
***
@
@@
8
ERα
actin
B
NAc
ERβ Normalized Density
1.4
1.2
1
.8
.6
.4
.2
0
#
@
@
ERβ
actin
C
BNST
c-fos Normalized Density
.6
.5
.4
.3
.2
.1
0
*
8
6
7
Immediate
Short-Term
Long-Term
c-Fos
actin

Figure 3.2. Scatterplots of mounting frequency and ERα levels in the NAc (A) and MeA (B) of male rats two weeks after treatment with oil (white) or E2 dissolved in oil (grey) beginning immediately (Immediate, left), one month (Short-Term, middle), or three months (Long-Term, right) after castration. Among the Oil groups, mounting frequency was positively correlated with ERα levels in the NAc of Short-Term rats. NS = no significant correlation between mounting frequency and ERα levels.

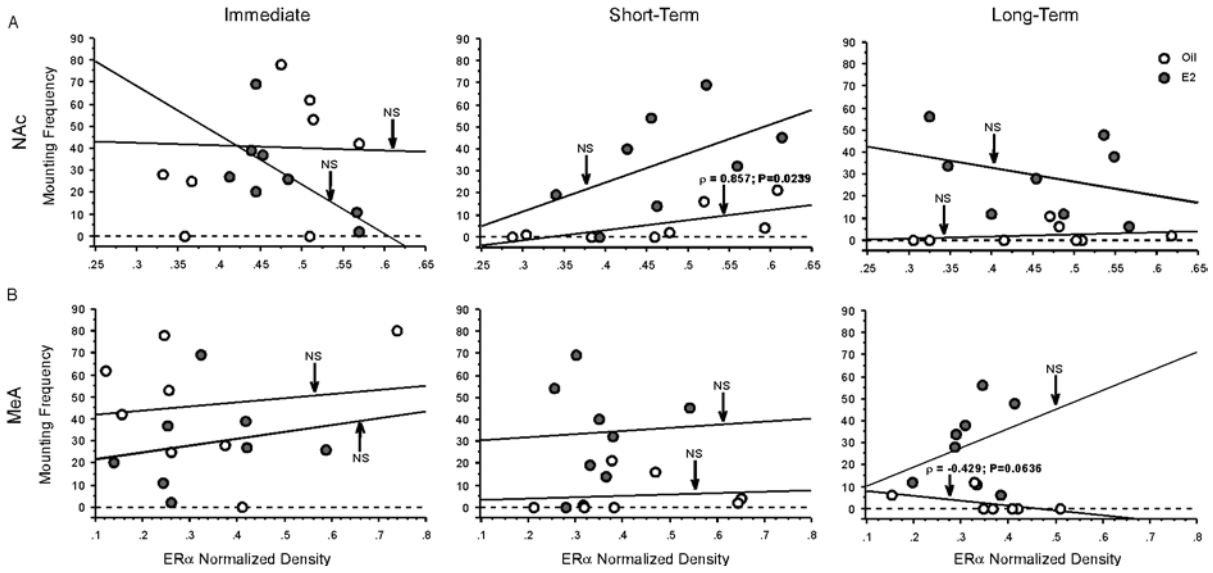

A
Immediate
Short-Term
Long-Term
NAc
Mounting Frequency
Oil
E2
NS
ρ = 0.857; P=0.0239
B
MeA
Mounting Frequency
ρ = -0.429; P=0.0636
ERα Normalized Density

Figure 3.3. Scatterplots of mounting frequency and ERβ levels in the NAc (A), MeA (B), POA (C), and BNST (D) of male rats two weeks after treatment with oil (white) or E2 dissolved in oil (grey) beginning immediately (Immediate, left), one month (Short-Term, middle), or three months (Long-Term, right) after castration. Mounting frequency was negatively correlated with ERβ levels in the POA of the Short-Term E2 group and positively correlated with ERβ levels in the MeA of the Long-Term E2 group. NS = no significant correlation between mounting frequency and ERβ levels.

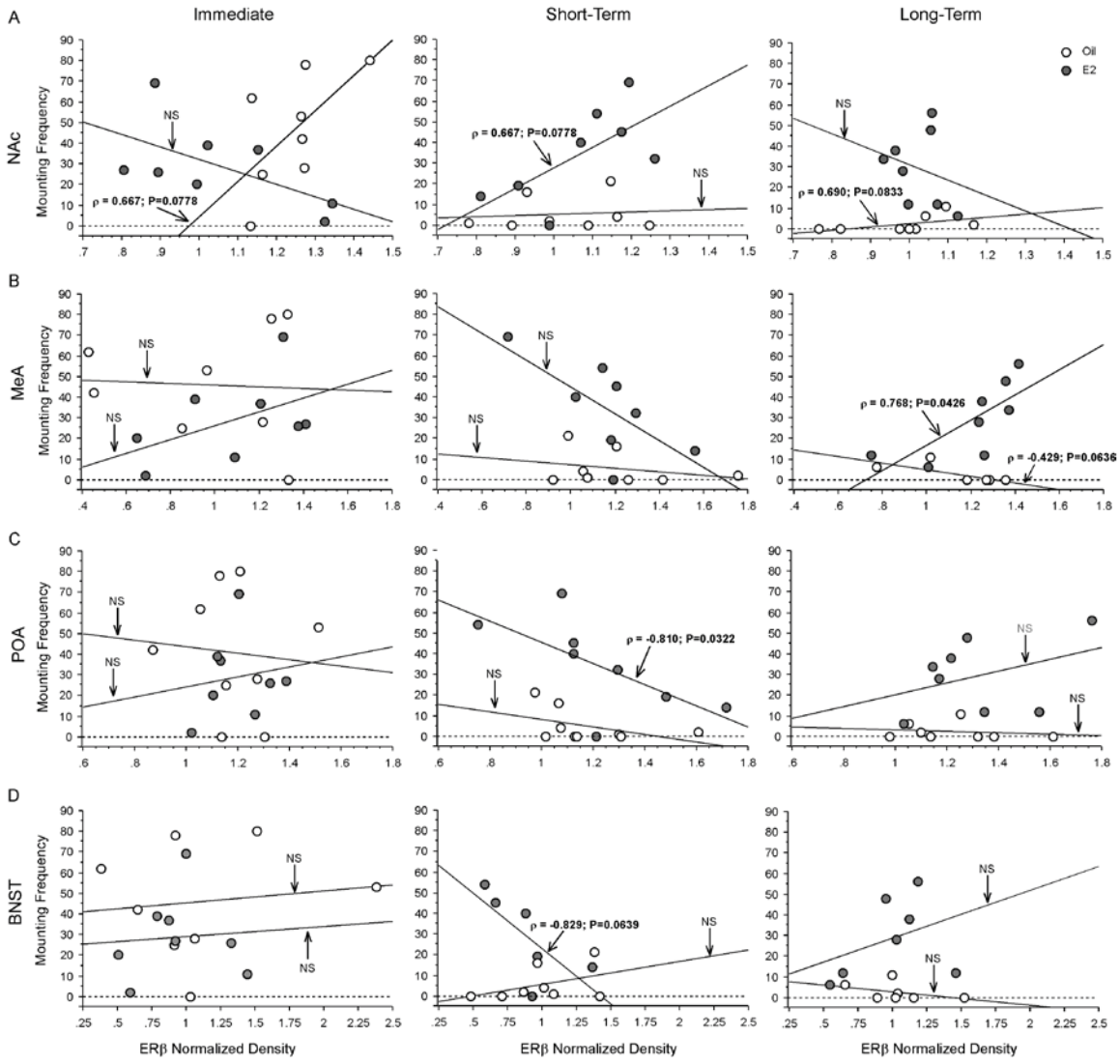

A
Immediate
Short-Term
Long-Term
Oil
E2
NAc
MeA
POA
BNST
Mounting Frequency
NS
ρ = 0.667; P=0.0778
ρ = 0.690; P=0.0833
ρ = 0.768; P=0.0426
ρ = -0.429; P=0.0636
ρ = -0.810; P=0.0322
ρ = -0.829; P=0.0639
ERβ Normalized Density

Figure 3.4. Scatterplots of mounting frequency and c-Fos density in the NAc (A), NAs (B), POA (C), and BNST (D) of male rats two weeks after treatment with oil (white) or E2 dissolved in oil (grey) beginning immediately (Immediate, left), one month (Short-Term, middle), or three months (Long-Term, right) after castration. Mounting frequency was positively correlated with c-Fos levels in the NAc and POA of the Short-Term E2 rats. NS = no significant correlation between mounting frequency and c-Fos levels.

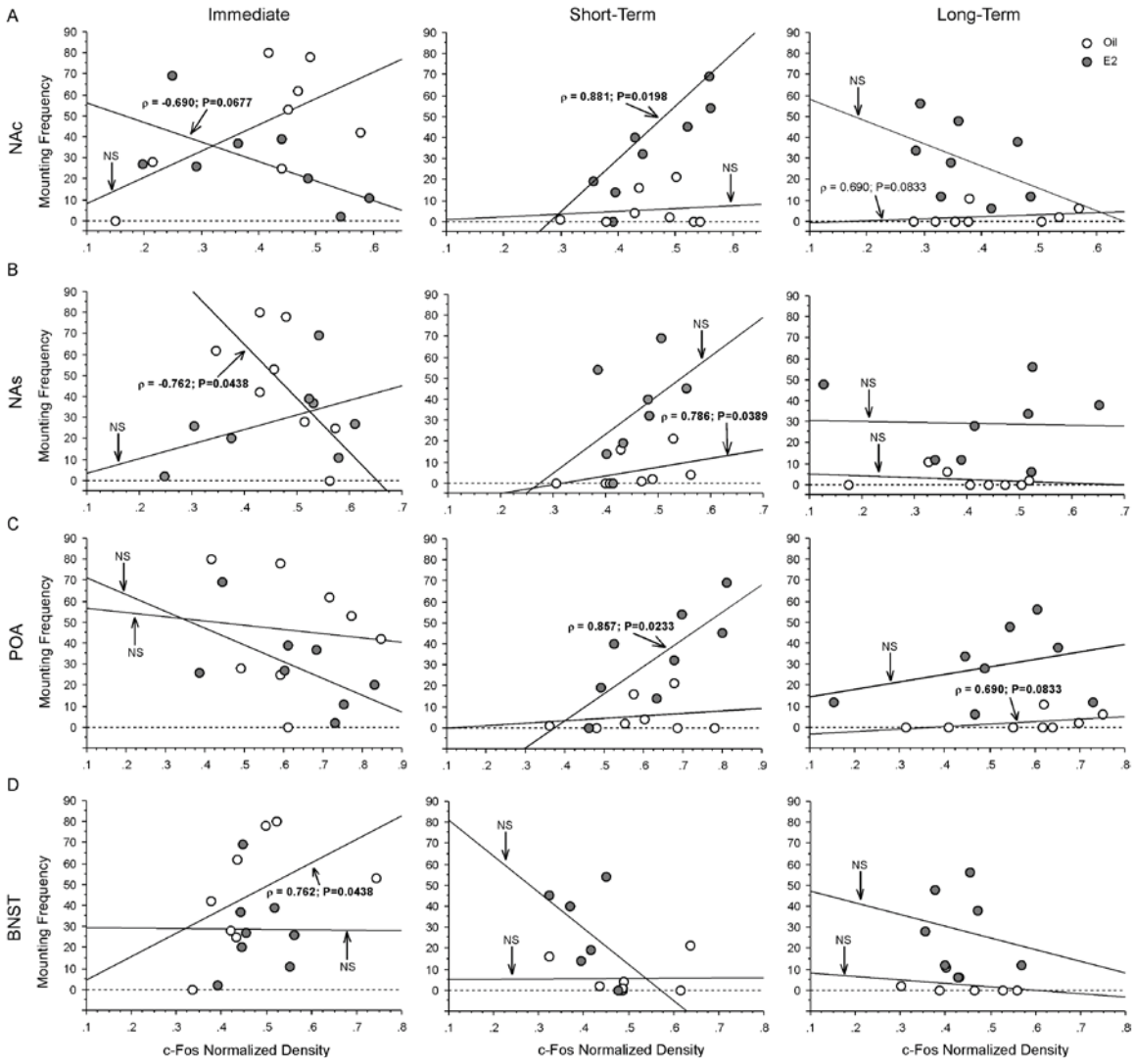

A
Immediate
Short-Term
Long-Term
Oil
E2
NAc
Mounting Frequency
ρ = -0.690; P=0.0677
NS
ρ = 0.881; P=0.0198
NS
NS
ρ = 0.690; P=0.0833
B
NAs
Mounting Frequency
ρ = -0.762; P=0.0438
NS
NS
ρ = 0.786; P=0.0389
NS
NS
C
POA
Mounting Frequency
NS
NS
ρ = 0.857; P=0.0233
NS
ρ = 0.690; P=0.0833
D
BNST
Mounting Frequency
ρ = 0.762; P=0.0438
NS
NS
NS
NS
NS
c-Fos Normalized Density
c-Fos Normalized Density
c-Fos Normalized Density

Table 3.1. Information on the antibodies used

Peptide/ Protein Target (Molecular Weight)	Immunogen	Name of Antibody	Manufacturer, Catalog No.	Species in which the antibody was raised; Clonality	Dilution Used
ERα (67 kDa)	Synthetic peptide conjugated to KLH derived from within residues 200-300 of human ERα	Anti- ERα	Abcam, ab37438	Rabbit, polyclonal	1:100
ERβ (58kDa)	Ala-Glu-Pro-Gln-Lys-Ser-Pro-trp-Cys-Glu-Ala-Arg-Ser-Leu-Glu-His	Anti-ERβ	Abcam, ab3577	Rabbit, polyclonal	1:20000
c-Fos (41 kDa)	Met-Met-Phe-Ser-Gly-Phe-Asn-Ala-Asp-Tyr-Glu-Ala-Ser-Ser	Anti-c-Fos	Abcam, ab7963	Rabbit, polyclonal	1:1000
Actin (42 kDa)	Ser-Gly-Pro-Ser-Ile-Val-His-Arg-Lys-Cys-Phe	Anti-Actin	Sigma-Aldrich, A 2066	Rabbit, polyclonal	1:1000

3.4 DISCUSSION

There are several main findings of this study. Firstly, ERα levels in the POA declined, instead of showing a trend of an increase, when castrated male rats received E2 either one or three months after castration, and this reduction was more prominent in the long-term (i.e., three months) castrates. Secondly, ERβ levels in the POA and the MeA were correlated with mounting frequency in rats that received E2 at one and three months after castration, respectively. In addition, mating-induced c-Fos induction in the POA was not affected by E2 treatment regardless of the timing of onset of that treatment. Collectively, these data suggest that the action of E2 on ERα in the POA regulates mounting behaviour. Furthermore, independence of mating-induced c-Fos induction in the POA from the interval between castration and E2 administration is consistent with our previous observation that E elevates mounting frequency to similar levels regardless of when the treatment is started after castration. These data provide evidence that ERβ may have a role in restoring sexual behaviour in castrated male rats.

This study has some limitations. Although western blot analyses help to reveal the overall abundance of protein in each brain area, it does not allow examination of the distribution of cells that express the proteins in a specific area. As an example (discussed further in section 3.4.2.), not all cells in the POA are likely to express ERs and/or c-Fos. In addition, the ERα antibody used in this study is not specific to ERα, as indicated by multiple bands, and recognized several proteins including ERα. While this non-specificity is not a major issue in western analyses and, in fact, I confirmed with uterine tissue that the ERα antibody detected a band that corresponds to the known molecular weight of ERα. However, the signal of the ERα band was relatively weak despite using a high antibody concentration throughout the experiment.

3.4.1 THE ACTION OF E IN THE POA

Our results using western analysis suggest that E2 activates mounting behaviour in castrated male rats mainly by acting on ERα in the POA, the main integrative site in

neuronal circuits that control male sexual behaviour (Hull and Rodrigues-Manzo, 2010). E2 downregulated ERα levels in the POA, consistent with the notion that the main effect of E2 on mating is mediated primarily through its action in this brain region. Similarly, E2 treatment reduces ERα mRNA levels in the POA of castrated male rats (Handa et al., 1996). The reduction of ERα levels in the POA is most prominent in long-term castrates in the present study. This could be because prolonged androgen deprivation elevates ERα mRNA (Handa et al., 1996) and protein (our study) levels in the POA, increasing the availability of ERα for ligand binding. Therefore, treating long-term castrates with E2 results in a great amount of ERα that binds to E2, which leads to an increase in the autoregulation (decrease) of ERα over time.

The autoregulation of ERα appears to be site-specific as ERα levels in the brain areas other than the POA remained constant after 2 weeks of E2 treatment, regardless of the interval between castration and E2 administration. The observed lack of changes in ERα levels in the MeA agrees with a previous report on ERα mRNA in the MeA of orchiectomized rats (Lauber et al., 1991). In contrast, although E2 treatment is known to reduce ERα mRNA levels in the BNST of castrated male rats (Handa et al., 1996), we did not observe ERα downregulation at the protein level in our study.

3.4.2 Mating-Induced c-Fos in the POA is not Affected by E2 Treatment

The patterns of c-Fos induction in the POA may help explain why male rats that underwent early and late E2 treatments after castration showed similar levels of mounting behaviour (Wibowo and Wassersug, 2013b). In the present study, mating activity resulted in similar levels of c-Fos in the POA regardless of the treatment (i.e., E2 or control) or the timing of E2 administration after castration (data not shown). Similarly, as previously reported by others, there were approximately equal numbers of mating-induced c-Fos-immunoreactive neurons in the POA, BNST and MeA of orchiectomized rats treated with either oil or E2 (Baum and Wersinger, 1993). This suggests that, though mating behaviour induces c-Fos-immunoreactive neurons in all of the three areas (Robertson et al., 1991; Baum and Everitt, 1992), the expression of c-Fos after a sexual encounter does

not appear to be dependent on E2's direct action on c-Fos positive neurons in these brain areas. In fact, only about 30% of c-Fos immunoreactive cells in those three areas express ERs but approximately 50% of ER neurons are positive for c-Fos (Greco et al., 1998) [only ERα, and not ERbeta, was analyzed in that study].

As suggested by Greco *et al*. (1998), E2 may have a modulatory or permissive role that helps control mounting behaviour; i.e., neurons in the MeA, POA and BNST are always activated by sexual encounters, even when rats are castrated, but mounting will only occur in the presence of E2. The equivalent c-Fos levels in the POA of E2-treated rats despite the differences in time since castration may explain the similar activation of mounting behaviour in castrated male rats treated with E2 after short- and long-term castration.

3.4.3 Possible Role of ERβ in Regulating Male Sexual Behaviour

We observed that the ERβ levels in the POA of Short-Term E2 rats and in the MeA of Long-Term E2 rats were correlated with mounting behaviour, raising the possibility that ERβ plays a role in male sexual behaviour. Currently, the activation of ERα but not ERβ is thought to be important for male mouse sex behaviour (Rissman, 2008). Gonad-intact male mice lacking ERβ (ERβKO) have similar mating behaviours to wildtype mice (Ogawa et al., 1999; Kudwa et al., 2005). However, that could be attributed to the presence of functional ERα and androgen receptors (ARs) in ERβKO mice. The ERβKO mice in those studies were intact, so sex steroids could still bind to ARs or other types of ERs to activate sexual behaviour. In order to confirm the definitive role of ERβ in male sexual behaviour, one needs to test the effect of ERβ-specific agonists on sex behaviour of castrated male rats.

In contrast to ERβKO mice, ERαKO male mice displayed normal mounting behaviour but lacked intromission and ejaculation (Ogawa et al., 1997). Since the mice in that study were surgically intact, the mounting behaviour displayed by those mice could be due to sex steroids acting on ARs or another ER type (e.g., ERβ). In a study by Wersinger *et al*.

(1997), ERαKO mice lacked mounting behaviour, but these mice were castrated and given supplemental testosterone that did not reach physiological plasma testosterone levels. Therefore, the mounting deficit in these ERαKO mice (Wersinger et al., 1997) was less likely due to the absence of ERα *per se* but rather due to low levels of sex steroids.

Additional piece of evidence supporting the role of ERβ in regulating copulatory behaviour comes from a study using quails; treating castrated quails with ERβ-selective agonists stimulates mounting behaviour as efficiently as treating them with ERα-selective agonists and diethylstilbestrol (Seredynski et al., 2011).

In the present study, treating castrated rats with E2 did not influence the expression of ERβ protein in any of the brain areas studied. Similarly, absence of E2 treatment effect on ERβ in the POA and BNST of female rats has been reported (Patisaul et al., 1999; Hrabovszky et al., 2000; Shima et al., 2003). However, the numbers of ERβ-immunoreactive cells in the POA and the BNST were reduced following E2 treatment (Greco et al., 2001; Kallo et al., 2001; Nomura et al., 2003), suggesting that E2 may affect the transcription or translation process of ERβ mRNA in these areas. In contrast, in the MeA, the abundance of ERβ mRNA (Osterlund et al., 1998) and the number of cells expressing ERβ mRNA (Shima et al., 2003) are reduced after E2 treatment, whereas the number of ERβ immunoreactive cells is not reduced (Greco et al., 2001). It is possible that the lack of change in the number of ERβ immunoreactive cells in the MeA may be due to the different treatment regimes followed in those studies [i.e., single 10 µg E2-benzoate injection by Greco *et al*. (2001) vs. subcutaneous Silastic implant containing crystalline E2 by Shima *et al*. (2003) or subcutaneous E2 pellets by Osterlund *et al.* (1998)]. Wibowo and Wassersug (2013a) discussed how the effect of E on male rat sexual behaviour may be influenced by the treatment regime (Appendix B).

3.4.4 Interpretation of the Results of Correlation Analyses

Our efforts to correlate the ER and c-Fos data from brain nuclei with sexual behaviour met challenges for a variety of reasons. There are several methodological issues to

consider. In this study, there were only a few statistically significant correlations and these were seen in the groups that received delayed (one month or three months) E2 treatment after castration, but this is likely because of small sample sizes. Another possible explanation is that two weeks of androgen deprivation might simply not have been long enough to lead to changes in the expression of ERs or mating-induced c-Fos as well as mounting behaviour. These methodological considerations notwithstanding, we also offer alternative explanations as discussed below.

Both the oil and E2 treated groups showed similar degrees of individual differences in ERβ levels across the brain areas studied. However, the majority of the statistically significant correlations were from the E2 groups. This poses a question: why do E2-treated rats with low ERβ levels in the POA show elevated mounting behaviour whereas oil-treated rats with similar levels of ERβ in the POA displayed little mounting behaviour?

Another issue is that the correlation trends between mounting behaviour and ERβ from the Short-Term and Long-Term E2 groups were almost opposite to each other. For example, short-term castrates with low ERβ in the MeA showed frequent mounting, but long-term castrates with high ERβ in the MeA also showed similarly frequent mounting (see Figure 3.5 for a schematic representation of the relationship between ERβ levels in the MeA, POA and BNST and the mounting behaviour of the animals).

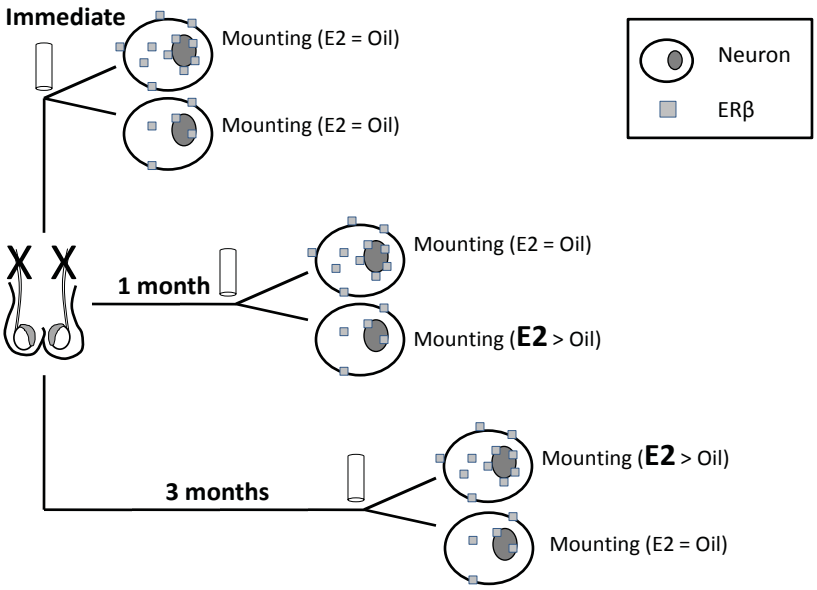
We have no obvious explanations for the different correlation patterns observed between the mounting behaviours of Short- and Long-Term E-treated rats and their ERβ levels. However, due to the complexity of the endocrine system, the dynamic of physiological effects associated with hormonal treatment may vary with time. For example, administering luteinizing hormone-releasing hormone agonists to men promptly elevates their testosterone levels, but prolonged treatment leads to reduced testosterone levels (Singer et al., 2008). In females, E increases progesterone receptors during the follicular phase but after ovulation, elevated progesterone levels downregulate ER expression (Bagamasbad and Denver, 2011). Possibly, there is a factor (or a combination of factors)

whose levels change with time after male castration, and these changes shift the direction of ERβ autoregulation. Castration may alter the levels of some molecules in the nervous system but these effects may be reversed with prolonged state of castration; e.g., some hormones are elevated shortly after castration but reduced after long-term androgen-deprivation, or vice versa (see Caruso et al. (2010) as an example). Therefore, E may change the direction of ERβ autoregulation depending on when it is administered with the goal to maintain normal cellular response.

3.4.5 Conclusions

In conclusion, the present study provides evidence suggesting that E2 acts on ERα in the POA to activate mounting behaviour in castrated male rats. Furthermore, the equal levels of c-Fos protein in the POA of E2-treated castrated rats may explain why mounting behaviour is elevated to a similar extent in short- and long-term castrated male rats. Lastly, we suggest that ERβ may have a role in stimulating male sexual behaviour.

Figure 3.5. Schematic representation of the possible relationships between mounting frequency and ERβ levels in the MeA, POA, and BNST of male rats at two weeks after treatment with oil (Oil) or estradiol dissolved in oil (E2) beginning immediately (top), one month (middle), or three months (bottom) after castration. The tubes represent the times when the rats were implanted with a Silastic tube containing either Oil or E2. The drawing of a cell to the right of each tube represents a neuron from one of the nuclei studied after two weeks of treatment. The number of small squares in each cell represents the relative density of ERβ in and on the cells. As a key feature of this schematic, two relative densities of ERβ receptors are shown for each timing interval to reflect individual variation observed among animals (the mechanisms for this variation are currently unknown). To the right of each cell is a summary of the relationship between ERβ density and rat mounting frequency. For example, when treatment begins immediately after castration (top), high ERβ levels are correlated with a similar amount of mounting in both E2 and oil treated animals. In contrast, when treatment begins three months after castration (bottom), high ERβ levels are correlated with higher mounting in the E2 group compared to the oil group.


Immediate
Mounting (E2 = Oil)
Mounting (E2 = Oil)
Neuron
ERβ
1 month
Mounting (E2 = Oil)
Mounting (E2 > Oil)
3 months
Mounting (E2 > Oil)
Mounting (E2 = Oil)

Chapter 4: MODULATION OF ESTROGEN RECEPTORS BY ESTRADIOL IN THE PELVIC FLOOR MUSCLES OF CASTRATED MALE RATS

Abstract

Estrogen (E) treatment may have biological benefits among steroid-deprived males and females. However, based on the "Critical Period Hypothesis", E administration in females can positively affect neuronal tissues only when initiated within a critical period from the onset of steroid deprivation (e.g., menopause or ovariectomy). One proposed explanation for the fact that delayed E treatment is not as effective as early treatment is that prolonged steroid-deprivation alters the autoregulatory mechanism of estrogen receptors (ERs). Whether the Critical Period Hypothesis also applies to the autoregulation of ERs in non-neuronal tissue is unknown. In this study, we investigated how the timing of E treatment after castration influences the abundance of ERs in the pelvic floor muscles (PFM) of male rats.

Male rats were treated with oil (as a control) or estradiol for two weeks starting immediately, one month (short-term), or three months (long-term) after castration. After the treatment, the rats were euthanized and their bulbocavernosus and levator ani muscles were collected.

Long-term (3 months) castration led to PFM atrophy, and E treatment failed to reverse the muscle shrinkage. The PFM of long-term castrates either with or without E treatment had a higher percentage of small muscle fibers than those that were more recently castrated.

We found that E increased ERβ levels in the bulbocavernosus of the short-term castrates but not of the immediate or long-term castrates. In contrast, ERα in the levator ani increased in rats receiving immediate E treatment after castration. There was also a trend

of an increase in ERα levels in the bulbocavernosus with E treatment in immediate castrates, though this did not reach statistical significance.

In sum, our data suggest that the autoregulation of ERs in PFM is disrupted after long-term androgen deprivation. It is possible that the modulatory effect of E on the function of PFM is also attenuated in males that have been deprived of androgen for a long time.

Publication Information

This manuscript is currently in preparation for publication. EW, RWC and RJW were involved in study design. EW and HJC ran the experiments and analyzed the data. RWC provided critical feedback on the experimental methods. EW wrote the first draft of the manuscript and all authors provided editorial input.

4.1 INTRODUCTION

Sex steroid deprivation impairs many physiological functions in both males and females. Due to low circulating steroids, androgen-deprived men and menopausal or surgically ovariectomized women can experience symptoms like sexual dysfunction, hot flashes, osteoporosis, and cognitive decline (Wibowo et al., 2011; Scott et al., 2012). In both sexes, estrogen (E) replacement can positively affect steroid-deprived individuals (Wibowo et al., 2011; Scott et al., 2012). However, according to the "Critical Period Hypothesis," E treatment is beneficial to neuronal tissues in females, only if started within a critical period close to the onset of steroid deprivation (e.g., menopause or surgical ovariectomy). To our knowledge, no study has investigated whether the same is true for E treatment in non-neuronal tissue.

Some non-neuronal tissues, such as the pelvic floor muscles (PFM), express steroid receptors and are hormone-sensitive (Sengelaub and Forger, 2008). Castration in male rodents causes the PFM to atrophy, and androgen-replacement can reverse the muscle shrinkage (Sengelaub and Forger, 2008). In contrast to androgens, the role of E in the function of PFM is not well-established. E treatment to male rats does not reverse PFM atrophy following castration (Verhovshek et al., 2010). However, exogenous E2 administration soon after castration restores the normal electromyographic properties of PFM in castrated male rats (Holmes and Sachs, 1992; Fargo et al., 2003; Foster and Sengelaub, 2004). Whether administering E2 to rats that have been castrated for a long time can still restore PFM excitability is not known.

Effects of E2 on PFM are mediated through the action of E on estrogen receptors (ERs) present on these muscles (Dube et al., 1976; Rudolph and Sengelaub, 2013). ERs autoregulate themselves in the presence of E, but to our knowledge, the autoregulation of ERs in PFM has not been studied.

In the present study, we investigated how E2 administration influences the expression of ERα and ERβ in the PFM of castrated rats. As autoregulation of ERs on the PFM may be

altered after long-term castration, we also tested how the duration of androgen deprivation affects the autoregulation of ERs in the PFM in response to E2.

4.2 Materials and Methods

4.2.1 Animals, Surgery and Oil/Estradiol Administration

PFM were collected from the animals used in a previous study, and the detailed experimental design, surgery and treatment protocols were described previously (Wibowo and Wassersug, 2013b). In brief, adult male Long-Evans rats (Charles River Canada, St. Constant, QC, Canada, 275-300 g at the time of arrival) were housed singly under a reversed 14:10 light:dark cycle at 23 ± 1ºC ambient temperature, with food and water available *ad libitum*. Animal handling protocols followed the guidelines of the Canadian Council on Animal Care and were approved by the Dalhousie University Committee on Laboratory Animals.

Male rats were gonadectomized under anesthesia (104 mg/kg ketamine, 4.8 mg/kg xylazine, and 0.9 mg/kg acepromazine, i.p) and were given an analgesic (Ketoprofen, 5 mg/kg, s.c.) and an antibiotic (Baytril, 5 mg/kg, s.c.) after the surgery.

Castrated rats received either oil or E2 (dissolved in oil) treatment (n = 24 per treatment) through a Silastic implant (1.6 mm inner diameter, 3.2 mm outer diameter; 35 mm in length; Dow Corning Corporation, Midland, MI). Each implant contained either sesame oil (60 μL; Catalog No. S3547, Sigma-Aldrich) for the oil groups, or 230 μg of 17β-E2 (Catalog No. E8875; Sigma-Aldrich) in 60 μL sesame oil for the E2 groups. This E2 dose elevated the plasma E2 level in the male rats to that of proestrus female rats (Wibowo and Wassersug, 2013b).

Each treatment group was further randomly subdivided into 3 groups (n = 8 per group) according to the timing of Silastic tube implantation after castration: immediately (Immediate), after one month (Short-Term), or after 3 months (Long-Term). Rats in the

Immediate groups received the Silastic implant (s.c) immediately after castration during the same surgery, whereas rats in the Short-Term and Long-Term groups were implanted with a Silastic tube 1 month and 3 months after castration, respectively, under isoflurane anesthesia (4% for induction, 2% for maintenance; 1 L/min) mixed with oxygen.

4.2.2 Tissue Collection and Preparation

Following 14 days of treatment, the rats were euthanized by anesthetic overdose (208 mg/kg ketamine, 9.6 mg/kg xylazine, and 1.8 mg/kg acepromazine, i.p). The two pelvic floor muscles, the bulbocavernosus (BC) and levator ani (LA) muscles, that are important for erection in males but LA appears to be more important in maintaining pressure in the penile bulb during erection than the BC (Holmes and Sachs, 1994). Both muscles were quickly dissected out and separated from each other. In rats, there are other PFM, but they were not analyzed. The two muscles were collected from each side in each rat. Muscle tissues from one side were frozen at -80°C until they were used for western analysis. The PFM from the opposite side were post-fixed by immersing in 4% paraformaldehyde solution for histology.

4.2.3 Hematoxylin and Eosin Staining

LA muscles were processed for standard hematoxylin and eosin staining. The muscles were dehydrated in a graded alcohol series, embedded in paraffin wax, and transversally sectioned at 10 μm. After deparaffining, the sections were rehydrated in a graded alcohol series and stained with hematoxylin and eosin. The sections were dehydrated and coverslipped. Images of the cross sections of muscle fibers were captured using a microscope (Zeiss Axioplan 2) and analyzed using AxioVision (Zeiss). Only muscle fibers that were clearly outlined and intact were used for analysis. On average, we measured 71.0 ± 24.1 muscle fibers per muscle per rat. The number of muscles measured per group is stated on the figure legend of Figure 4.2. We measured the cross-sectional area of individual muscle fibers, counted the number of muscle fibers in various size classes (with cross-sectional of 0-400, 400-800, or >800μm^2), and divided those numbers

by the total number of muscle fibers measured per rat. This calculation was done to estimate the extent of muscle fiber atrophy in relation to the gross muscle shrinkage. Only fibers along the periphery of the muscle, where they have the best fixation, were analyzed to ensure that the same relative area was sampled for each rat.

4.2.4 Western Blot

BC and LA tissues were dissected and placed in homogenization buffer (0.32 M sucrose in 0.1 M PBS with one complete mini protease inhibitor tablet (Catalog No. 04 693 124 001, Roche) per 10 mL, homogenized for 2 minutes and centrifuged for 10 minutes at 13,000 X g.

Extracted proteins (5 µg per lane, determined by Bio-Rad protein assay) were mixed with Laemmli sample buffer in 1:4 ratio, then heated at 95°C for 10 minutes. The protein samples were fractionated in 7.5% SDS-polyacrylamide gels for 20 minutes at 75 V, followed by 1 hour at 175 V. We used a multi-strip western blot technique as described by Aksamitiene *et al*. (2007) to allow us to compare samples from all 48 animals on one membrane. Briefly, we divided each gel into two strips containing protein either between 40-60 kDa or 60-80 kDa. The strips containing the same molecular weight range were then placed on a single filter paper and transferred to a PVDF membrane for 4 hours at 100 V.

4.2.5 Immunolabeling

The membranes were incubated in primary antibodies at 4°C overnight after 1 hour blocking at room temperature in a 5% skim milk solution in Tris-buffered saline with 0.1% TWEEN® 20 (TBST). The primary antibodies and their concentrations used were as follows: mouse monoclonal anti-ERα (ab2746, Abcam, 1:1000), rabbit polyclonal anti-ERβ (ab3577, Abcam,1:2000), and mouse monoclonal anti-β-tubulin (T4026, Sigma-Aldrich, 1:5000) antibodies. After rinsed in TBST, the membranes were incubated for 1 hour at room temperature in chicken anti-rabbit IgG-HRP (sc-2963, Santa Cruz

Biotechnology, 1:10000) and chicken anti-mouse IgG-HRP (sc-2962, Santa Cruz Biotechnology, 1:100). The membranes were then washed in TBST. An ECL 2 kit (Product No. 80196, Pierce) and Typhoon 9410 scanner were used to detect bands on the membranes.

4.2.6 Densitometry and Statistical Analyses

ImageJ 1.46r was used to measure the band density (RawIntDen value) of each protein. Each band density for ERα, ERβ and tubulin was subtracted by the background density. To obtain a normalized band density for ERα and ERβ, the density value of each band was divided by that of the loading control (tubulin).

Statview 5.0 (SAS Institute Inc., Cary, NC) and Prism 4.03 (GraphPad Software, San Diego, CA) were used for statistical analyses. Normalized band densities and the muscle fiber size data were analyzed using Mann-Whitney and Kruskal-Wallis tests, followed by Dunn post hoc test when statistical significance was reached. Probabilities less than 0.05 were considered statistically significant.

4.3 RESULTS

4.3.1 SIZE OF THE PFM

In the absence of E2, the BC from long-term castrated rats had significantly lower lengths, widths, and weights than those from the rats in Immediate Oil group (Kruskal-Wallis Test, $H = 9.9, 9.0, 15.5$, $P < 0.01, 0.05, 0.001$; Long Term < Immediate Oil, $P < 0.01, 0.05, 0.001$ respectively; Figure 4.1). In addition, the Short-Term Oil group had significantly lower BC weight than the Immediate Oil group (Kruskal-Wallis Test, $H = 15.5$, $P < 0.001$; Short-Term < Immediate Oil, $P < 0.05$).

Two weeks of E2 treatment did not significantly alter the length, width or weight of the BC regardless of when the treatment was started after castration (Figure 4.1).

Among E2-treated rats, long-term castrates had significantly lower BC width and weight than rats immediately after castration (Kruskal-Wallis Test, $H = 11.4, 18.1$, $P < 0.01, 0.001$; Long-Term < Immediate E2, $P < 0.01, 0.001$ respectively). Additionally, the Long-Term E2 group also had significantly lower BC weights than Short-Term E2 group (Kruskal-Wallis Test, $H = 18.1$, $P < 0.001$; Long-Term < Short-Term E2, $P < 0.05$).

4.3.2 Cross-Sectional Area of Muscle Fibers in the Levator Ani

An analysis of cross-sectional areas of the muscle fibers between rats treated with E2 or oil in the Immediate, Short-Term or Long-Term groups showed no effects of E2 treatment (all $P > 0.05$). Therefore, we combined the data from both the treatment groups at each time interval after castration, as shown in Figure 4.2. We found that the Long-Term group had a significantly higher proportion of small muscle fibers (i.e., a cross sectional area of 0-400 μm^2) than the Immediate or Short-Term groups (Kruskal-Wallis Test, $H = 10.3$, $P < 0.01$; Long-Term > Immediate, Long-Term > Short-Term, $P < 0.05$ for both). The long-Term group had a smaller proportion of medium sized muscle fibers (i.e., cross-sectional area between 400-800 μm^2) than the other two groups (Kruskal-Wallis Test, $H = 10.4$, $P < 0.01$; Long-Term > Immediate, Long-Term > Short-Term, $P < 0.01, 0.05$ respectively). Similarly, the long-Term group had a smaller proportion of large muscle fibers (i.e., with cross-sectional area of more than 800 μm^2) than the other two groups, with significant difference between the Short-Term and Long-Term groups (Kruskal-Wallis Test, $H = 6.5$, $P < 0.05$; Long-Term > Short-Term, $P < 0.05$). Sample photographs of the muscle fibers from the three timing intervals can be found in Figure 4.3.

4.3.3 Changes in Estrogen Receptors

To compare how the duration of androgen-deprivation (regardless of treatment) affected the PFM, we combined data from both the oil and E2 groups. The abundance of ERβ increased after long-term (three months) androgen deprivation in both BC and LA muscles (Kruskal-Wallis Test, $H = 9.5, 10.84$, $P < 0.01$ for both; Long-Term > Immediate

groups, $P < 0.01$ for both, Figure 4.4A). The same trend of increase was observed in the expression of ERα in the BC and LA, with a significant difference between ERα levels in the LA of Short-Term and Immediate groups (Kruskal-Wallis Test, $H = 8.9$, $P < 0.05$; Short-Term > Immediate groups, $P < 0.05$, Figure 4.4B).

Next, to compare how the oil and E2 treatments (regardless of the timing interval from castration to the onset of treatments) affected the PFM we combined and analyzed the data according to treatment groups. Two weeks of E2 treatment did not affect the expression levels of ERβ in the BC or LA muscles (Figure 4.5A). However, the same treatment significantly increased ERα levels in both muscles (Mann-Whitney Test, $P < 0.05$ for both, Figure 4.5B).

When the data on the groups were analyzed separately according to when the treatment was started, administering E2 to Immediate and Long-Term castrates did not affect the expression levels of ERβ in the BC muscle (Figure 4.6A). However, administering the same E2 treatment to Short-Term castrates significantly increased the ERβ protein levels (Mann-Whitney Test, $P < 0.01$). Among the E2 groups, ERβ levels in the BC were higher in the Long-Term groups compared to the Immediate groups (Kruskal-Wallis Test, $H = 6.4$, $P < 0.05$; Long-Term > Immediate groups, $P < 0.05$, Figure 4.6B). In contrast, though there is a trend of increase, ERα levels in the BC were not significantly affected by E2 treatment regardless of when the treatment was started (Figure 4.6C).

In the LA muscle, without E2 treatment, ERβ levels did not vary among the Immediate, Short-Term and Long-term groups (Figure 4.6B). Furthermore, administering E2 treatment did not elevate ERβ levels regardless of when treatment was initiated after castration (Figure 4.5B). Among the E2 groups, ERβ levels in the LA were higher in the Long-Term than the Immediate group (Kruskal-Wallis Test, $H = 9.5$, $P < 0.01$; Long-Term > Immediate groups, $P < 0.01$, Figure 4.6B). Without E2 treatment, ERα levels in the LA increased over time (Kruskal-Wallis $H = 12.2$, $P < 0.01$; Immediate < Short-Term Oil, Immediate < Long-Term Oil, $P < 0.05$, $P < 0.01$ respectively, Figure 4.6D).

However, E2 administration increased ERα levels in the LA of the Immediate group (Mann-Whitney Test, $P < 0.05$) but not in Short- or Long-Term castrates.

Figure 4.1. The length (A), width (B), and weight (C) of one side of the BC muscle two weeks after treatment of oil (white) or E2 dissolved in oil (grey) beginning immediately (Immediate), one month (Short-Term), or three months (Long-Term) after castration (n = 8 per group). The Long-Term Oil group had a lower BC length, width, and weight than the Immediate Oil group; but all three parameters were similar to the Long-Term E2 group. * indicates a significant difference from the Immediate group of the same treatment, $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. $^{\#}$ indicates a significant difference from the Short-Term group of the same treatment, $P < 0.05$.

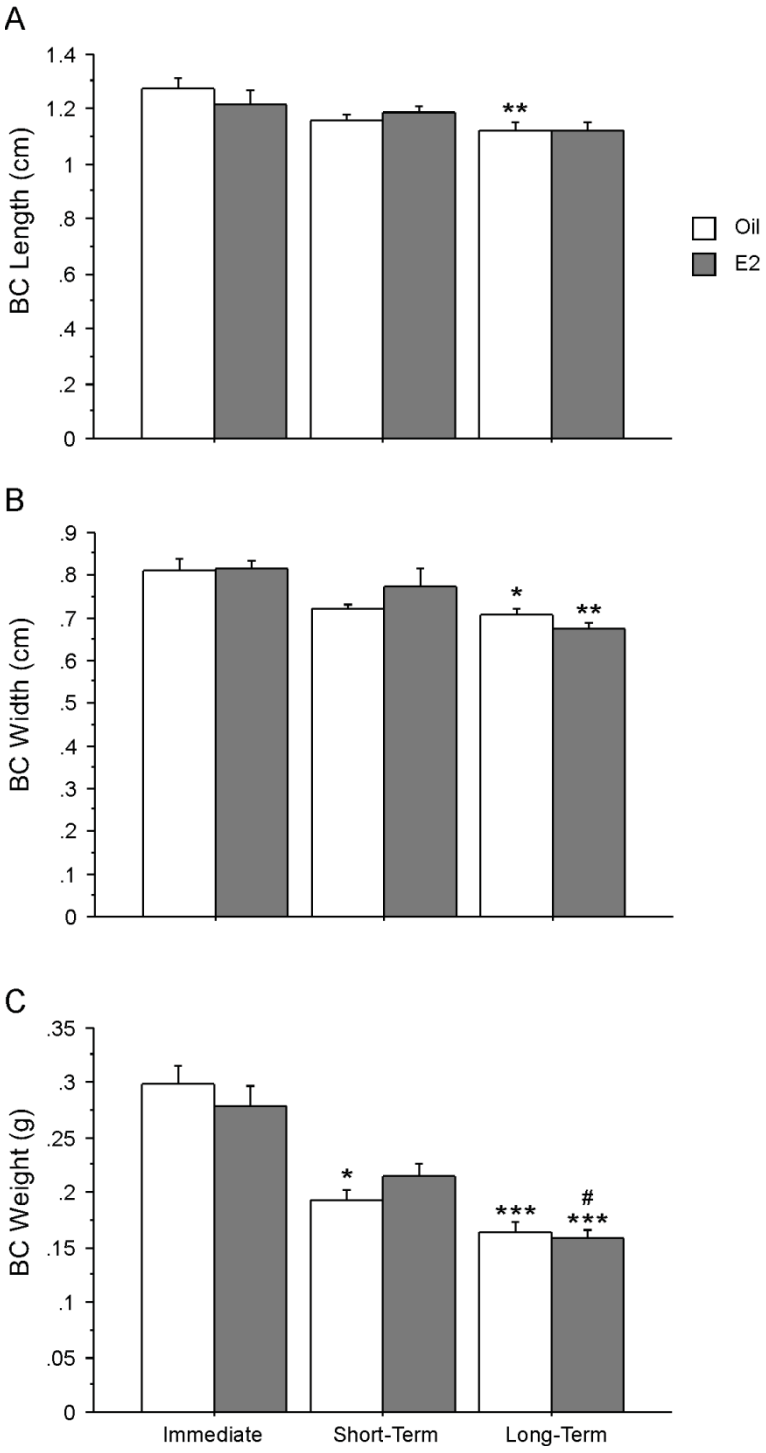

A
BC Length (cm)
1.4
1.2
1
.8
.6
.4
.2
0
**
Oil
E2
B
BC Width (cm)
.9
.8
.7
.6
.5
.4
.3
.2
.1
0
*
**
C
BC Weight (g)
.35
.3
.25
.2
.15
.1
.05
0
*
***
#
***
Immediate
Short-Term
Long-Term

Figure 4.2. The percentage of muscle fibers in three size classes from the LA muscle of rats in the Immediate (white), Short-Term (grey), or Long-Term (black) groups (n = 7, 6, 5 respectively). The sum of the 3 fiber sizes for each group is 100%. In the long-Term group there were more muscle fibers in the 0-400 μm^2 range than the Immediate or Short-Term group. In contrast, Immediate and Short-Term groups had more fibers with cross sectional area of > 400 μm^2. * indicates a significant difference from the Immediate group of the same treatment, $P < 0.05$, ** $P < 0.01$.

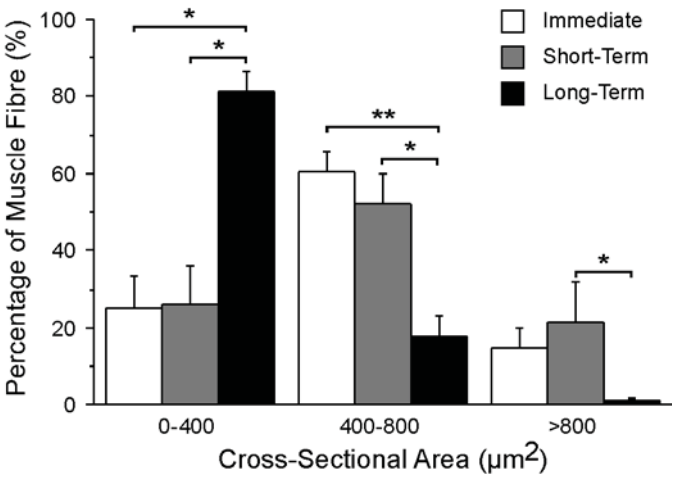

Immediate
Short-Term
Long-Term
Percentage of Muscle Fibre (%)
100
80
60
40
20
0
*
*
**
*
*
0-400
400-800
>800
Cross-Sectional Area (μm2)

Figure 4.3. Representative images of transverse sections of LA muscles of animals from the Immediate (left), Short-Term (middle) and Long-Term (right) groups. Sections were stained with hematoxylin and eosin.

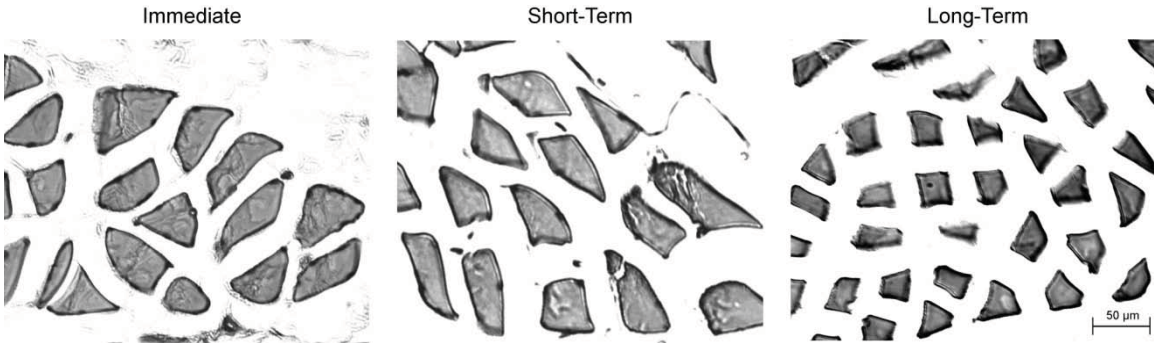

Immediate
Short-Term
Long-Term
50 µm

Figure 4.4. The abundance (Mean + SEM normalized optical density) of ERβ (A) and ERα (B) in the BC (left) and the LA (right) muscles of male rats from Immediate (white), Short-Term (grey), or Long-Term (black) groups (n = 16 per group). Data from rats of the oil and E2 groups are combined based on the timing interval between castration and treatment. In both BC and LA, ERβ levels increased after 14 weeks of castration. ERα level in the LA increased after 6 weeks of castration (B).* indicates significant difference from the Immediate group, $P < 0.05$, ** $P < 0.01$.

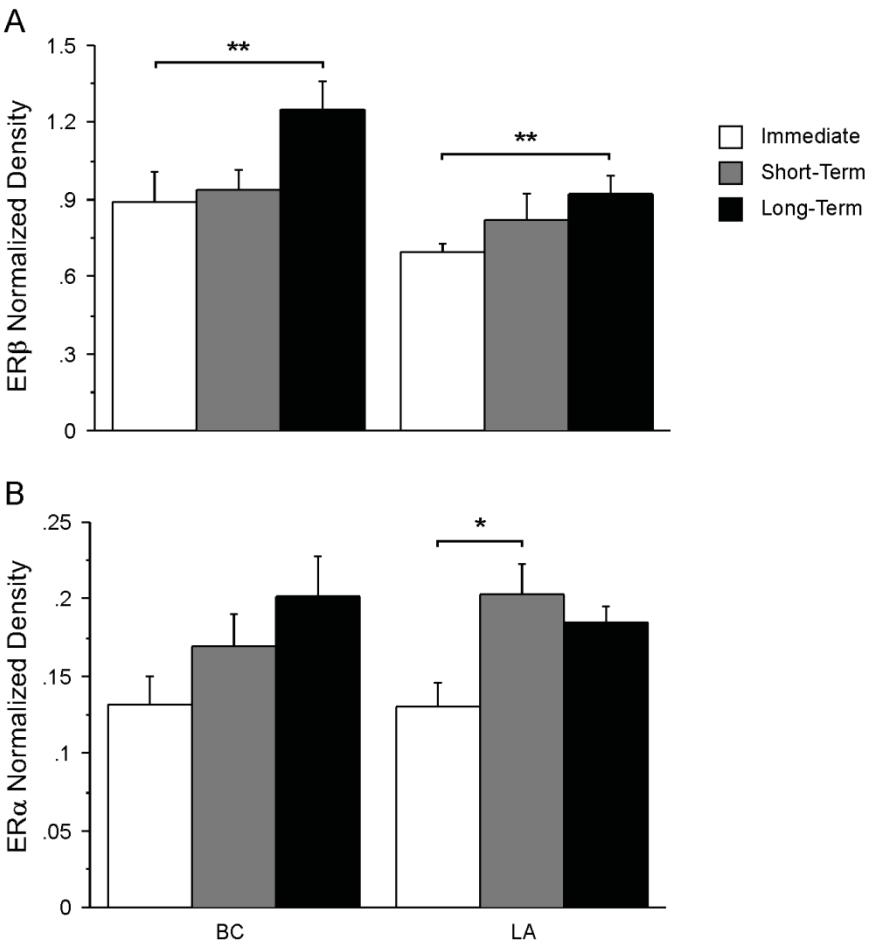

A
1.5
1.2
.9
.6
.3
0
ERβ Normalized Density
**
**
Immediate
Short-Term
Long-Term
B
.25
.2
.15
.1
.05
0
ERα Normalized Density
*
BC
LA

Figure 4.5. The abundance (Mean + SEM normalized optical density) of ERβ (A) and ERα (B) in the BC (left) and the LA (right) muscles of male rats after 2 week treatment with oil (white) or E2 dissolved in oil (grey) (n = 24 per group). The data are combined into treatment groups (oil or E2) regardless of the timing interval between castration and treatment. E treatment increased ERα levels in both BC and LA muscles. * indicates significant difference from the Oil, $P < 0.05$.

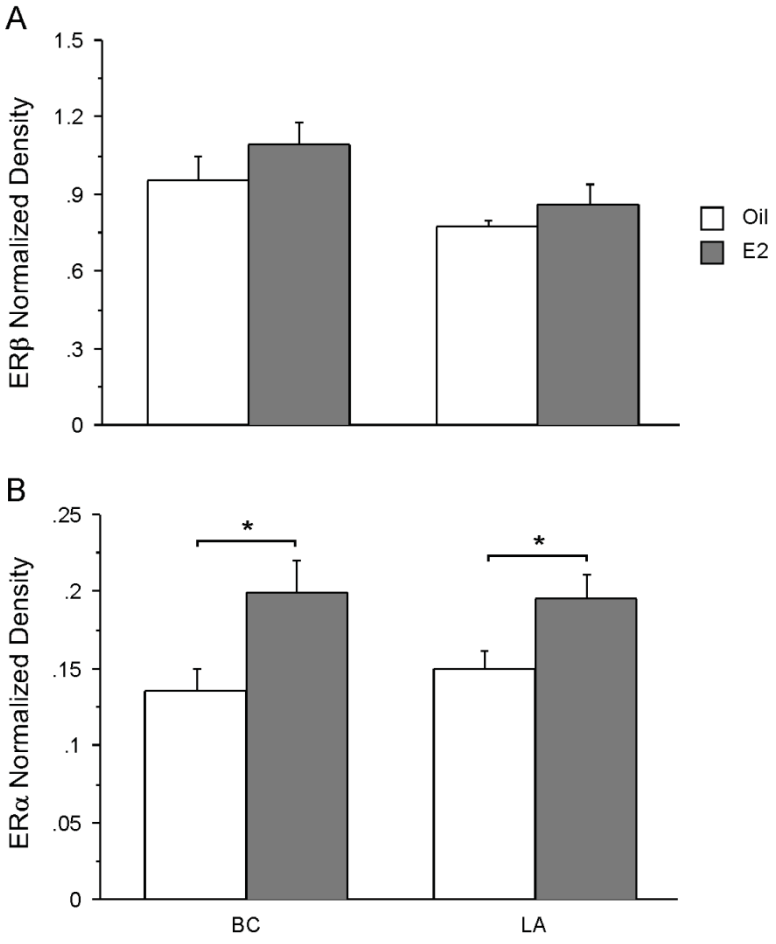
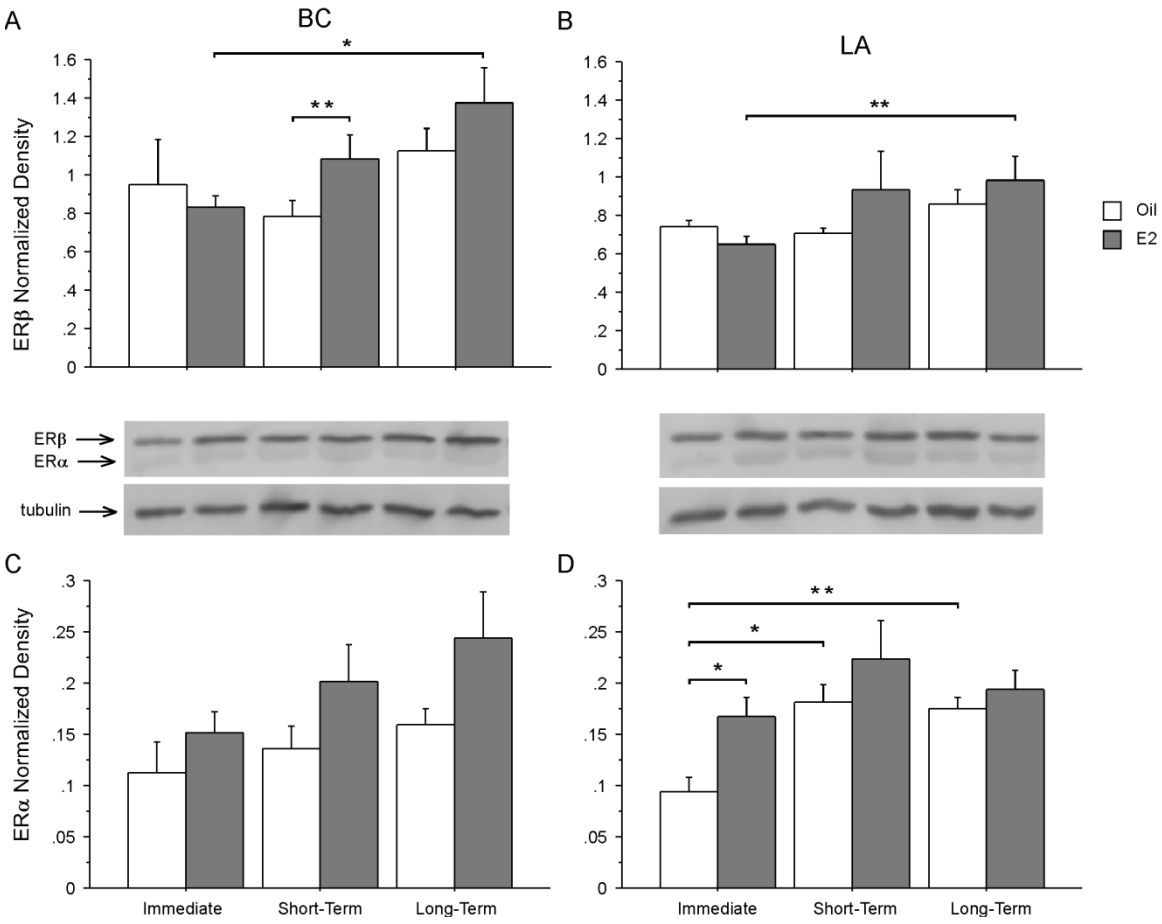

A
1.5
1.2
.9
.6
.3
0
ERβ Normalized Density
Oil
E2
B
.25
.2
.15
.1
.05
0
ERα Normalized Density
*
*
BC
LA

Figure 4.6. The abundance (Mean + SEM normalized optical density) of ERβ (A, B) and ERα (C, D) in the BC (left) and LA (right) muscles of male rats at two weeks after treatment with oil (white) or E2 dissolved in oil (grey) beginning immediately (Immediate), one month (Short-Term), or three months (Long-Term) after castration (n = 8 per group). Representative images of the Western blot and loading control (tubulin) are between the top and bottom graphs. Each band corresponds to the treatment group indicated in the bar graph directly above and below it. E treatment increases ERβ levels in the BC of the Short-Term group and ERα levels in the LA of the Immediate group. * indicates significant difference from each other, $P < 0.05$. $^{\#}$ indicates that the difference approaches significance between the groups, P-value is between 0.1 and 0.05.


A
BC
B
LA
ERβ Normalized Density
1.6
1.4
1.2
1
.8
.6
.4
.2
0
*
**
**
Oil
E2
ERβ
ERα
tubulin
C
D
ERα Normalized Density
.3
.25
.2
.15
.1
.05
0
*
*
**
Immediate
Short-Term
Long-Term

4.4 DISCUSSION

As expected, we found that prolonged androgen deprivation led to the atrophy of both BC and LA muscles, and E2 treatment failed to reverse this muscle shrinkage, regardless of the timing of the treatment relative to castration. Furthermore, the muscle shrinkage was also demonstrated in terms of the thickness of individual muscle fibers, as indicated by higher percentages of muscle fibers with smaller cross-sectional areas in the long-term castrates. At a molecular level, ERα and ERβ levels in both muscles were higher in the long-term castrates than the immediate groups. Following E2 treatment, ERα but not ERβ levels in the BC and LA were elevated. When further analyzed, E2 only elevated ERβ levels in the BC of short-term castrates and ERα in the LA of the immediate castrates, suggesting that long-term androgen deprivation alters the autoregulatory mechanism of ERs in both muscles in a receptor subtype- and muscle-specific manner. In sum, our data indicate that the Critical Period Hypothesis as applied to E sensitivity of neural tissue also applies to PFM that are involved in erection and ejaculation.

4.4.1 PFM MORPHOLOGY

The atrophy of the PFM after castration in the present study is not surprising as the morphology of PFM depends on androgen (Sengelaub and Forger, 2008). Consistent with the muscle shrinkage, castration reduces the muscle fiber diameter in LA muscles, and with a longer period of androgen-deprivation, the muscle shrinkage becomes more prominent. In fact, the androgen-dependence of PFM is known to be greater than in other skeletal muscles because the PFM contain more androgen receptors than other skeletal muscles (Dubois et al., 2012).

Shrinkage of the PFM after castration may contribute to the impairment of both erection and ejaculation with castration. However, the reduced myofibril size *per se* may not be the only factor that interrupts normal PFM function. Other parameters including neuromuscular junction size, number of acetylcholine receptors, excitability of the

muscle, and activity of motoneurons that innervate PFM also decline after castration (Sengelaub and Forger, 2008).

We also found that E2 cannot restore the morphology of the PFM. This finding is in accordance with previous studies showing that dihydrotestosterone, but not E2, is the testosterone metabolite that has anabolic properties in the PFM (Forger et al., 1992; Verhovshek et al., 2010). This finding may explain why androgen-deprived males, who may regain some sexual interest with E2 therapy (Wibowo and Wassersug, 2013a), do not regain erection with the E2 treatment (O'Hanlon et al., 1981).

4.4.2 Autoregulation of Estrogen Receptors in the PFM

We showed that long-term castrates have more ERs in the PFM than those that were castrated more recently. Increases in the expression of ERs may be due to muscle atrophy as the muscle weight and muscle fiber sizes decrease with time after castration (discussed below). Based on our histological data, a large proportion of the muscle fibers in the LA of the long-term castrates were small (thin) muscle fibers. We do not know if the abundance of ER per muscle fiber (density of ERs) declines too as the muscle atrophies.

We noticed that the weight of the BC from the Long-Term rats was approximately 50% less than that of the Immediate rats (Figure 4.1.).When the western blots were run, the same amount of muscle tissue was sampled from the Long-Term and Immediate groups. Assuming that the amount of ERs in each muscle fiber remain unchanged in relation to the time since castration, the amount of ERs in the BC of the Long-Term groups should have been twice that of the Immediate groups (i.e., there should be a 100% increase in the ERs of the Long-Term rats). However, we observed that the ERβ and ERα in the BC of the Long-Term rats were only approximately 30% and 60% higher than those in Immediate groups, respectively (Figure 4.4). This suggests that the ER content in the BC may actually decrease with prolonged castration.

Despite this caveat, to our knowledge, this is the first report showing that ERα, but not ERβ, was increased in both PFMs after E2 treatment. E treatment has been known to cause the autoregulation of ERs and depending on the tissue type, autoinduction or autorepression may occur (Bagamasbad and Denver, 2011). In our case we observed an upregulation.

4.4.3 Critical Period Hypothesis of ER Autoregulation in the PFM

In this study, ERβ levels in the BC were increased only in Short-Term castrates whereas ERα levels in the LA were increased only following immediate E2 treatment after castration; E2 treatment failed to affect ER levels in Long-Term castrates. These findings suggest that prolonged androgen deprivation disrupts the autoregulatory mechanism of ERs in the PFM. Similarly, an absence of ER autoregulation after long-term steroid deprivation has been reported in the female rat hippocampus (Bohacek and Daniel, 2009).

The loss of ER autoregulation in long-term castrates may suggest that the effect of exogenous E on PFM function is attenuated in males that have been androgen-deprived for a long time. However, in the first place, the role of E itself in PFM is not well defined despite the fact that ERs are present in PFM (Dube et al., 1976; Rudolph and Sengelaub, 2013). In castrated male rats, although E can improve PFM excitability (Holmes and Sachs, 1992; Fargo et al., 2003; Foster and Sengelaub, 2004), E does not restore erection (O'Hanlon et al., 1981).

Possibly, E is involved in other PFM functions such as maintaining continence. ER expression in LA is altered in postmenopausal women with stress urinary incontinence (Copas et al., 2001; Zhu et al., 2004), and there is evidence that administering E can improve this condition (Rechberger and Skorupski, 2007). In skeletal muscles other than the PFM, E therapy in post-menopausal women has been shown to influence contractile properties such as twitch characteristics, force generated, muscle fatigue and muscle repair (Enns and Tiidus, 2010). To our knowledge none of these parameters has been studied in the PFM of E-treated castrated males. In addition, proper muscle tone in the

PFM may not only be important in continence function but also in orgasm as PFMs normally contract during orgasm in both sexes (Bohlen et al., 1980; Bohlen et al., 1982).

In conclusion, our study indicates that the Critical Period Hypothesis for E treatment on neural tissues also applies to the PFM. Castration (androgen-deprivation) alters the autoregulatory mechanism of ERs in the PFM. Presumably, the effects of E on the PFM are attenuated after long-term castration. More research is needed to determine the effects of E on PFM functions in males and how the timing of E treatment may influence these effects.

Chapter 5: CHANGES IN ESTROGEN RECEPTOR LEVELS IN THE HIPPOCAMPUS AND PREFRONTAL CORTEX FOLLOWING ESTRADIOL TREATMENT IN CASTRATED MALE RATS: IMPLICATIONS FOR THE CRITICAL PERIOD HYPOTHESIS

Abstract

Administering exogenous estrogen (E) treatment to steroid-deprived males and females has positive effects on various functions. According to the "Critical Period Hypothesis" for females, E administration can only provide benefit when the treatment is started close to the onset of steroid deprivation. Beginning E treatment soon after menopause or ovariectomy maximizes the beneficial effects of E on cognitive, sexual, and cardiovascular functions.

One proposed explanation for the critical period is that the autoregulatory mechanism of estrogen receptors (ERs) is altered after prolonged steroid deprivation, but remains functional when E is administered early. In support of this notion, in female rats, early (but not late) E administration after ovariectomy causes upregulation of ERα in the hippocampus. However, ERα in the prefrontal cortex is upregulated only when E treatment is delayed. Whether the same responses occur in the male rat nervous system had not been explored.

Here we investigated whether the timing of estradiol (E2) treatment after castration in male rats influences the regulation of ERs in the hippocampus and prefrontal cortex. We found that ERα and ERβ protein levels in the hippocampus are downregulated when E2 is administered early (but not late) after castration. In contrast, only delayed E2 treatment downregulated ERα in the prefrontal cortex.

Our data indicate that, as in females, the effects of E2 on the maintenance of ERα in the hippocampus and prefrontal cortex are consistent with the Critical Period Hypothesis. However, unlike in females, ERα in both brain areas is downregulated in response to E2

treatment, suggesting that there are sex differences in the timing at which E2 modulates the expression of ERα in the hippocampus and prefrontal cortex after castration.

Publication Information

This manuscript is currently in preparation for publication. EW, RWC and RJW were involved in study design. EW and HJC ran the experiments and analyzed the data. RWC provided critical feedback on the experimental methods. EW wrote the first draft of the manuscript and all authors provided editorial input.

5.1 INTRODUCTION

Sex steroid deprivation impairs various physiological functions in both adult males and females. Prostate cancer patients on androgen deprivation therapy and post-menopausal or ovariectomized women who have low estrogen (E) levels experience negative symptoms such as hot flashes, osteoporosis, sexual problems, cognitive decline and increased cardiovascular morbidity risks (Wibowo et al., 2011; Scott et al., 2012).

E replacement may reverse some of the deleterious effects of steroid deprivation in both sexes (Wibowo et al., 2011; Scott et al., 2012). Based on the "Critical Period Hypothesis" in females, E only has neuroprotective effects when the treatment is started during a critical period near the onset of menopause or surgical ovariectomy (Scott et al., 2012). Early E, but not delayed E, treatment after steroid deprivation is beneficial for cognitive (Scott et al., 2012), cardiovascular (Scott et al., 2012), and sexual (Czaja and Butera, 1985) functions in females.

The "Critical Period Hypothesis" may be explained by changes in the autoregulatory mechanism of estrogen receptors (ERs) that are likely to occur after long-term steroid deprivation. E binds to ERs to exert its effect and, in order to maintain optimal physiological responses, ERs autoregulate their expression in the presence of E (Bagamasbad and Denver, 2011). In female rats, long-term deprivation of ovarian steroids alters the autoregulatory mechanism of ERα in the hippocampus and prefrontal cortex (PFC) (Bohacek and Daniel, 2009). However, normal ER autoregulation is preserved when E is administered soon after ovariectomy (Bohacek and Daniel, 2009). Whether the timing of E administration impacts the autoregulatory mechanism of ERα in the hippocampus or PFC of castrated male rats was not known.

The hippocampus and PFC are two brain areas involved in cognitive function, which include respectively spatial and working memories (Gillies and McArthur, 2010). Castration in males impairs both hippocampal- and PFC-dependent tasks. However, E treatment may reverse these effects (Gillies and McArthur, 2010). Studying how ERs

change in the hippocampus and PFC after E treatment may bring some insight to how E effects cognitive performance.

In the present study, we investigated the effects of early versus late estradiol (E2) treatment after castration on the expression of ERα and ERβ in the hippocampus and PFC of male rats.

5.2 Materials and Methods

5.2.1 Animals, Surgery, and Oil/Estradiol Administration

Brains were collected from adult male rats that were used in a previous sexual behavioural study (Wibowo and Wassersug, 2013b). The detailed experimental design, surgery, and treatment protocols are described in that paper. In brief, male Long-Evans rats (Charles River Canada, St. Constant, QC, Canada, 275-300 g at the time of arrival) were housed individually under a reversed light cycle (14:10 light:dark) at 23 ± 1ºC ambient temperature. Food and water were available *ad libitum*. Animal handling protocols followed the guidelines of the Canadian Council on Animal Care and were approved by the Dalhousie University Committee on Laboratory Animals. All efforts were made to minimize animal suffering, and to reduce the number of animals used.

The male rats were castrated under anesthesia (104 mg/kg ketamine, 4.8 mg/kg xylazine, and 0.9 mg/kg acepromazine, i.p). Following surgery the rats received an analgesic and an antibiotic (Ketoprofen, 5 mg/kg, s.c., and Baytril, 5 mg/kg, subcutaneous, respectively), and were returned to the animal colony.

The castrated rats were randomly assigned to either oil (as a control) or E2 (dissolved in oil) treatment group (n = 24 per treatment group). Animals in each treatment group were further divided into 3 groups (n = 8 per group) according to when the treatment began after castration: immediately (Immediate), one month (Short-Term), or 3 months (Long-Term).

A Silastic tube (1.6 mm inner diameter, 3.2 mm outer diameter; 35 mm in length; Dow Corning Corporation, Midland, MI) was implanted subcutaneously on the back of each rat. Each tube contained either sesame oil (60 μL; Catalog No. S3547, Sigma-Aldrich) for the oil groups, or 230 μg of 17β-E2 (Catalog No. E8875; Sigma-Aldrich) in 60 μL sesame oil for the E2 groups. This E2 dose was chosen to raise the plasma E2 level of the castrated male rats to a level similar to proestrus E2 levels in female rats (Wibowo and Wassersug, 2013b).

Rats in the Immediate groups were implanted with a Silastic tube immediately after castration during the same surgery. Rats in the Short-Term and Long-Term groups were implanted with a Silastic tube at 1 month and 3 months after castration, respectively, under isoflurane anesthesia (4% for induction, 2% for maintenance; 1 L/min) mixed with oxygen.

5.2.2 Tissue Collection and Preparation

Two weeks after Silastic tube implantation, the male rats were euthanized with anesthetic overdose (208 mg/kg ketamine, 9.6 mg/kg xylazine, and 1.8 mg/kg acepromazine, i.p). Their brains were quickly removed and frozen at -80°C.

Brains were cryosectioned at 300 μm. The entire hippocampus, including both the dorsal and ventral hippocampus, was microdissected from each rat. The prefrontal cortex (PFC) was sampled using a 1.0 mm Harris MicroPunch™ (Catalog No. 69035-10, Electron Microscopy Sciences) according to the technique in Palkovitz and Brownstein (1988). Brain tissue samples were immediately placed in either 40 μL (PFC) or 400 μL (hippocampus) of homogenization buffer (0.32 M sucrose in 0.1 M PBS with one complete mini protease inhibitor tablet (Catalog No. 04 693 124 001, Roche) per 10 mL The samples were homogenized for 2 minutes then centrifuged for 10 minutes at 13,000 X g. Lastly, the supernatant was collected and the Bio-Rad protein assay was used for protein determination.

5.2.3 Western Blot

Approximately 20 µg of protein was mixed with Laemmli sample buffer in a 1:1 ratio and heated at 95°C for 10 minutes. The protein samples were then separated in 7.5% SDS-polyacrylamide gels for 20 minutes at 75 V, followed by 1 hour at 175 V. We performed a multi-strip western blot technique as described by Aksamitiene *et al*. (2007) to quantitatively compare samples from all animals on one membrane. Briefly, we ran 8 gels (each gel contained samples from 6 animals) and divided each gel into two strips depending on their molecular weight ranges: one between 40-60 kDa (c-Fos band was detected in this range) and a second between 60-80 kDa (ERs bands were detected in this range). For each brain area, 8 strips with proteins of the same molecular weight range were assembled on a single filter paper and transferred to a PVDF membrane (IPVH00010, Millipore) over 4 hours at 100 V.

5.2.4 Immunolabeling

All membranes were blocked for 1 hour at room temperature in a solution containing 5% skim milk in Tris-buffered saline with 0.1% TWEEN® 20 (TBST). After blocking, the membranes were incubated in primary antibodies for two days at 4°C. The primary antibodies included: a rabbit polyclonal anti-ERα antibody (ab37438, Abcam; 1:100), a rabbit polyclonal anti-ERβ antibody (ab3577, Abcam; 1:20,000), and a rabbit polyclonal anti-actin antibody (A2066, Sigma-Aldrich; 1:1000). Following primary antibody incubation, the membranes were washed in TBST and then incubated for 1 hour at room temperature in a chicken anti-rabbit IgG-HRP (sc-2963, Santa Cruz Biotechnology; 1:10000). After an additional rinse, the bands were visualized using an ECL 2 kit (Product No. 80196, Pierce) and imaged with a Typhoon 9410 scanner (Amersham Biosciences).

5.2.5 Densitometry and Statistical Analyses

ImageJ 1.46r was used to quantify the density (RawIntDen value) of each protein band. Each protein band density was subtracted by the background density. To obtain a normalized band density, the ERα and ERβ bands were divided by the density of the loading control (actin) band.

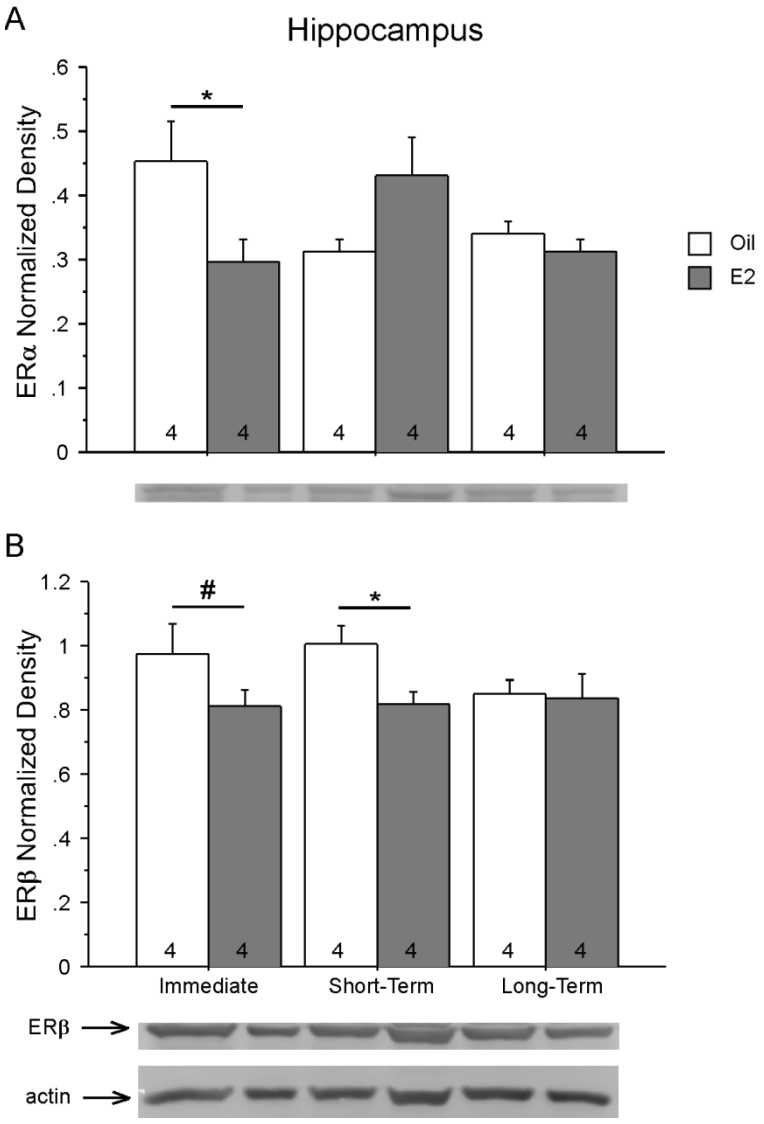
Statview 5.0 (SAS Institute Inc., Cary, NC) was used for all statistical analyses. The Mann-Whitney *U* test was used to compare differences in the normalized density values between treatment groups. To test for the effects of the time intervals between castration and treatment, we performed Kruskal-Wallis tests. Probabilities less than 0.05 were considered statistically significant. We also noted differences that approached statistical significance ($0.05 < P < 0.1$).

5.3 Results

In the hippocampus, after 2 weeks of E2 treatment, the Immediate E2 group had lower ERα levels than the Immediate Oil group (Mann-Whitney test, $P < 0.05$, Figure 5.1A). However, no effects of E2 treatment were observed in the Short-Term or Long-Term groups. In addition, the E2 groups had lower ERβ levels than the Oil groups under the Immediate and Short-Term, but not the Long-Term, conditions. This difference was significant under the Short-Term condition (Mann-Whitney test, $P < 0.05$, Figure 5.1B).

Contrary to the findings in the hippocampus, in the PFC, 2 weeks of treatment had no effects on the levels of ERα or ERβ under either the Immediate or Short-Term conditions (Figure 5.2). However, under the Long-Term condition, ERα (but not ERβ) levels were significantly lower in the E2 group than the Oil group (Mann-Whitney test, $P < 0.01$).

Figure 5.1. The abundance (normalized optical density, mean + SEM) of ERα (A) and ERβ (B) in the hippocampus of male rats after two weeks of treatment with either oil (white) or E2 dissolved in oil (grey) beginning immediately (Immediate), one month (Short-Term), or three months (Long-Term) after castration. The number of rats in each group is indicated at the bottom of each bar. Representative images from the Western blots are shown below; each band corresponds to the bar directly above it. A representative image of the loading control (actin) is shown at the bottom of the figure, in B. ERα was only downregulated in rats receiving the Immediate E2 treatment (A). In addition, E treatment downregulated ERβ levels in the Short-Term rats (B). * indicates that two groups are significantly different from each other, $P < 0.05$. # indicates that the difference between the groups approaches significance, $P = 0.08$.


A
Hippocampus
ERα Normalized Density
.6
.5
.4
.3
.2
.1
0
*
4
4
4
4
4
4
Oil
E2
B
ERβ Normalized Density
1.2
1
.8
.6
.4
.2
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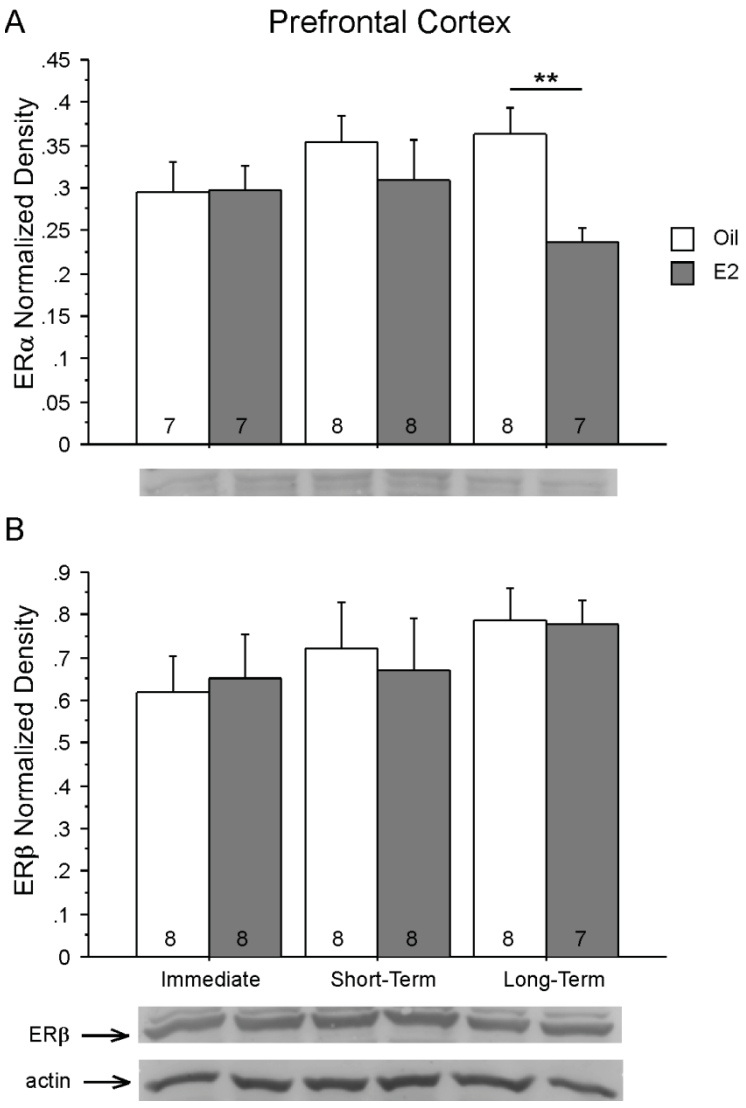
*
4
4
4
4
4
4
Immediate
Short-Term
Long-Term
ERβ
actin

Figure 5.2. The abundance (normalized optical density, mean + SEM) of ERα (A) and ERβ (B) in the PFC of male rats after two weeks of treatment with oil (white) or E2 dissolved in oil (grey) beginning immediately (Immediate), one month (Short-Term), or three months (Long-Term) after castration. The number of rats in each group is indicated at the bottom of each bar. Representative images from the Western blots are shown below; each band corresponds to the bar directly above it. A representative image of the loading control (actin) is shown at the bottom of the figure, in B. ERα was downregulated in the Long-Term rats treated with E2. ** indicates that two groups are significantly different from each other, $P < 0.01$.


A
Prefrontal Cortex
ERα Normalized Density
.45
.4
.35
.3
.25
.2
.15
.1
.05
0
**
Oil
E2
7
7
8
8
8
7
B
ERβ Normalized Density
.9
.8
.7
.6
.5
.4
.3
.2
.1
0
8
8
8
8
8
7
Immediate
Short-Term
Long-Term
ERβ
actin

5.4 DISCUSSION

Our data demonstrate that the timing of androgen deprivation after castration influences the autoregulation of ERα and ERβ in the hippocampus and PFC, and that the effective timing is different between the two brain regions. After castration hippocampal ERs were downregulated after treating E2 to immediate (ERα) and short-term (ERβ) castrates, but not to long-term castrates. In contrast, E2 only downregulated ERα in the PFC of the long-term castrates. These data suggest that the "Critical Period Hypothesis" applies to the autoregulation of ERs in the hippocampus and PFC of male rats but how the duration of androgen-deprivation affects ER autoregulation varies between the two areas.

By comparing our results in male rats to those from female rats (Bohacek and Daniel, 2009), both similarities and differences emerge in how the timing of E2 administration affects the regulation of ERα and ERβ in the PFC and hippocampus. In both male (our study) and female (Bohacek and Daniel, 2009) rats, immediate E2 treatment after gonadectomy leads to the autoregulation of hippocampal ERα, but not when the treatment is delayed. In contrast, ERα in the PFC autoregulates its receptors only when E2 is administered after a long delay (three months) between gonadectomy and treatment. Therefore, in both sexes, prolonged steroid deprivation appears to disrupt the autoregulatory mechanism of hippocampal ERα, but sensitizes the autoregulatory mechanism of ERα in the PFC.

It is important to note that the direction of autoregulation differs between sexes; i.e., following E2 treatment, ERα in the hippocampus and PFC is downregulated in gonadectomized male rats (our study), but upregulated in female rats (Bohacek and Daniel, 2009). The opposite ERα autoregulation patterns between sexes in both brain regions is likely due to sexual differentiation of the brain during fetal development, which causes permanent changes that eventually influence how each sex responds to hormonal treatment in adulthood (McCarthy, 2008). Molecular, anatomical, and biochemical responses to E2 have been documented in both brain areas in both sexes, and there are sex differences. For example, E2 increases dendritic spine density, acetylcholine release

and NMDA receptors in the female hippocampus, but fails to do so in the male hippocampus (Gillies and McArthur, 2010). In addition, there are sex differences in how E2 affects hippocampal- and PFC-dependent cognitive tasks (Gillies and McArthur, 2010).

In term of ERβ, in both sexes E2 administration does not result in substantial changes to the expression of ERβ in the PFC (Bohacek and Daniel, 2009). However, sex difference exists in how ERβ respond to E2 treatment in the hippocampus; i.e., early E2 treatment downregulates ERβ in the male (the present study) but not in female (Bohacek and Daniel, 2009) hippocampus. Treating male rats with ERβ agonists at two weeks post castration has been shown to improve performance in hippocampal-dependent tasks (Lagunas et al., 2011). Whether the same treatment produces similar behavioural results in male rats that have been long-term castrated is not known.

Currently, the mechanisms by which the timing of E2 adminstration in castrated males affects behaviour is not well-understood. The hippocampus and PFC are two areas that are involved in diverse neural functions, most notably cognition. For example, the hippocampus is involved in spatial memory whereas the PFC is involved in working memory (Gillies and McArthur, 2010). Evidence in females supports the "Critical Period Hypothesis" for cognitive function; i.e., beginning E therapy soon after steroid deprivation (e.g., ovariectomy or menopause) preserves memory function, but not when treatment is delayed (Scott et al., 2012). Some men undergo androgen-depriving treatments that indirectly suppress E2—notably advanced prostate cancer patients and male-to-female transsexuals. Cognition in these individuals may be affected due to low circulating steroids (Jamadar et al., 2012), and high dose E treatments may be cognitively benefial (cf. Beer et al. (2006) for prostate cancer patients and Miles et al. (Miles et al., 1998; Miles et al., 2006) for the transsexuals). However, whether early E administration after androgen suppression is better than late treatment in improving cognitive function in androgen-deprived males remains uninvestigated (Wibowo et al., 2011).

In conclusion, our study supports the "Critical Period Hypothesis," suggesting that the effect of E2 on the autoregulation of ERs in the hippocampus and PFC depends on when the treatment is started after castration. Furthermore, we show that there is a sex difference in how ERs from both the hippocampus and PFC autoregulate themselves in response to E2. These findings suggest that sex difference exists in how E modulates hippocampal- and PFC-dependent behaviours. However the extent to which early versus late E2 administered to castrated males affects hippocampal- and PFC-dependent neural processes remains to be investigated.

Chapter 6: ESTRADIOL TREATMENT MODULATES SPONTANEOUS SLEEP AND RECOVERY SLEEP AFTER SLEEP DEPRIVATION IN CASTRATED MALE RATS

Abstract

Exogenous estradiol (E2) is used occasionally to treat the side effects associated with androgen-deprivation in men, but its effects on sleep patterns have received little attention. We examined whether E2 modulates sleep patterns and recovery from sleep loss in castrated male rats. Adult male rats were castrated and implanted subcutaneously with Silastic tubes containing either oil (Oil) or E2 dissolved in oil (E2). Sham-operated male rats (Intact) were implanted with oil-filled tubes. All rats were also implanted with EEG and EMG electrodes for sleep/wake recordings. After two weeks, polysomnographic recordings were made before, during, and following 6 h of sleep deprivation (SD). At baseline, the Oil group showed sleep and EEG patterns similar to those in the Intact group. Compared to these groups, the E2 group spent more time awake during the dark (active) phase, and showed higher EEG theta power (a measure of cortical activation) during wake and rapid eye movement (REM) sleep in both the light and dark phases. Following SD, the E2 group showed a larger increase from baseline in REM sleep amount, compared to the Oil group. The Oil group showed prolonged rebound in non-REM sleep and EEG delta power, and reduced REM sleep rebound, compared to the other two groups. These results indicate that E2 treatment in castrated male rats promotes baseline wakefulness during the active phase, and facilitates recovery of REM sleep after acute sleep loss. The possible benefit of E2 treatment for improving sleep quality in androgen-deprived men remains to be investigated.

Publication Information

This chapter has previously been published as: Wibowo E, Deurveilher S, Wassersug RJ, Semba K (2012) Estradiol treatment modulates spontaneous sleep and recovery after sleep deprivation in castrated male rats. Behav Brain Res 226:456-464. EW performed the experiments, analyzed the data and prepared the manuscript.

6.1 INTRODUCTION

Men on androgen deprivation therapy (ADT) for the treatment of prostate cancer commonly reported sleep disturbances and daytime fatigue (Stephens et al., 2007; Kyrdalen et al., 2010; Hanisch et al., 2011). Estradiol (E2) is occasionally prescribed to them to alleviate some of the side effects of this treatment (Engstrom, 2008; Wibowo et al., 2011). In another population of androgen-deprived genetic males, namely male-to-female transsexuals, high doses of E2 increase light (stage 1) non-rapid eye movement (NREM) sleep, but do not affect deeper stages of NREM or REM sleep (Kunzel et al., 2011). In light of the fact that sleep problems are common for prostate cancer patients on ADT (Stephens et al., 2007; Hanisch et al., 2011) and that E2 therapy is used to facilitate transitioning to female for male-to-female transsexuals (Wassersug and Gray, 2011), we set out to investigate how E2 might affect sleep patterns in castrated male rats, a rodent model of androgen-deprived males.

Although circulating levels of E are much lower in males than in females, E2 can be locally produced from circulating testosterone by aromatizing enzymes synthesized in the brain (Naftolin et al., 1975). Increasing evidence supports a role of E2 in a variety of functions in both male and female brains, including synaptic plasticity, neurotransmission, and neuroprotection (Gillies and McArthur, 2010). These actions of E2 are likely mediated by both nuclear and membrane estrogen receptors (ERs) that are widely distributed in both the male and female brains, including sleep-wake regulatory nuclei such as the basal forebrain, hypothalamus/preoptic area, dorsal raphe nucleus and locus coeruleus (Simerly et al., 1990; Shughrue and Merchenthaler, 2001).

We investigated the effects of E2 treatment on baseline sleep, as well as recovery sleep after acute (6 h) SD, in castrated male rats that were subcutaneously implanted with E2-containing capsules that provided stable and relatively high levels of hormonal release. The stable hormonal levels allowed us to study the baseline sleep and recovery from SD against the same hormonal background. Previous studies from our laboratory using similar E2 treatment in ovariectomized female rats showed that E treatment promoted

baseline wakefulness and, following 6 h of SD, facilitated rapid-eye-movement (REM) sleep rebound while reducing sleep intensity (Deurveilher et al., 2009, 2011). In light of the sexual differentiation of the brain by gonadal hormones (McCarthy, 2008), we also asked whether the effects of E2 on sleep depend on the animal's sex.

6.2 Materials and Methods

6.2.1 Animals

Adult male Wistar rats (Charles River Canada, St. Constant, QC, Canada), weighing 250-350 g at the time of surgery, were housed under a 12/12 h light/dark cycle (lights on at 07:00 am) at 23 ± 1ºC ambient temperature, with rat chow and water available *ad libitum*. Animal handling protocols followed the guidelines of the Canadian Council on Animal Care and were approved by the Dalhousie University Committee on Laboratory Animals.

Rats were randomly assigned to 3 treatment groups (n = 7 per group): gonad-intact, sham-operated rats with oil-filled implants (Intact), castrated rats with oil-filled implants (Oil), and castrated rats with E2-filled implants (E2). As described in more detail below, E was delivered using sesame oil as the vehicle through a subcutaneous Silastic implant to provide a stable hormonal release (Deurveilher et al., 2009).

6.2.2 Surgery

Rats were anesthetized with a combination of 104 mg/kg ketamine, 4.8 mg/kg xylazine, and 0.9 mg/kg acepromazine, i.p. For castration, bilateral incisions were made through the skin and the dartos muscle, and the testes gently extruded through the incisions. The blood vessels and spermatic cord to each testis were clamped with hemostats, tied off with absorbable sutures, and excised distal to the ligature. The muscle layer of the scrotal wall was sutured and the skin closed using surgical adhesive. For sham operation (Intact group), an incision was made through the skin and the dartos muscle. Both layers were then sutured, leaving the testes intact.

After castration or sham operation and still under anesthesia, a small incision was made through the skin on the rat's back, and a Silastic implant (1.57 mm inner diameter, 3.18 mm outer diameter; 35 mm in length; Dow Corning Corporation, Midland, MI) was inserted subcutaneously. Each implant was pre-filled with either sesame oil (60 μL; Catalog No. S3547, Sigma-Aldrich, St Louis, MO) for the Intact and Oil groups, or 230 μg of 17β-E2 (Catalog No. E8875; Sigma-Aldrich) in 60 μL sesame oil for the E2 group. The dosage of E2 was established based on a pilot study that used castrated rats implanted with Silastic implants containing oil (n = 1) or 17β-E2 at 10.5 μg, 60 μg, and 230 μg (n = 4 rats per dose). These E doses were chosen based on our previous study using female rats (Deurveilher et al., 2009). The highest dose (230 μg) provided plasma E2 levels (21.9 pg/mL) similar to the E2 levels observed during proestrus in females, and was used in the present study. [Of note, male rats required a considerably higher dose of E2 than females (230 μg vs. 60 μg as in Ref. (Deurveilher et al., 2009)) to achieve similar plasma E2 levels]. The need for a higher dose may reflect higher metabolic rates of E2 in males than in females (Maggs et al., 1992) and higher body weights (mean of 309 g vs. 238 g for ovariectomized females used by Deurveilher et al. (2009)). Each implant was incubated in saline at 37°C for 24 hours before implantation to prevent an initial surge of estradiol (Karsch et al., 1973). After implantation, the skin incision on the back was sutured.

While still under anesthesia, each animal was placed in a stereotaxic apparatus and implanted with 2 miniature stainless steel screws serving as EEG electrodes, one over the frontal cortex (1 mm rostral to bregma and 2 mm right of the midline) and the other over the occipital cortex (6 mm caudal to bregma and 2 mm left of the midline). A third screw was placed over the cerebellum to serve as a ground electrode (3 mm caudal to lambda). A pair of fluorocarbon-coated stainless steel wires with a 2–3 mm exposure was inserted into the dorsal neck muscles to record the electromyogram (EMG). All electrodes were connected to a small connector (Plastics One Inc., Roanoke, VA) and the complete assembly was anchored to the skull with dental acrylic.

Following surgery, rats were given subcutaneous injections of the analgesic Ketoprofen (5 mg/kg) and the antibiotic Duplocillin (0.15 mL). All rats were monitored as they emerged from anesthesia. They were then returned to the animal colony where they were housed singly for further recovery.

6.2.3 Habituation and Polygraphic Recording

Ten days after surgery, rats were transferred individually to a clear Plexiglass chamber (40 × 30 × 40 cm) placed inside an individual sound-proof cubicle that was equipped with a fan and an incandescent light controlled by a timer that maintained the same 12/12 h light/dark cycle as in the colony room. On the next day, rats were connected to a flexible recording cable attached to a commutator (Plastics One Inc.) and remained connected for 3 days to adapt to the recording chamber and cable set-up prior to polygraphic recording.

A 24 h baseline EEG/EMG recording started at the mid-light phase at 1:00 pm. This was followed by 6 h of SD and a 42 h recovery period, for a total of 72 h of recording. For SD, gentle handling was used as in our previous study (Deurveilher et al., 2009). When behavioural signs of sleepiness were observed (i.e., the rats became immobile, adopting a sleep posture) or when slow waves were apparent in the EEG, the rats were kept awake by various interventions, including tapping the cage, introducing novel objects (i.e., plastic toys) into the cage, gently shuffling the bedding, and slowly moving the litter tray.

All EEG and EMG signals were amplified, band pass-filtered (EEG: 0.3–100 Hz; EMG: 10–100 Hz; Grass Telefactor, West Warwick, RI), and digitized at 256 Hz. The signals were acquired by using SleepSign (Kissei America, Irvine, CA) and stored on a computer for off-line analysis.

6.2.4 Sleep-Wake Scoring and Data Analyses

Behavioural states were scored automatically using the SleepSign software in consecutive 10-sec epochs with each epoch identified as wakefulness, NREM sleep, or REM sleep. Low-voltage, fast EEG activity with moderate to high EMG activity identified wakefulness. NREM sleep was defined by high-voltage, slow EEG activity, often dominated by delta waves (0.5-4 Hz), and low amplitude EMG. REM sleep was identified by EEG activity dominated by theta waves (4.5-8 Hz) with very low EMG activity with occasional phasic activities indicating muscle twitches. The automatic scoring was inspected visually off-line; in case of disagreement between visual and automatic scoring, scores from visual examination were used.

EEG power spectra in 0.5 Hz bins were obtained using fast Fourier transform (FFT; Hanning window) in 2-sec windows during artifact-free wake, NREM, and REM sleep epochs. The power values were then tallied in the following frequency ranges: delta (0.5-4 Hz), theta (4.5-8 Hz), sigma (8.5-13 Hz), beta (13.5-30 Hz), and gamma (30.5-50 Hz). Power values were averaged over a 10-sec epoch and expressed as absolute values, with the exception of the analysis of the time course of EEG delta power which used values normalized to the mean over a 24 h period to reduce inter-individual variability.

6.2.5 Blood Collection and Radioimmunoassay for Estradiol

At the end of the 72 h recording period, rats were given an overdose of anesthetics (208 mg/kg ketamine, 9.6 mg/kg xylazine, and 1.8 mg/kg acepromazine, i.p.). Blood samples were obtained by cardiac puncture in heparinized tubing and then centrifuged at 3000 rpm for 10 min. Plasma was collected and kept frozen at −80°C for later radioimmunoassay. Plasma concentration of E2 was determined by using commercial kits (DSL 4800 Ultra-Sensitive Estradiol RIA kit; Beckman Coulter). The detection limit of the assays was 3.3 pg/mL. All samples were assayed in duplicate (intra-assay coefficient of variation = 6%) and all assays were conducted in a single session.

6.2.6 Statistical Analyses

Statistical analyses were conducted with Statview 5.0 (SAS Institute Inc., Cary, NC) and SPSS 17.0 (SPSS Inc., Chicago, IL). Plasma 2E levels were analyzed using a non-parametric Kruskal-Wallis test. Sleep-wake parameters were analyzed using a one-way ANOVA. Differences between baseline and recovery in each group were assessed using one-way repeated measures ANOVA. Post hoc Tukey and Dunn's multiple comparison analyses were used to assess comparisons when main effects were significant in ANOVA and Kruskal-Wallis test, respectively. Probabilities of < 0.05 were considered statistically significant.

6.3 Results

6.3.1 Plasma E2 levels and Body Weight Gain

At the end of the experiment (i.e., 16-17 days after surgery), the E2 group had significantly higher E2 levels (26.0 [19.0] pg/mL; median [IQR]) than the Intact (6.1 [7.1] pg/mL) and Oil (6.4 [7.0] pg/mL) groups (Kruskal-Wallis test, $H = 10.4$, $P < 0.01$; E2 > Intact and Oil, $P < 0.05$). Conversely, the E2 group had a smaller gain in body weight (1 ± 2%, mean ± SEM) than the Intact (16 ± 4%) and Oil (13 ± 3%) groups ($F_{2,18} = 8.6$, $P < 0.005$; E2 < Intact and Oil, $P < 0.05$).

6.3.2 Baseline Amounts of Sleep-Wake States

Under baseline conditions, the Oil group showed similar amounts of wake, NREM, and REM sleep compared to the Intact group, either across the 24 h period or separately during the 12 h light or the 12 h dark phase (Figure 6.1). In contrast, the E2 group spent significantly more time awake (+79 to 86 min) and less time in NREM sleep (−85 to 90 min) than the other two groups during the 24 h baseline period (Group: $F_{2,18} = 5.7$ and 5.5, $P < 0.05$, for wake and NREM sleep, respectively; $P < 0.05$ vs. Intact and Oil; Figure 6.1A). The increase in wake and decrease in NREM sleep in the E2 group were

prominent during the dark phase (Group: $F_{2,18} = 6.7$ and 6.4, $P < 0.01$, respectively; $P < 0.05$ vs. Intact and Oil; Figure 6.1C). During the light phase, there were no significant differences in the amounts of wake or NREM sleep among the three groups (Figure 6.1B).

The amount of REM sleep over the 24 h baseline period did not significantly differ among the groups (Figure 6.1A). However, when examined separately for the light and dark phases, the E2 group had slightly but significantly less REM sleep (−6 min) than the Oil group during the dark phase (Group: $F_{2,18} = 4.5$, $P < 0.05$; $P < 0.05$ vs. Oil; Figure 6.1C). As a result of the decreases in the amounts of NREM and REM sleep in the dark phase, the E2 group had a significantly higher light:dark ratio for both NREM and REM sleep than the Oil group ($P < 0.05$ vs. Oil; Figure 6.1D).

6.3.3 Baseline Mean Duration and Numbers of Sleep-Wake Episodes

The increase in the amount of wakefulness in the E2 group was due to an increase in the mean duration, not the number, of wake episodes. During the 24 h baseline period, the E2 group had longer episodes of wakefulness (by 15-17%) than the other two groups (Table 6.1), and this increase was significant between the E2 and Intact groups (Group: $F_{2,18} = 4.1$, $P < 0.05$; $P < 0.05$ vs. Intact). These increases in wake episode duration occurred predominantly during the dark period (Table 6.1).

Conversely, the decrease in the amounts of NREM and REM sleep in the E2 group was associated with a non-significant decrease in the number of NREM sleep episodes (by 20% vs. Intact; Table 6.2) and in the mean duration of REM sleep episodes (by 6% vs. Intact; Table 6.1), respectively, during the dark phase.

6.3.4 Baseline EEG Power Spectra

The Oil group showed EEG power spectra similar to those from the Intact group during wake, NREM, and REM sleep in both the light and dark phases (Figure 6.2). In contrast,

the E2 group generally had higher theta (4.5-8 Hz) power values than the other two groups during wake and REM sleep. Specifically, in the dark phase, the E2 group had significantly higher theta power values than both the Intact and Oil groups during wake (+43 to 44%) and REM sleep (+70 to 102%; E2 > Intact and Oil, $P < 0.05$ for both wake and REM sleep; Figure 6.2D and F). Similarly, in the light period, the E2 group had higher EEG theta power (+53%) than the Intact group during wake ($P < 0.05$ vs. Intact; Figure 6.2A), and higher theta (+89 to 108%), as well as sigma (+38 to 39%), values than the Intact and Oil groups during REM sleep (E2 > Intact and Oil, $P < 0.05$; Figure 6.2C).

6.3.5 Sleep Deprivation

During the 6 h SD period, all groups were awake for 97-98% of the time. In each group, NREM sleep occurred for only 3-6 min in total on average, while REM sleep was completely absent. The number of interventions required to keep the rats awake increased across the hours in each group ($F_{5,90} = 56.36$, $P < 0.0001$), but the time course of this increase was similar among groups (Group × 1 h time interval, n.s.). Over the total 6 h of SD, the E2 group tended to require fewer interventions (88 ± 19, mean ± SEM) than the Intact (118 ± 22) and Oil (96 ± 10), but there was no significant group difference.

6.3.6 Recovery Sleep/Wake States following Sleep Deprivation

In the 12 h dark phase immediately following SD, all groups showed a significant increase in the amount of both NREM and REM sleep, compared to their time-matched 12 h baseline period. This sleep rebound was most prominent during the first 3 h period of the dark phase in all groups (Figure 6.3).

The E2 group had a smaller absolute amount of NREM sleep (−59 to 66 min over 12 h) than the other two groups during the recovery dark phase (Group: $F_{2,18} = 6.7$, $P < 0.01$; $P < 0.05$ vs. Intact and Oil; Figure 6.4B), which paralleled their reduced NREM sleep during the baseline dark phase (Figures 6.1C and 6.4B). However, the relative increase in NREM sleep from baseline was not significantly different among the three groups (+49

to 70 %; Figure 6.4D). The rebound increase in the amount of NREM sleep was due to an increase in the duration of NREM sleep episodes in all groups (+41 to 56 %). Compared to the Intact and E2 groups, the NREM sleep rebound in the Oil group lasted longer, with significant differences from baseline levels at the first and second 3 h points (Figure 6.3B middle panel).

Similar to NREM sleep time, the amount of REM sleep greatly increased after SD during the first 3 h of the dark phase in both the Intact and E2 groups (Figure 6.3C left and right panels). The amount of REM sleep thereafter declined but remained elevated above baseline levels, reaching statistical significance in the third 3 h interval in these two groups ($P < 0.05$ vs. baseline; Figure 6.3C left and right panels). In contrast, in the Oil group, the REM sleep amount did not show a significant increase from baseline during any 3 h interval after SD (Figure 6.3C middle panel).

Quantitatively, in contrast to NREM sleep, the absolute amount of REM sleep was similar among the three groups during the 12 h recovery dark phase (Figure 6.4C). However, the relative increase from baseline was higher in the E2 group (+127%) than the Oil group (+64%; $P < 0.05$; Figure 6.4D). The increase in the amount of REM sleep during the recovery dark phase was due to an increase in the number of REM sleep episodes in all groups (+46 to 69%), as well as in the duration of REM sleep episodes in the Intact and E2 groups (+18 to 42%; Tables 6.1 and 6.2).

Converse to the increases in NREM and REM sleep, the amount of wakefulness decreased below baseline levels during the recovery dark phase in all groups (Figures 6.3A and 6.4A). These decreases were due to a reduction in the duration of wake episodes in all groups (−20 to −28%; Table 6.1).

In the subsequent light phase (i.e., 12-24 h post-SD), there were no group differences in the amount of wake, NREM sleep, or REM sleep (data not shown).

6.3.7 Recovery EEG Power Spectra

All groups showed an increase in normalized NREM EEG delta power relative to baseline values in the first 3 h of the dark phase following SD ($P < 0.05$; Figure 6.5) but there was no significant group difference in the levels of NREM delta power. In the Intact (Figure 6.5A) and E2 groups (Figure 6.5C), NREM delta power returned to baseline in the second 3 h recovery period, whereas in the Oil group, the significant increase in delta power persisted in the second 3 h interval, albeit to a lesser degree, and returned to baseline levels in the third 3 h interval (Figure 6.5B). During the first 6 h of the next light phase (12-18 h post-SD), in the Intact group, NREM delta power was significantly lower than baseline levels (negative delta rebound) (Figure 6.5A). In addition, during the first 3 h of the next light phase (12-15 h post-SD), the E2 group had significantly higher NREM delta power than the other two groups (both $P < 0.05$; Figure 6.5C).

The baseline group differences in EEG power spectra during wakefulness and REM sleep largely persisted through the dark and light recovery periods (Supplementary Figure 6.1). Thus, compared to the Intact or Oil group, the E2 group had significantly higher REM theta power during both the dark (+112% vs. Intact) and light (+104% vs. Intact; +44% vs. Oil) phases ($P < 0.05$), and higher wake theta power (+59%) and REM sigma power (+29%) during the light phase ($P < 0.05$).

Figure 6.1. Amounts (min) of wake, non-rapid eye movement (NREM) sleep, and REM sleep during baseline recordings in intact and castrated male rats treated with oil or estradiol. Data are shown for the 24 h period (A), 12 h light phase (B), and 12 h dark phase (C), and for the light/dark ratio for each sleep-wake state (D). All values are expressed as means + standard error of the mean [SEM], with n = 7 per group. A-C: The gonad-intact males treated with oil (Intact, black) and the castrated males treated with oil (Oil, white) had similar amounts of wake, NREM and REM sleep in all the analyses. The castrated males treated with estradiol (E2, grey) spent more time in wakefulness and less time in NREM and REM sleep than the other two groups, particularly in the dark phase. # Significantly different from Intact; * different from Oil. All $P < 0.05$ (Tukey post hoc comparisons). D: There was a significant group difference in the ratio for NREM and REM sleep ($F_{2,18} = 5.8$ and 3.8, $P < 0.05$, respectively), and the E2 group had a significantly higher light/dark ratio than the Oil group for both sleep states. * Different from Oil, $P < 0.05$ (Tukey post hoc comparison).

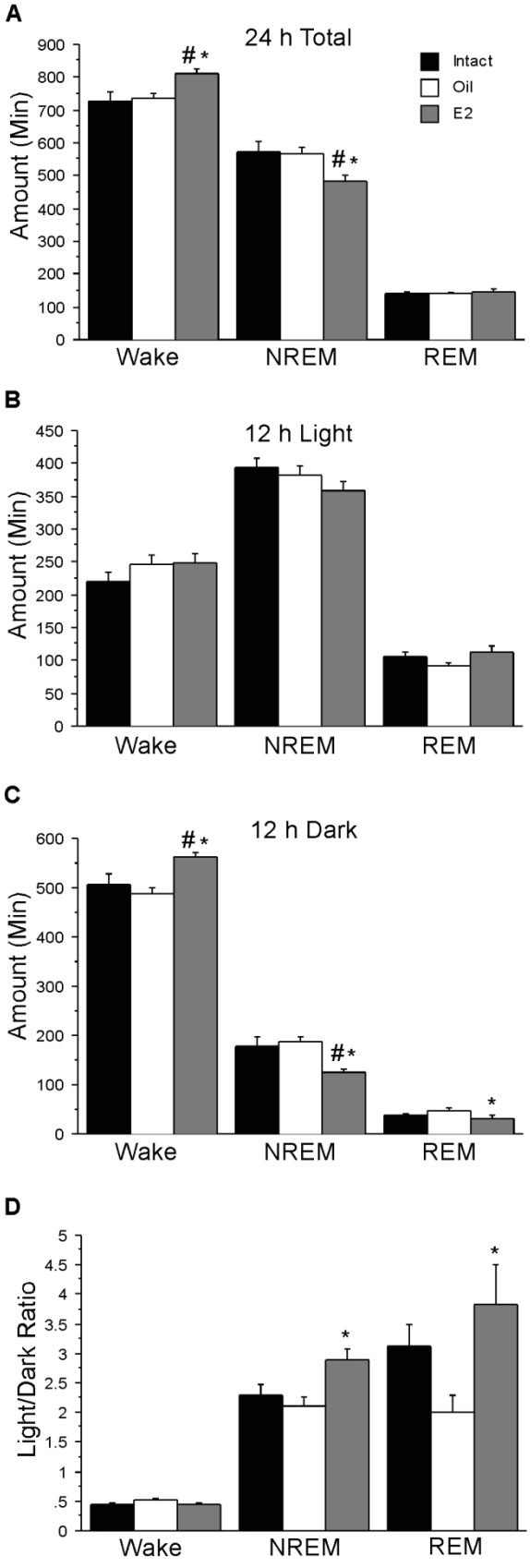

A
24 h Total
Intact
Oil
E2
Amount (Min)
900
800
700
600
500
400
300
200
100
0
#*
#*
Wake
NREM
REM
B
12 h Light
Amount (Min)
450
400
350
300
250
200
150
100
50
0
Wake
NREM
REM
C
12 h Dark
Amount (Min)
600
500
400
300
200
100
0
#*
#*
*
Wake
NREM
REM
D
Light/Dark Ratio
5
4.5
4
3.5
3
2.5
2
1.5
1
.5
0
*
*
Wake
NREM
REM

Figure 6.2. EEG power (mean + SEM, μV^2) in five frequency bands in baseline wake (A and D), NREM (B and E), and REM (C and F) sleep during the 12 h light (top row) and 12 h dark phases (bottom row) in the Intact (black), Oil (white), and E2 groups (grey) (n = 7 per group). In the dark period, the E2 group had higher theta power (4.5-8 Hz) during wake and REM sleep than the Intact and Oil groups (Group: $F_{2,17} = 4.6$ and 8.7, $P < 0.05$ and < 0.005, respectively, for wake and REM sleep). Likewise, in the light period, the E2 group had higher wake theta power than the Intact group (Group: $F_{2,17} = 4.8$, $P < 0.05$), and higher REM theta power than both the Intact and Oil groups (Group: $F_{2,17} = 11.5$ and 5.3, $P < 0.01$ and < 0.05, respectively). # Different from Intact; * different from Oil. All $P < 0.05$ (Tukey post hoc comparison).

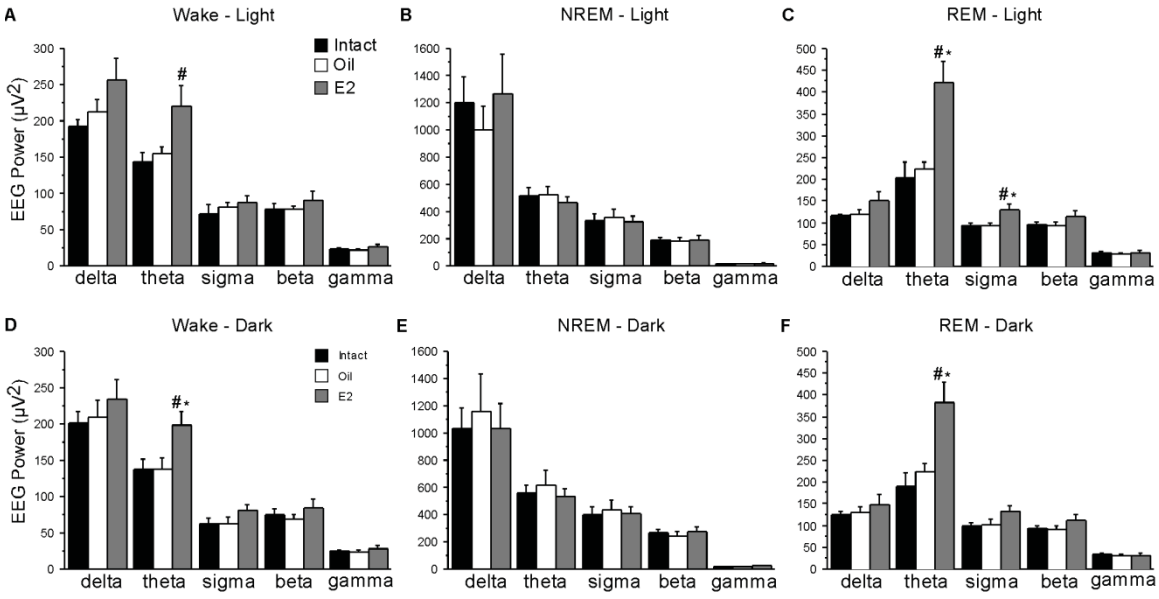

A
Wake - Light
Intact
Oil
E2
EEG Power (μV2)
delta
theta
sigma
beta
gamma
B
NREM - Light
C
REM - Light
D
Wake - Dark
E
NREM - Dark
F
REM - Dark

Figure 6.3. Time course of wake (A), NREM (B) and REM sleep (C) amounts (mean ± SEM, min) in 3 h intervals across the 24 h baseline period (white), 6 h sleep deprivation (SD) period, and following 18 h recovery period (black) in the Intact (left), Oil (middle) and E2 (right) groups (n = 7 per group). A and B: In all groups, wake amounts (A) decreased, while NREM amounts (B) increased, after SD compared to baseline. The Oil group showed significantly higher NREM sleep amounts relative to baseline during the first 6 h of recovery, while the increased NREM sleep amount lasted only for the first 3 h in the other two groups. C: In contrast, the Intact and E2 groups showed REM sleep rebound at most 3 h intervals during the initial 12 h of recovery, while the Oil group showed values similar to baseline. The thick black bar above the x-axis indicates the 12 h dark phase, whereas the thinner black bar at the top of each panel indicates the SD period for 6 h. * Different from corresponding baseline. All $P < 0.05$ (Tukey post hoc comparison).

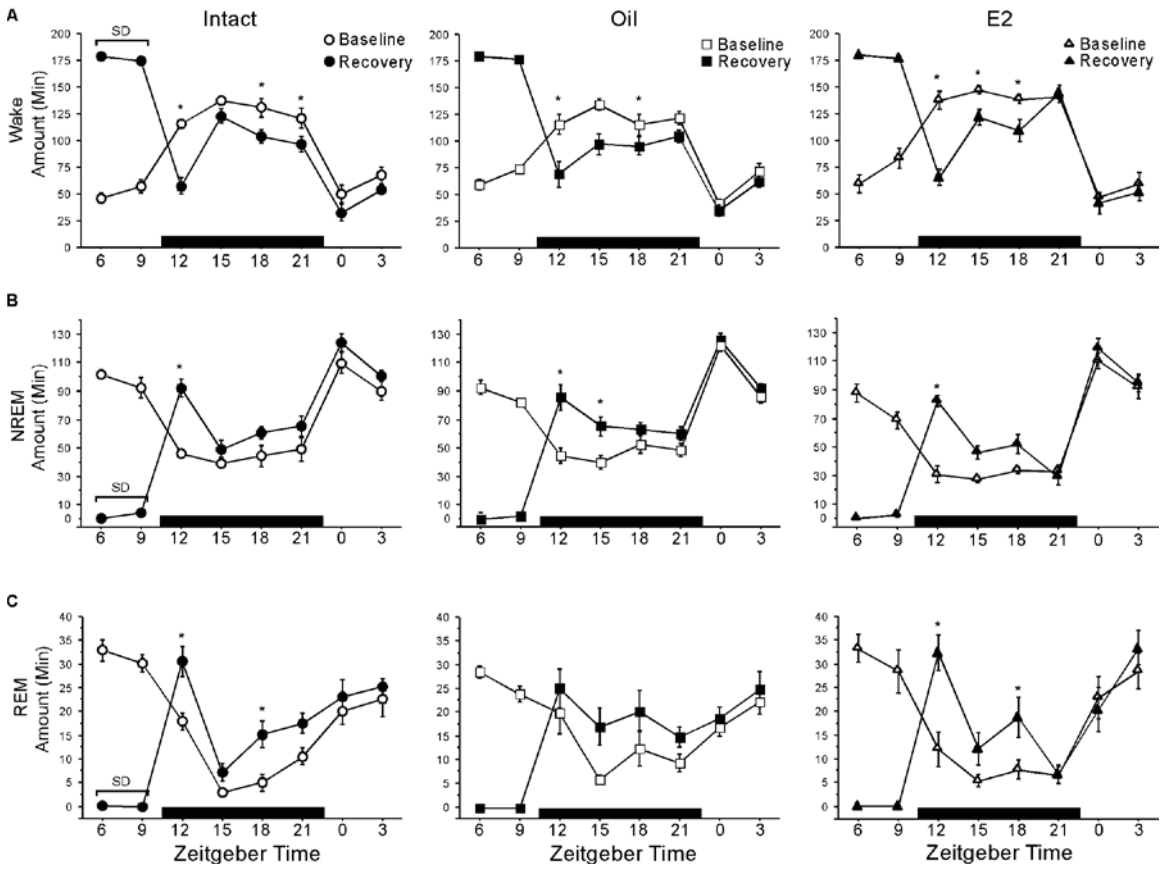

A
Intact
Oil
E2
SD
Baseline
Recovery
Wake
Amount (Min)
B
NREM
Amount (Min)
C
REM
Amount (Min)
Zeitgeber Time
Zeitgeber Time
Zeitgeber Time

Figure 6.4. Amount (mean + SEM, min) of wake (A), NREM (B) and REM sleep (C) during the 12 h recovery dark phase immediately after 6 h of SD (dashed bars) and the corresponding 12 h baseline dark period (white bars), as well as the percentages of change (D), in the Intact (left), Oil (middle), and E2 groups (right) (n = 7 per group). A-C: All groups showed a rebound in NREM and REM sleep amounts following SD (B and C). While the recovery REM sleep amount was similar among the groups, the recovery NREM amount was lower in the E2 group than the other two groups, as was the case during the baseline period. [a] Different from corresponding baseline; # = different from Intact; * different from Oil, $P < 0.05$ (Tukey post hoc comparison); $P < 0.05$ (paired t-test). D: Percentage of change from baseline in the amount of wake, NREM and REM sleep during the 12 h recovery dark phase. A significant group difference was found for REM sleep only ($F_{2,18} = 3.6$, $P < 0.05$). The E2 group had a larger REM sleep rebound relative to baseline than the Oil group. * Different from Oil, $P < 0.05$ (Tukey post hoc comparison).

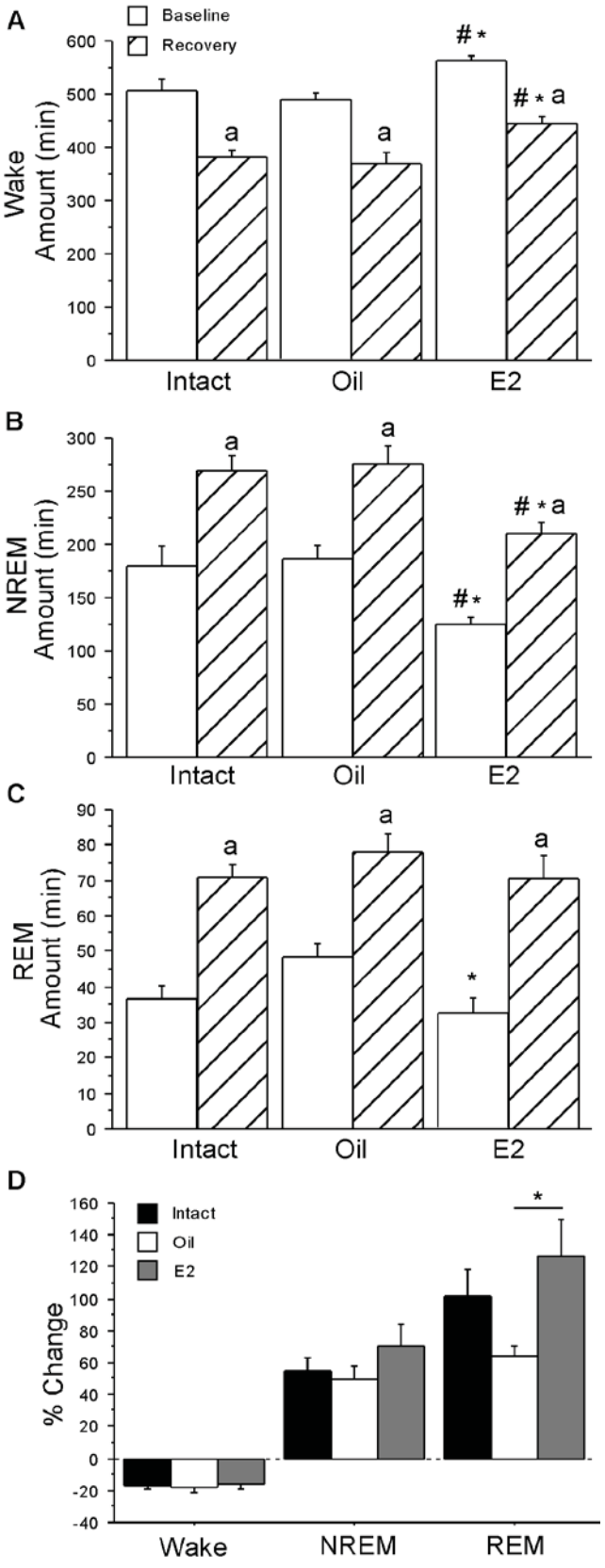

A
Baseline
Recovery
Wake Amount (min)
600
500
400
300
200
100
0
a
a
#*
#*a
Intact
Oil
E2
B
NREM Amount (min)
300
250
200
150
100
50
0
a
a
#*
#*a
Intact
Oil
E2
C
REM Amount (min)
90
80
70
60
50
40
30
20
10
0
a
a
a
*
Intact
Oil
E2
D
% Change
160
140
120
100
80
60
40
20
0
-20
-40
Intact
Oil
E2
*
Wake
NREM
REM

Figure 6.5. Time course of normalized NREM EEG delta power (mean ± SEM) in 3 h intervals across the 24 h baseline period (white) and during the 18 h recovery period (black) immediately following 6 h of SD in the Intact (A), Oil (B), and E2 (C) groups. NREM delta power was normalized to the 24 h average baseline at 3 h intervals. All groups showed a large rebound in NREM delta power in the first 3 h of recovery. The Oil group showed significantly elevated NREM delta power compared to baseline during the first 6 h of recovery (B), while the increased NREM delta power lasted only for the first 3 h in the other two groups (A and C), as was the case for NREM sleep amounts (see Figure 6.3). In the Intact group, but not the other two groups, there was a decrease in delta power below baseline levels ("negative rebound") during the first 6 h of the subsequent light phase (starting 12 h post-deprivation). The black bar on the x-axis indicates the dark phase.* Different from corresponding baseline, $P < 0.05$ (Tukey post hoc comparisons).

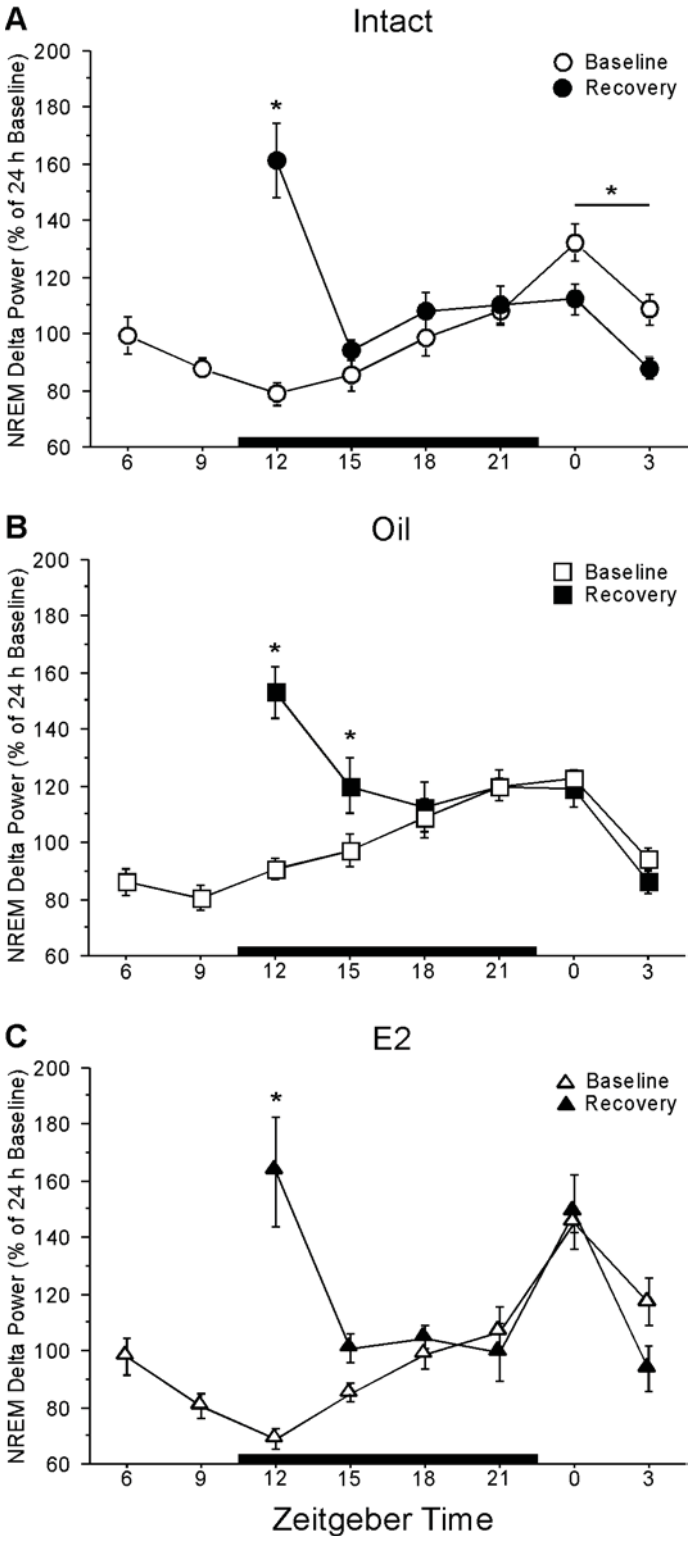

A
Intact
Baseline
Recovery
NREM Delta Power (% of 24 h Baseline)
200
180
160
140
120
100
80
60
6
9
12
15
18
21
0
3
*
*
B
Oil
Baseline
Recovery
NREM Delta Power (% of 24 h Baseline)
*
*
C
E2
Baseline
Recovery
NREM Delta Power (% of 24 h Baseline)
*
Zeitgeber Time

Table 6.1. Mean duration (s) of episodes of wake, NREM and REM sleep during baseline and recovery sleep after sleep deprivation

Stage, Group, Condition	24 h Total	12 h Light	12 h Dark
Wake			
Intact			
Baseline	123 ± 5	72 ± 6	188 ± 19
Recovery	95 ± 4[b]	60 ± 3[b]	143 ± 10[b]
Oil			
Baseline	126 ± 4	70 ± 4	225 ± 30
Recovery	101 ± 5[b]	52 ± 2	158 ± 16[b]
E2			
Baseline	149 ± 11[a]	76 ± 8	284 ± 47
Recovery	119 ± 15	68 ± 7	205 ± 30
NREM sleep			
Intact			
Baseline	98 ± 8	128 ± 10	66 ± 7
Recovery	106 ± 4	113 ± 7	100 ± 6[b]
Oil			
Baseline	98 ± 5	108 ± 4	86 ± 9
Recovery	114 ± 8[b]	112 ± 9	119 ± 13[b]
E2			
Baseline	92 ± 10	110 ± 11	66 ± 11
Recovery	99 ± 8	103 ± 8	96 ± 9[b]
REM sleep			
Intact			
Baseline	100 ± 6	113 ± 9	77 ± 4
Recovery	94 ± 5	107 ± 6	106 ± 7[b]
Oil			
Baseline	88 ± 5	90 ± 5	88 ± 10
Recovery	87 ± 7	96 ± 7	98 ± 8
E2			
Baseline	96 ± 7	108 ± 5	72 ± 12
Recovery	89 ± 8	100 ± 9	97 ± 10[b]

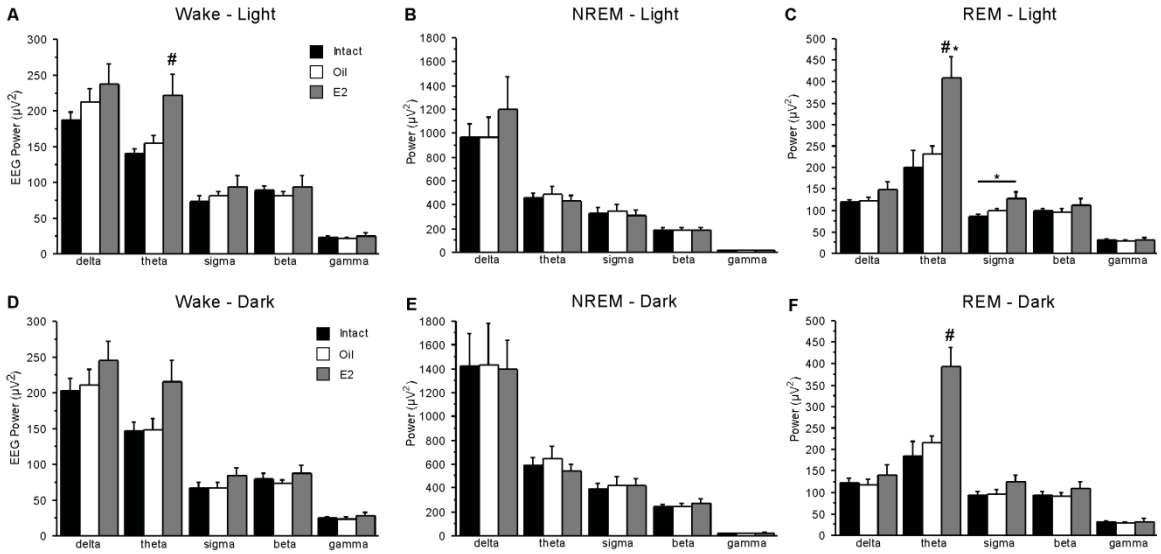
All values are expressed as mean ± SEM. All $P < 0.05$ (Tukey post hoc comparison).
[a] Different from Intact.
[b] Different from the corresponding baseline.

Table 6.2. Mean number of episodes of wake, NREM and REM sleep during baseline and recovery after sleep deprivation

Stage, Group, Condition	24 h Total	12 h Light	12 h Dark
Wake			
Intact			
Baseline	357 ± 14	189 ± 12	168 ± 12
Recovery	380 ± 7	216 ± 9[a]	164 ± 9
Oil			
Baseline	352 ± 10	214 ± 4	138 ± 12
Recovery	361 ± 20	216 ± 11	145 ± 12
E2			
Baseline	337 ± 26	205 ± 16	133 ± 14
Recovery	363 ± 27	221 ± 15	142 ± 17
NREM sleep			
Intact			
Baseline	355 ± 13	190 ± 12	165 ± 12
Recovery	380 ± 6	218 ± 9[a]	163 ± 9
Oil			
Baseline	350 ± 11	214 ± 4	136 ± 12
Recovery	362 ± 19	217 ± 11	145 ± 12
E2			
Baseline	333 ± 26	204 ± 16	128 ± 14
Recovery	364 ± 28	224 ± 15	141 ± 18
REM sleep			
Intact			
Baseline	86 ± 3	57 ± 3	29 ± 3
Recovery	98 ± 4	57 ± 5	41 ± 3[a]
Oil			
Baseline	97 ± 5	62 ± 5	35 ± 4
Recovery	110 ± 7[a]	60 ± 5	49 ± 5[a]
E2			
Baseline	94 ± 9	64 ± 6	30 ± 5
Recovery	115 ± 12[a]	70 ± 8	45 ± 5[a]

All values are expressed as mean ± SEM. All $P < 0.05$ (Tukey post hoc comparison).
* Different from the corresponding baseline.

Supplementary Figure 6.1. EEG power (mean + SEM, μV^2) in five frequency bands in wake (A and D), NREM (B and E), and REM (C and F) sleep during the 12 h light (top) and 12 h dark (bottom) phases of the recovery period following 6 h of SD in the Intact (black), Oil (white), E2 (grey) groups. Note that the dark phase (bottom) represents the first 12 h period after SD, and the light phase (top) represents the second 12 h period after SD. The E2 group had significantly higher REM theta power (4.5-8 Hz) than the Intact group during both the dark and light phases of the recovery period (Group: $F_{2,17} = 11.6$ and 8.7, $P < 0.001$ and < 0.005, respectively), as was observed during the baseline recording (see Figure 6.2). In addition, similar to the baseline (Figure 6.2), the E2 group had higher wake theta and REM sigma power (8.5-13-Hz) than the Intact group during the recovery light phase (Group: $F_{2,17} = 5.3$ and 4.4, $P < 0.05$, respectively). $^{\#}$ Different from Intact; * different from Oil. All $P < 0.05$ (Tukey post hoc comparisons).


A
Wake - Light
EEG Power (μV²)
Intact
Oil
E2
#
delta
theta
sigma
beta
gamma
B
NREM - Light
Power (μV²)
C
REM - Light
#*
*
D
Wake - Dark
E
NREM - Dark
F
REM - Dark

6.4 Discussion

There are three major findings in this study. First, castration in adult male rats did not significantly affect baseline sleep/wake patterns or the EEG when examined 2 weeks post-surgery. During recovery from 6 h of SD, however, it prolonged slightly both the rebound in NREM sleep time and the increase in NREM EEG delta power, while reducing REM sleep rebound. Second, a 2-week E2 treatment in castrated rats increased baseline amount of wakefulness at the expense of both NREM and REM sleep during the dark (active) phase - thereby increasing diurnal variation (light:dark ratio) of NREM and REM sleep - and enhanced baseline EEG theta power during wake and REM sleep in both the light and dark phases. Finally, E2 treatment in castrated males enhanced REM (but not NREM) sleep rebound, and tended to increase NREM delta power rebound following 6 h of SD. These findings indicate that E2 modulates the patterns of both baseline and recovery sleep as well as the EEG in castrated male rats.

A relatively high dose of E2 was administered in this study, producing plasma levels that are above normal for males. These high E2 levels, nonetheless, may be clinically relevant, as some patients with prostate cancer and male-to-female transsexuals take high doses of E2 that produce serum E2 concentrations comparable to the highest levels of E2 during the menstrual cycle in women (Purnell et al., 2006; Abel et al., 2011). In addition, these high E2 levels allowed for comparison of E2 effects on sleep regulation between male (this study) and female rats (Deurveilher et al., 2009, 2011).

6.4.1 Castration does not Affect Baseline Sleep but Alters the Time Course of Recovery Sleep following Sleep Deprivation

Castration did not affect baseline amounts of wake, NREM and REM sleep or the EEG associated with these states, suggesting that withdrawal of androgens in adult male rats does not modulate baseline sleep, at least at 2 weeks post-surgery. This finding is consistent with previous studies in rats (Peder, 1987) and mice (Paul et al., 2006),

whereas another study in rats reported increased REM sleep during the dark period after castration (Yamaoka, 1980). This discrepancy may be related to differences in the strain of rats used (Wistar [present study and (Peder, 1987)] vs. Sprague-Dawley (Yamaoka, 1980)), light/dark cycle (LD12:12 [present study and (Peder, 1987)] vs. LD14:10 (Yamaoka, 1980)), and/or difference in time interval between castration and EEG recording (two weeks in the present study vs. > 4 weeks in Ref. (Yamaoka, 1980)). The lack of an effect of castration on baseline sleep is consistent with the absence of effects of castration on the neuronal activity in certain sleep/wake-regulatory nuclei in adult male rats at approximately two weeks post-surgery (Muschamp et al., 2007; Hadjimarkou et al., 2008).

In contrast to the lack of an effect on baseline sleep, castration altered the time course of sleep recovery following 6 h of SD, prolonging the rebound in NREM sleep amount and NREM delta power by ~ 3 h compared to the gonad-intact rats. Furthermore, castration suppressed the initial REM rebound, and permitted only modest and non-significant REM sleep rebound over the first 12 h recovery period. These effects were not previously reported in castrated male mice after 6 h of SD (Paul et al., 2006) or castrated male rats subjected to 4 days of REM SD (Peder, 1987). Differences in species and SD protocols may account for these differences.

6.4.2 Estradiol Promotes Wake and Decreases both NREM and REM Sleep during the Dark Period at Baseline

E2 treatment in castrated rats promoted wakefulness at the expense of NREM and REM sleep particularly during the dark (active) period, resulting in a greater light:dark ratio for both NREM and REM sleep. The increase in wake amount was due to longer wake episodes, suggesting that E promotes wake maintenance. The reduction in NREM and REM sleep amounts with E treatment was mainly due to a reduction in the number of NREM episodes and in the duration of REM sleep episodes, respectively. Collectively, these observations suggest that E2 opposes NREM initiation and REM sleep maintenance, while promoting wakefulness, during the baseline dark phase, consistent

with general arousal-promoting effects of E2 in rodents (Pfaff et al., 2002). These effects of E2 may be due to E2-induced activation of neurotransmitter systems that are known to promote wake and/or inhibit REM sleep (Serova et al., 2002; Mong et al., 2003; Hiroi et al., 2006; Hadjimarkou et al., 2008; Devidze et al., 2010).

These significant effects of E2 on sleep regulation in castrated male rats were somewhat unexpected in light of early studies which reported no effects of single or two E2 injections on sleep (Branchey et al., 1973; Yamaoka, 1980). However, plasma E levels were not reported in these studies, and the possibility cannot be excluded that the E2 doses were not sufficient to affect sleep regulation. The difference in treatment strategies (2-week implants vs. 1-2 subcutaneous injections) and the type of E2 used (17β-E2 vs. E2 benzoate) may have also contributed to this discrepancy. Interestingly, unlike adult castrated rats, neonatally castrated male rats, when tested as adults, responded to two daily injections of E2 benzoate plus progesterone by reducing both NREM and REM sleep (Branchey et al., 1973). Since brain sexual differentiation occurs during perinatal period (McCarthy, 2008), neonatal castrates would have partially feminized brains. This likely explains the fact that their response to E2 (i.e., the activational effects of E2) in adulthood resembles those observed in ovariectomized female rats treated with E2. The modulation of sleep by E2 in male rats castrated as adults is a new finding, and the genetic and hormonal factors that play a role in this modulation require further investigation.

6.4.3 Estradiol Enhances EEG Theta Power during Wake and REM Sleep at Baseline

E2 treatment increased EEG theta power (4.5-8 Hz) during wake and REM sleep in both the light and dark phase at baseline. EEG theta activity during wakefulness is associated with exploratory behaviour in rodents (Vanderwolf, 1975), and one possible explanation for the increased EEG theta power during wakefulness is an increase in locomotor activity. However, a 2-week E2 treatment that increased E levels to female's proestrous levels (as in the present study) did not affect spontaneous locomotor activity in castrated

male rats (Mitsushima et al., 2009). In addition, increased locomotor activity cannot explain increased theta power during REM sleep. As EEG theta power during REM sleep has been proposed as a measure of REM sleep intensity and need (Borbely et al., 1984), it is possible that the E2-induced reduction of the baseline amount of REM sleep has been compensated for by an increase in the intensity of REM sleep. The increased theta power following E2 administration may be due, at least in part, to E2's action at theta-generating brain regions such as the basal forebrain and hippocampus, which contain membrane and nuclear ERs (Shughrue et al., 2000; Miettinen et al., 2002; Hazell et al., 2009; Hammond et al., 2011).

In addition to theta power, E2 enhanced baseline EEG sigma power (8.5-13 Hz) particularly during REM sleep in the light (inactive) phase. Similarly, administration in men of dehydroepiandrosterone, which can act as a precursor of the gonadal steroids including E2 (Labrie, 1991), increased sigma power during REM sleep (Friess et al., 1995). The functional significance of sigma power in REM sleep, however, is unclear.

6.4.4 Estradiol Promotes REM Sleep Rebound following Total Sleep Deprivation

Although E2 treatment did not affect the amount of NREM sleep rebound, the relative increase in REM sleep from baseline was larger in the E2 group than in the Oil group, suggesting a role for E2 in promoting recovery REM sleep. Further, the amplitude and the duration of elevation were similar between the E2 group and the Intact group, suggesting that E2 treatment restored the REM sleep recovery pattern in castrated rats to that seen in gonad-intact rats. Neurons in the median preoptic nucleus contain ERα (Perez et al., 2003) and have been suggested to be involved in REM sleep homeostasis (Gvilia et al., 2006). Thus, E may promote REM sleep rebound by acting at this nucleus.

6.4.5 Comparison of the Effect of E2 on Sleep Patterns and the EEG between Male and Female Rats

The effects of E2 on sleep modulation in female rats have been studied in some detail (Branchey et al., 1971; Deurveilher et al., 2009, 2011; Schwartz and Mong, 2011). Among these studies, the SD protocols and E2 regimen used in this study for male rats were identical to those used for female rats in our recent studies (Deurveilher et al., 2009, 2011). This, in conjunction with the similar levels of plasma E2 levels attained in the male rats, allows for comparison, with due cautions, of E2's effects on sleep/wake states and the EEG between gonadectomized male and female rats. There are both similarities and differences.

The effects of E2 on the EEG differ between males and females. At baseline, E2 elevates the theta power during both wake and REM sleep in castrated males (present study), but not during REM sleep in ovariectomized females (Deurveilher et al., 2011) (theta power during wake was not analyzed in that study). Following SD, E2 reduces NREM delta power rebound in ovariectomized females (Deurveilher et al., 2009), but not in castrated males (present study). Sex differences in the densities of ERs in EEG-regulatory brain regions, such as the basal forebrain and hippocampus (Cahill, 2006; Gillies and McArthur, 2010), may be responsible, at least in part, for the different effects of E2 on the EEG of males and females.

In contrast to EEG patterns, the effects of E2 on sleep/wake states were largely similar between males and females. For both sexes E2 promotes wake and reduced REM sleep at baseline particularly during the dark phase, which might suggest similar actions of E2 on the suprachiasmatic nucleus, the site of principal circadian clock in mammals (Rusak and Zucker, 1979; Moore and Leak, 2001) which contains ER (Shughrue et al., 1997; Vida et al., 2008), or ER-rich nuclei that project to this nucleus (Karatsoreos and Silver, 2007). Following SD, E2 promotes REM sleep rebound in both sexes. The presence of ER in the sleep-wake regulatory areas, such as the basal forebrain and brainstem, in both sexes may

account for some of the effects of E2 on sleep homeostasis that are common to males and females.

Overall, these results suggest a sex-specific activational effect of E2 on the EEG, but sex-independent modulation of sleep/wake states by E2. This differentiation was unexpected as the mechanisms underlying EEG and sleep/wake regulations are normally closely linked to each other. It is particularly noteworthy that the amount and the intensity of rebound NREM sleep are differentially modulated by E2 between the sexes. The sex differences in the effect of E2 on both EEG and NREM rebounds are likely due to sexual differentiation in the brain that arose during the perinatal period, which subsequently determined responses to hormones in the adults (Wallen, 2009).

6.4.6 Clinical Implications

Some six hundred thousand men at any time in North America are on ADT for the treatment of prostate cancer. Among the most common side effects reported by these patients are daytime fatigue and sleep disturbance (see section 6.1) which have been associated with hot flashes and night sweats (as is commonly noted in postmenopausal women). Hot flashes occur in 70-80% of patients taking luteinizing hormone-releasing hormones (LHRH) agonists for ADT; supplemental E2 is sometimes prescribed to reduce hot flashes and night sweats in men on ADT (Engstrom, 2008).

Our data show that, in androgen-deprived rats, E2 promotes wakefulness during the active (dark) phase and promotes recovery of REM sleep after sleep loss. Whether E2 treatment might similarly reduce fatigue during the active (day) and help recover REM sleep following sleep loss for men on ADT is unknown and warrants clinical assessment. This would be consistent with evidence that supplemental E can reduce tiredness (Polo-Kantola et al., 1998) and increase vigilance (Saletu et al., 1995) in post-menopausal women, although there are also studies that did not replicate these findings (Dzaja et al., 2005).

Caveats are due, however, in generalizing the current findings to castrated men. First, castration itself did not affect baseline amounts of wake, NREM or REM sleep in rats (present study and Refs. (Peder, 1987; Paul et al., 2006), but see Ref. (Yamaoka, 1980)). In contrast, men who are androgen-deprived using LHRH agonists show less deep (stage 4) NREM sleep than those on LHRH agonists in combination with testosterone (Leibenluft et al., 1997). The only study that investigated the effect of E2 on sleep in a genetic male population reported little effects on sleep (see section 6.1). In castrated male rats, however, we found that E2 treatment reduces NREM sleep and, to a lesser extent, REM sleep. These differences may reflect different methods of androgen deprivation and species differences, such as the fact that one species is diurnal and the other one is nocturnal. In addition, sociocultural factors in humans cannot be excluded.

6.5 CONCLUSIONS

Administration of a relatively high dose of E2 to castrated male rats alters baseline sleep patterns and the ability to recover sleep after acute sleep loss, as well as the EEG associated with these behavioural states. E2 promotes wakefulness and reduces both NREM and REM sleep during the dark period at baseline. In addition, E2 increases the EEG theta power in both wake and REM sleep at baseline irrespective of the diurnal phase. Furthermore, E2 appears to reverse the negative impact of castration on the ability to recover REM sleep in male rats. These effects of E2 are not identical to those observed in female rats. It remains to be seen whether supplemental E2 is beneficial for symptom management in androgen-deprived men who commonly experience sleep disturbance and daytime fatigue.

Chapter 7: GENERAL DISCUSSION

Androgen-deprivation via castration has detrimental effects on male rats, including loss of sexual motivation (indicated by mounting behaviour) and the inability to recover REM sleep after sleep deprivation. Some of the impairments associated with castration can be alleviated with E administration. Specifically, I demonstrate that E elevates mounting behaviour and helps castrated rats regain REM sleep after sleep deprivation. Furthermore, I also show that E may regulate ERs in brain areas important for sexual behaviour and cognitive function as well as in the PFM that plays a role in erectile function. These findings suggest that although E is normally present at low levels in males, E may still have a role in normal male functions. Information on the effects of E in males in general is scarce, and my findings help expand the current knowledge on the neurobehavioural effects of E in males. In addition, my studies provide pre-clinical data suggesting potential benefits of E therapy for PCa patients who experience deleterious effects from ADT.

7.1 Sex Differences in Estrogen Effects

In this thesis I observed how estrogen affects male behaviours, and found a number of differences compared to how E affects females. Such sex differences are likely due to sexual differentiation of the brain, i.e., when the brain is developing gonadal hormones organize neural circuits in a sex-specific manner (Wallen, 2009). As a consequence, gonadal hormone administration in adulthood does not always produce the same result between sexes.

A large proportion of research on T is conducted in males, whereas most research on E is done on females. This reflects both the primary physiological functions of these hormones in each sex and the differences in serum concentrations; i.e., T is normally present at higher levels in males and E is naturally present at higher levels in females (Becker et al., 2005; Hughes, 2007). However, there are genetic females that go on T therapy (such as female-to-male transsexuals) and there are genetic male populations on

E therapy (such as PCa patients on ADT, male-to-female transsexuals, and men who have aromatase gene mutations). Overall, however, the side effect profile of T administered to women or E administrated to men is poorly documented compared to what is known about the effects of T in men or E in women. In general the physiological effect of cross-sex hormonal treatments is underinvestigated.

Currently, the majority of clinical data on the effects of E are based on studies with females because women naturally experience ovarian hormone deprivation after menopause. As such, some women elect to take hormone replacement therapy (Scott et al., 2012). Substantial evidence shows that E therapy can improve sleep quality, urogenital health, cognition, and cardiovascular function in some post-menopausal women (Dzaja et al., 2005; Lachowsky and Nappi, 2009; Scott et al., 2012). However, based on my findings and other studies (Gillies and McArthur, 2010), some of the positive effects of E therapy might extend to males whereas others would not. The differences in the effects of E in male versus female rats, noted so far, suggest that the benefits and risks of E therapy for human males on ADT need to be carefully assessed to determine the precise functions and behaviours.

7.2 Clinical Implications

Some of the results from my studies may have important clinical implications for a number of quality-of-life issues experienced by PCa patients who are on ADT. Much of what we now know about the effect of E in rodents is currently uninvestigated in humans.

Previous studies indicate that the majority of PCa patients on high-dose E therapy as a primary ADT had sexual dysfunction (Ellis and Grayhack, 1963; Bergman et al., 1984; Choi et al., 1998). However, problems with libido *per se* were not explicitly addressed in those studies and only coital sex was considered a sexual activity. Whether the reduced frequency of coital sex reported in those studies was due to low libido or erectile dysfunction is not known. No study so far has investigated if the frequency of sexual fantasies, the number of erotic dreams, or the extent of non-coital sexual activities

changes with E administration in androgen-deprived men. My study, as well as many other animal studies (Appendix B), has shown that E may increase some sexual interest in androgen-deprived (castrated) males. If E can help restore some libido in PCa patients on ADT, the patients may be able to maintain some sexual intimacy with their partners. This could improve the quality of life of the patients and their partners as well. Interestingly, Bergman *et al*. (1984) reported that 7 out of 10 PCa patients, who received high dose E treatment, retained sexual activities although they did not reach orgasm. In sum, how E affects libido in androgen-deprived men needs to be more thoroughly assessed as previous studies on the sexuality of PCa patients focused on erectile potency, leaving other areas of male sexuality unexplored.

Sleep problems are common in PCa patients on ADT (Stephens et al., 2007; Hanisch et al., 2011; Savard et al., 2012), but data on how E replacement affects sleep in androgen-deprived men are scarce. A relatively brief interval of androgen deprivation may not impair baseline sleep patterns, as in my rodent study, two weeks after castration baseline sleep-wake behaviour was not altered. In contrast, patients on ADT in previous studies, who reported sleep disturbance (Stephens et al., 2007; Savard et al., 2012), had been androgen-deprived for months. It is possible that this is a cumulative effect of insufficient sleep over this period, and my observation that following sleep deprivation, castrated rats had altered sleep recovery is relevant because it suggests that the homeostatic sleep regulation, particularly with respect to REM sleep recovery, was affected by castration. These findings suggest that E can help androgen-deprived males recover REM sleep. While this effect has not been studied in men, hormone-replacement therapy increases their REM sleep, improves subjective sleep quality, and reduces nocturnal awakening in some post-menopausal women (Dzaja et al., 2005; Polo-Kantola, 2011). Furthermore, E may also help maintain sleep by reducing nocturnal awakening due to hot flashes (Engstrom, 2008). In sum, PCa patients on ADT may have better sleep when they receive E therapy.

E also promotes wakefulness in castrated male rats during the dark period (when nocturnal rodents are normally more active). Daytime fatigue is another side effect of

ADT that can affect daily functioning of PCa patients (Storey et al., 2012). Though currently uninvestigated, my findings from rodents suggest that E may help androgen-deprived men to stay more awake during the day, for example, by reducing fatigue, daytime sleepiness or by increasing alertness. Following directly from my research, a Phase 2 clinical trial has just been funded by Prostate Cancer Canada to investigate the impact of supplemental E on the sleep quality and daytime fatigue of PCa patients on ADT in British Columbia (personal communication, R. Wassersug).

Lastly, my findings show that long-term androgen deprivation disrupts the autoregulation of ERs in the PFM. This suggests that the effect of E treatment on the PFM may be diminished in long-term castrates. However, more research is needed to determine the role of E in the PFM of males, for example, in continence control or orgasm. Studies in women indicate that the PFM is involved in continence (Sartori et al., 2011), and in both sexes the PFM contracts rhythmically during orgasm (Bohlen et al., 1980; Bohlen et al., 1982). While E cannot reverse the PFM atrophy caused by castration, the fact that E can maintain the excitability of the PFM (Holmes and Sachs, 1992; Fargo et al., 2003; Foster and Sengelaub, 2004) suggests that E has a role in PFM function. Further work is needed to document how voiding routines of men vary between those that are on ADT and those that are on ADT in combination with E. Additionally, while many men report loss of orgasm after ADT (Higano, 2012), some men on ADT can reach orgasm through alternative sexual practices (Gray and Klotz, 2004; Warkentin et al., 2006). Whether administering E supplementation to men on ADT may increase their chances of achieving orgasm is not known, and warrants investigation.

7.3 The "Critical Period Hypothesis" for the Effects of Estrogen in Males

In my studies, I observed that some of the effects of E in male rats vary depending on when the treatment is initiated after castration. In a similar vein, based on the "Critical Period Hypothesis" in females, there is a critical period after menopause or ovariectomy when E maximally benefits females (Scott et al., 2012; Daniel, 2013). The results of my

studies suggest that the "Critical Period Hypothesis" for E treatment may also apply to at least some functions in males.

Cognition is one particular function in males that may be sensitive to the timing of E treatment, as it is in females (Daniel, 2013). In support of this notion, as previously shown in E-treated ovariectomized female rats (Bohacek and Daniel, 2009), I found that long-term castration alters the autoregulation of ERα in two brain areas that are involved in cognition. Daniel (2013) proposed that E may improve female cognitive performance by modulating the cholinergic system in the hippocampus via its action on ERα. Some studies have similarly suggested that E therapy can improve cognition in androgen-deprived men (Miles et al., 1998; Beer et al., 2006; Miles et al., 2006) and male animals (Gillies and McArthur, 2010). Following on this, future studies should explore whether the restorative effects of E on male cognition depend on when the treatment is initiated after androgen deprivation. If early E treatment brings more cognitive protection than delayed E treatment, as has been shown in females, such a finding would strengthen the case for men to start E therapy as soon as ADT commences.

To date, the mechanism underlying the variation in the protective role of E with time after hormone-deprivation has not been investigated. Brinton et al. (2008) proposed a "Healthy Cell Bias Hypothesis," that states that E can be neuroprotective when administered to 'healthy' cells, but not to cells with extensive damage (e.g., after long-term steroid deprivation). Previous studies indicate that androgen deprivation exacerbates the production of reactive oxygen species from the mitochondria, which can eventually cause oxidative stress to cells (Razmara et al., 2007; Shiota et al., 2011). E administration reduces oxidative stress in the mitochondria (Razmara et al., 2007), and has been shown to have an antioxidant effect on the livers of recently ovariectomized rats, but not in long-term ovariectomized rats (Lopez-Grueso et al., 2013). Possibly, without early hormonal intervention, the oxidative damage to cells becomes substantial and, thus, these cells fail to respond to E. Since the DNA-binding domain of ERs is sensitive to oxidative insult (Liang et al., 1998; Whittal et al., 2000), the elevated oxidative stress after castration may disrupt the DNA-binding domains of ERs. Without proper DNA-binding domains, the E-

ER complex will not be able to bind to the E response element in the DNA, and thus will be unable to activate gene transcription. Currently, the mechanism responsible for how the duration of androgen deprivation alters the effectiveness of E treatment is not known. Future studies will need to address how cellular physiology changes in relation to the time since gonadal hormone deprivation, if we are going fully to understand the molecular basis for the "Critical Period Hypothesis" in both males and females.

Some of the positive effects of E on androgen-deprived males, at least in terms of elevating sexual interest, appear to be insensitive to when the treatment is started after ADT. This finding suggests that PCa patients who have been on ADT for many years may still benefit from E therapy. Therefore, although some of the restorative effects of E (e.g., possibly cognition) may diminish in long-term androgen-deprived men, beginning E treatment after long-term ADT may still potentially have some positive effects for PCa patients.

Currently, some PCa patients on ADT take supplemental E to counteract hot flashes. However, E therapy is often not started until the patients experience severe hot flashes. It may be preferable to begin E treatment soon after beginning ADT to help the patients avoid experiencing the full severity of some of the side effects including hot flashes (Engstrom, 2008) and osteoporosis (Ockrim et al., 2004; Morrison et al., 2011). Furthermore, commencing E treatment together with ADT may also reduce the detrimental 'flare' phenomenon that often happens in the first few weeks of ADT because of elevated plasma T levels (Bubley, 2001).

Future research is needed to determine how long E treatment (either early or delayed treatment) after castration can sustain the restorative effects of E on male functions. As an example, to follow up my study (Chapter 2), it would be interesting to evaluate the rat's mounting behaviour several times over a longer period of time instead of only assessing it once after two weeks of E2 treatment. Furthermore, knowing that E2 downregulates ERα in the POA, continuous E2 treatment may potentially influence ER downregulation. For example, if ERα is further downregulated with long-term E2

treatment, it may potentially dampen the restorative effects of E in mounting. If that is so, it would be worthwhile to compare how intermittent and continuous dosing of E influences the effectiveness of E2.

7.4 Cautionary Considerations for Estrogen Therapy

Although the previous sections discussed various beneficial effects of E therapy in PCa patients on ADT, E therapy also has some potential risks that need to be acknowledged. A more detailed discussion of this topic is available in Appendix B.

Despite evidence emphasizing the beneficial effects of E, many physicians are reluctant to prescribe E to PCa patients because of the cardiovascular morbidity associated with elevated plasma E2 levels. However, data to date suggest that this risk is not higher than that from LHRH agonists (Hedlund et al., 2008; Abel et al., 2011; Langley et al., 2011).

Another negative side effect is that E therapy in males causes gynecomastia, which has both psychological and social implications (Wassersug and Oliffe, 2009; Wassersug and Gray, 2011). Furthermore, though rare, high-dose E in men may elevate their risk of breast cancer (Karlsson et al., 2006). In vitro studies raise the possibility that E may promote the carcinogenesis of PCa at the cellular level through the activation of ERα (Ho et al., 2011; Nelles et al., 2011). Currently, the risks of cardiovascular morbidity and gynecomastia remain the primary reasons PCa patients do not take E therapy.

7.5 Conclusion

My research described in this thesis provides evidence of the positive effects of E on a number of neurobehavioural measures in androgen-deprived males. Follow-up research on PCa patients is warranted as offering E therapy to PCa patients may dampen some of the negative side effects of ADT. While some benefits of E may not depend on when the treatment is initiated after castration, starting E therapy early after ADT should still be

considered as E may help PCa patients avoid some of the detrimental side effects of ADT.

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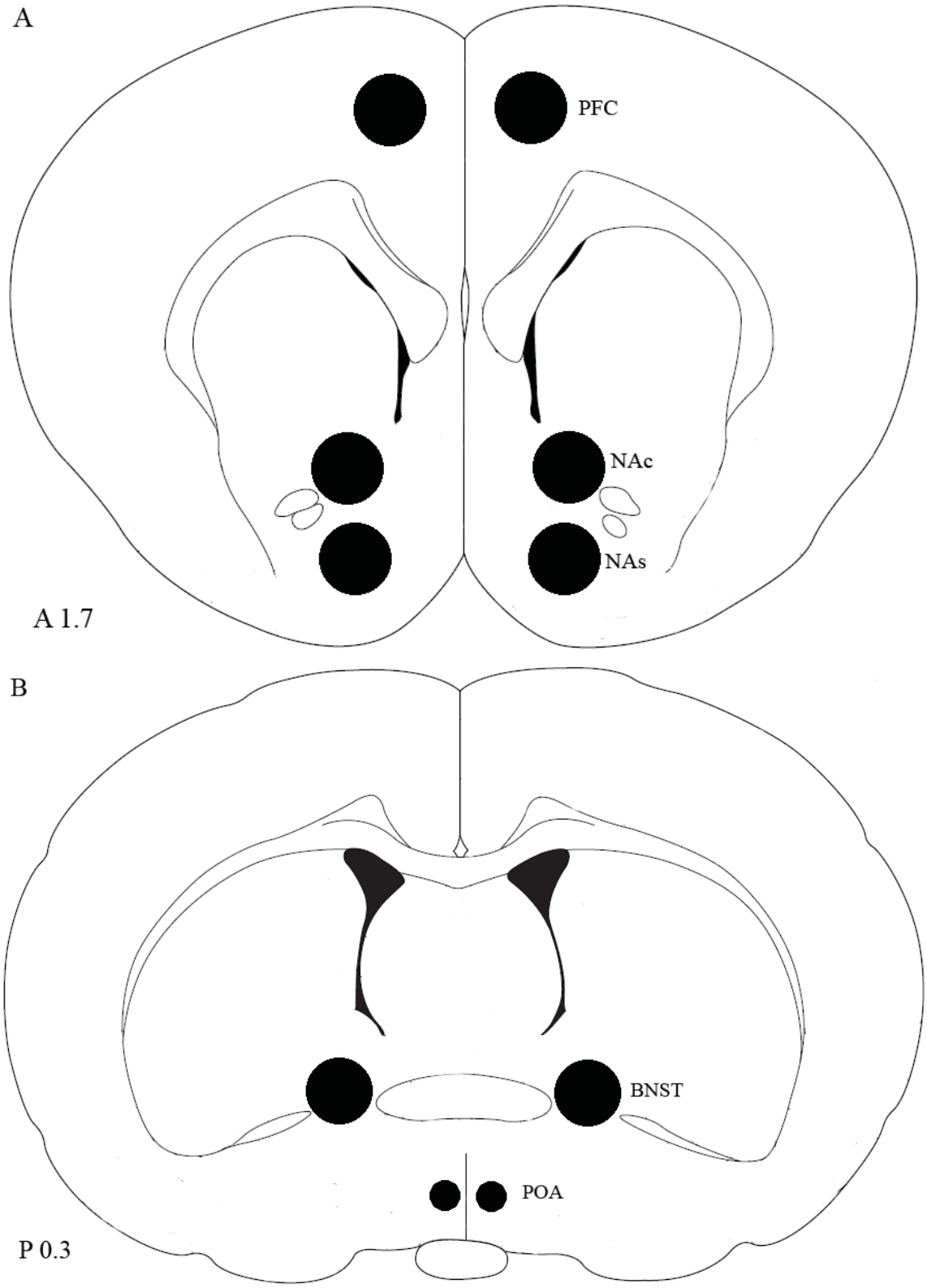
Appendix A: LOCATIONS OF MICROPUNCHES



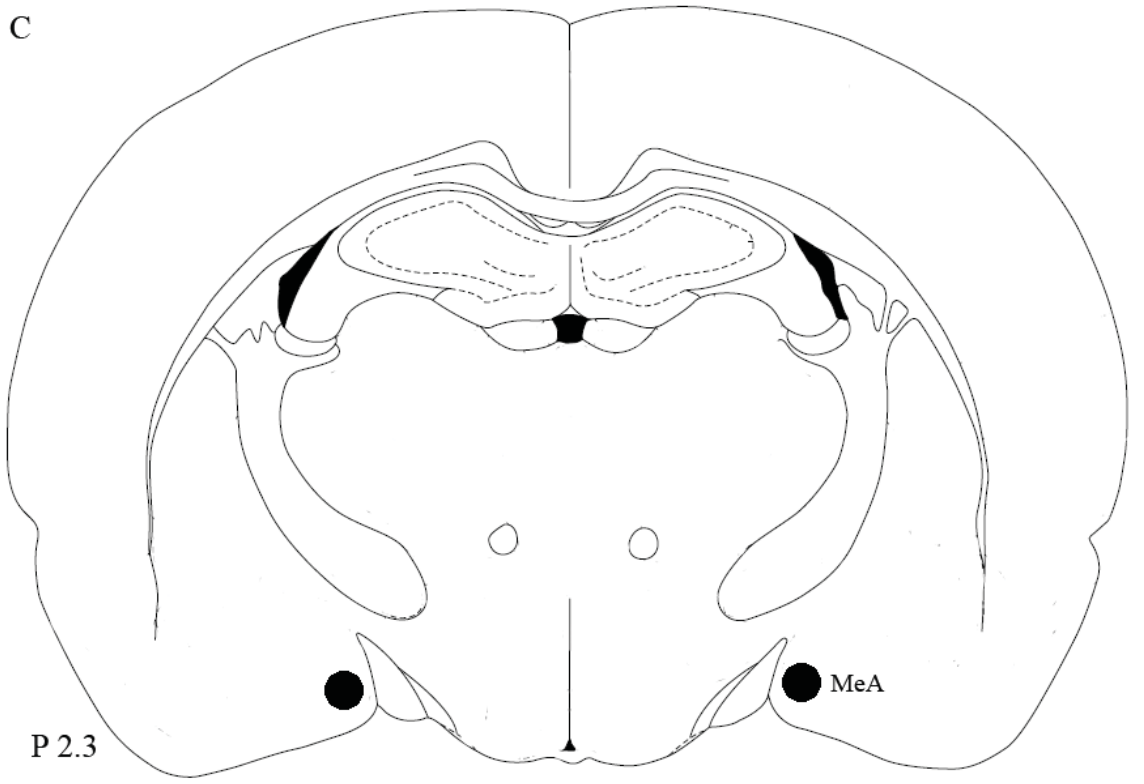


Figure A.1. Locations of micropunches (black circles) shown on coronal sections for the following brain areas: prefrontal cortex (PFC), core area of nucleus accumbens (NAc), shell area of nucleus accumbens (NAs), preoptic area (POA), bed nucleus of the stria terminalis (BNST), and medial amygdala (MeA). Micropunches were obtained bilaterally from 300 μm sections. The number at the bottom left corner of each figure indicates a distance in millimeters anterior (A) or posterior (P) from bregma. The images are modified from Paxinos and Watson (1998).

Appendix B: THE EFFECT OF ESTROGEN ON THE SEXUAL INTEREST OF CASTRATED MALES: IMPLICATIONS TO PROSTATE CANCER PATIENTS ON ANDROGEN DEPRIVATION THERAPY

Abstract

Androgen deprivation therapy (ADT) for prostate cancer (PCa) treatment causes sexual dysfunction. We review here the effects of estrogen on the sexual performance of androgen-deprived males. The major findings are:

1. Estrogen receptors are present in brain centers that are important for sexual behaviour; as well as in male reproductive organs, in a pattern suggesting that estrogen may have some role in orgasmic function and genital skin sensitivity.

2. Estrogen restores sexual interest above castrate levels in many vertebrates including reptiles, birds and mammals; but multiple factors contribute to the magnitude of this effect.

3. Data from castrated men, aromatase-deficient men, male-to-female transsexuals, and men on antiandrogens all suggest that estrogen can maintain some libido in androgen-deprived men.

We discuss the general benefits of estrogen therapy to quality of life of men on ADT, the potential risks of this treatment, and possible treatment regimes for estrogen therapy in males. Unless contraindicated, we propose that PCa patients on ADT would benefit from supplemental parenteral estrogen.

Publication Information

This chapter has previously been published as: Wibowo E, Wassersug RJ (2013) The effect of estrogen on the sexual interest of castrated males: Implications to prostate cancer patients on androgen-deprivation therapy. Crit Rev Oncol Hematol. 87:224-238. EW reviewed all papers, made the tables and prepared the draft manuscript.

B.1 INTRODUCTION

There are various situations where genetic males are therapeutically androgen-deprived. The most common reason for androgen deprivation therapy (ADT) is to slow down prostate cancer (PCa)'s growth. In addition, as part of sex reassignment surgery, male-to-female transsexuals (MtFs) are also androgen-deprived. ADT can be achieved by either surgical or chemical castration. Currently, luteinizing hormone-releasing hormone (LHRH) agonists are the most frequently used agents for ADT in the PCa patient population. However, other agents including high-dose estrogen (E), high-dose ketoconazole, abiraterone, and LHRH antagonists can also be used to achieve a castrate level of testosterone. Single-agent antiandrogen therapy is also used as a form of ADT, but does not lower serum testosterone levels.

In most cases, ADT impedes sexual function; reducing libido and causing erectile dysfunction (Mazzola and Mulhall, 2012). These effects distress patients and psychologically impact their intimate partners, reducing the quality of life for both (Elliott et al., 2010). While treatments for erectile dysfunction are available, currently there is no treatment for loss of libido subsequent to ADT. Yet, loss of erections due to ADT does not mean a cessation in sexual activity (Wibowo et al., 2012b). For example, men can still achieve orgasm without an erect penis.

ADT not only depletes androgens in men, but also estrogens. This is because estrogen in males is derived from testosterone. Some males on ADT receive E therapy. For MtFs, E therapy can aid in body feminization (breast development) and, for PCa patients, supplemental E can alleviate some of the more intense adverse events, such as hot flashes (Engstrom, 2008). Additional benefits of E treatment for androgen-deprived men include improving bone mineral density (Ockrim et al., 2004) and lipid profiles (Purnell et al., 2006). In one study, treatment with E also improved some aspects of cognitive function (Beer et al., 2006).

Previously we reviewed papers suggesting that E can, to some extent, elevate sexual interest in castrated males (Wibowo et al., 2011). We have since confirmed this with a study of castrated male rats with and without estradiol (E2) treatment (Wibowo and Wassersug, 2013b). Here, we provide a more extensive literature review on how E influences sexual interest in androgen-deprived males for a wealth of species, ranging from amphibians to mammals including humans. In addition, we discuss the potential effect of E on peripheral tissues that are related to sexual function, such as genital skin and pelvic floor muscles that are important in achieving an orgasm. We then discuss the pros and cons of E therapy as well as various dosing regimes—factors that need to be considered in clinical settings.

B.2 Estrogen Receptor

E induces its effects by acting on estrogen receptors (ERs) that are widely distributed throughout the body. In the tetrapod brain, ERs are present in areas that control male sexual behaviour, most notably the medial preoptic area, medial amygdala and the bed nucleus of stria terminalis (Kruijver et al., 2003; Pak and Handa, 2008). Intracranial E implants in those specific areas of the brain have been shown implicitly to increase sexual behaviour in castrated males of many vertebrate species (see Suppl. Tables A.1 and A.2). The equivalent brain areas in humans also express ERs. Replicating the results observed in animals by implanting E into human brains would be excessively invasive; however, there is evidence that castrated men on E therapy maintain better libido than those not receiving supplemental E (Wibowo et al., 2011).

The mechanism for how E elevates sexual interest in castrated men has not been extensively investigated. In imaging studies, the preoptic area and medial amygdala are activated during sexual arousal by both visual (Ferretti et al., 2005) and olfactory stimulation (Savic et al., 2005; Huh et al., 2008) although not necessarily by tactile stimulation (Georgiadis et al., 2009). However, no study has explored if these activation patterns in response to sexual stimuli change after E treatment in androgen-deprived men.

E may also influence sexual behaviour by acting on peripheral tissues. Indeed, ERs are present in male reproductive organs although their function remains enigmatic (Mowa et al., 2006). They may not be related to erectile function *per se* because in both castrated men and other male mammals, E treatment does not restore erectile function. We discuss in later sections how E may potentially modulate pelvic floor muscle function and genital skin physiology.

B.3 Estrogen and Male Sexual Behaviour

B.3.1 Animal Studies

Dating back to the early 1900s, there were studies showing that ovarian grafts in capons (Goodale, 1918) or injecting placental extract into castrated male rats (Nissen, 1929) increased sexual activity. These were the first observations to suggest that female reproductive organs contained some substances that could positively influence sexual behaviour in castrated males. The first natural estrogen, estrone, was identified in 1929-30 and within a decade, Ball (1937) provided the first direct evidence that E elevates male sexual interest by injecting estradiol benzoate (EB) into castrated male rats. Since then, studies on other species ranging from amphibians to mammals, have explored the effect of E on sexual behaviour in castrated males (see Suppl. Tables A.1 and A.2), but rats remain by far the most studied species.

Administering E to castrated male rats increases mounting behaviour, however, the extent to which E changes libido varied among studies (Suppl. Table B.1). One factor likely to contribute to the varying results is the age at which castration is performed. Rats castrated at birth (Pfaff and Zigmond, 1971) or prepubertally (Södersten, 1975) have less restoration in their sexual behaviour than those castrated in adulthood (Pfaff and Zigmond, 1971; Södersten and Larsson, 1975). This is likely because sexual differentiation of the brain, which first occurs during the perinatal period (McCarthy, 2008) then again during puberty (Schulz et al., 2009), requires aromatizable androgen.

Males castrated at birth or before puberty are not fully masculinized, resulting in less developed sexual behaviour.

Other factors which may influence the effects of E on sexual behaviour include the dose (e.g., (Larsson et al., 1973a); (Luttge et al., 1975) vs. (Paup et al., 1975)) and type of estrogen (Ball, 1939; Larsson et al., 1976). A dose too low (e.g., injection of ≤ 5μg EB/day in rats) is not optimal in reinstating copulatory behaviour. Interestingly, daily injection of high dose E restores all copulatory behaviours in castrated adult rats including ejaculation (Södersten, 1973) even though the erectile reflex is not fully restored (O'Hanlon et al., 1981). The mechanism to account for this is unclear since pelvic floor muscles, that are important for ejaculation, atrophy following castration, and E cannot restore their gross morphology (Nowacek and Sengelaub, 2006).

The method of E administration is similarly an important factor in determining how extensively E raises sexual interest because different methods are associated with different fluctuations in plasma E2 levels. For example, daily injections of E lead to a sharp increase in plasma E2 levels that rapidly decline within 12 hours (Isaksson et al., 2011). Thus, several weeks of daily injections are required for males to reach the equivalent plasma E2 levels found in proestrous females (Strom et al., 2008). In contrast, the use of a Silastic tube (i.e., slow-release implant) to administer E results in supraphysiological plasma E2 levels which stabilize to proestrous levels within 24 hours (Isaksson et al., 2011). However, the E2 content in the implanted tubes declines over time resulting in a gradual reduction in the plasma E2 levels over several weeks (Strom et al., 2008). Thus, if sustained dosing is the goal, Silastic tubes containing E need to be replaced every several weeks.

Different methods of E administration are reflected in difference in the male rats' behaviour. For example, several weeks are required to activate mounting in all castrates by daily injection of high dose EB (Södersten, 1973), whereas this is achieved more quickly with Silastic tube implants (McGinnis and Dreifuss, 1989).

It is also important to note that many factors influence normal sexual behaviour in rats; for example, housing condition (e.g., the number of animals per cage (Beach, 1942a)), previous sexual experience (Attila et al., 2010), and the strain of the animal (Sachs, 1996). Undoubtedly these variables can influence how much sexual behaviour can be restored by E after castration in male rodents.

In Suppl. Table B.2, we review studies on 24 tetrapod species, excluding rats. In 18 of these species, 12 of which are mammalian, E elevated sexual interest after castration, as indicated by copulatory and/or courtship behaviours. Studies to date with castrated amphibians fail to indicate that E restores sexual activity (Kelley and Pfaff, 1976; Andreoletti et al., 1983; Deviche and Moore, 1988). Of note, though the E dose used in those studies was very high and the authors reported some mortality associated with the treatment. Whether lower E doses or a different type of E (only E2 has been tested) would produce different results needs further clarification. It is also possible that a complete restoration of sexual behaviour in amphibians does not depend solely on gonadal steroids. For example, in castrated newts E only restored courtship behaviour in combination with vasotocin (Moore and Miller, 1983).

Studies in reptiles and avian species show more inconsistent results. Only in castrated green anole lizards, chickens and Japanese quails, does E treatment increase copulatory behaviour (see Suppl. Table B.2). However, in other reptilian and avian species, E increased courtship behaviour but not copulatory behaviour. These observations suggest that some sexual interest can be restored by E in castrated males of these species. The only exception is the zebra finches, in which neither copulatory nor courtship behaviours have been restored with E administration. However, this could be dose-related as the implant used in that study had a relatively small volume compared to the implant used in Japanese quails. In fact, Watson et al. (1990) showed that restoration of sexual activity in castrated quails by E is dose-dependent. To date, different doses of E have not been tested in castrated zebra finches.

As shown in Suppl. Table B.2, the majority of mammalian castrated males (12 out of 13 species, excluding rats) increase sexual activity above castrate level following E treatment. The only exception is the rhesus monkeys (Phoenix and Chambers, 1982; Michael et al., 1990). However, this could be dose-related as high EB doses (higher than 5µg/kg/day) cause penile, scrotal sac and perineal edema in rhesus monkeys (Michael et al., 1990). E-induced genital edema was also observed with baboons injected with EB (Gilbert, 1944) and rhesus monkeys with estradiol dipropionate (Kamal et al., 1985), but not in chimpanzees receiving oral α-estradiol or ethinyl estradiol (Clark, 1946, 1949). These findings suggest that the swelling of peripheral tissue in some castrated male primates given supplemental E may be associated with the method of administration or the type of E compound used. This warrants further investigation since different E compounds activate sexual behaviour in other mammals where E2 fails to restore copulatory behaviour after castration; e.g., in guinea pigs (compare (Antliff and Young, 1956) and (Alsum and Goy, 1974)) and in rabbits (compare (Foote et al., 1977)and (Agmo and Södersten, 1975; Beyer et al., 1975)) estrone, but not EB, can elevate sexual interest after castration.

Similar to what has been observed with rats, studies with other species have shown that various factors contribute to how much sexual behaviour can be restored after castration. Once again, relevant factors include age at castration (Romeo et al., 2002), type of E compound (D'Occhio and Brooks, 1976), and method of administration (Balthazart et al., 1985). Additionally, lighting conditions can be crucial for those species whose sexual activity varies seasonally in their natural habitat. For example, in green anole lizards, E increased copulatory activity when the amount of light per day increases, resembling the Spring season, but not under Fall lighting conditions (Latham and Wade, 2010). Other factors, such as the duration of the treatment, become important when daily injections are used (see results in deer mice (Clemens and Pomerantz, 1981) and in golden hamsters (Noble and Alsum, 1975; DeBold and Clemens, 1978)). This is because, as previously mentioned for rats, with daily injections it takes several weeks to elevate plasma E2 levels to those of proestrous females (Strom et al., 2008). Therefore, if this method of administration is used, experimenters should consider assessing the sexual behaviour

multiple times over several weeks, as the effects of E may require a prolonged time to be optimized. In sum, E can increase sexual activity in castrated males from a variety of tetrapod species and many factors contribute to how much sexual interest can be elevated by E.

B.3.2 Human Studies

E has been administered to genetic males who are androgen (and/or E) deprived (Wibowo et al., 2011). High doses of E reduce libido in intact men because E shuts down the hypothalamic-pituitary-gonadal axis by enhancing negative feedback inhibition (Martin-Du Pan, 2011). On the other hand, E may increase the libido of hormone-deprived men above castrate levels.

In Table B.1, we reviewed studies on prostate cancer patients who were on E-therapy. In those studies, there was always a subset of patients who maintained erectile function following PCa treatments, however, what determines if erectile potency can be preserved is not known. It is also important to add that erectile function does not always translate into the patients being sexually active. In Ellis and Grayhack (1963) and Choi et al. (Choi et al., 1998), 38 and 19 patients, respectively, were sexually potent prior to any treatment but only 26 and 7, respectively, were sexually active (more than 13 coitus/year) after treatment. Thus, pre-treatment sexual behaviour may influence whether patients remain sexually active after treatment.

In two studies (Ellis and Grayhack, 1963; Bergman et al., 1984), some men were sexually active prior to treatment and among these men, more patients on E therapy remained sexually active than those who were orchiectomized. Ellis & Grayhack (1963), however, only assessed sexual activity based on penile-vaginal intercourse. This was not the case in the Bergman et al. (1984) study as only 2 out of 12 patients retained erection. In contrast, Bergman et al. (1984) reported that 7 out of 10 E-treated men continued sexual activity even in the absence of orgasm. Petersen (1965) found that 8 out of 38 patients retained libido and 3 out of 26 patients maintained coital activity after E treatment, but the author

did not mention if the patients were involved in non-coital sexual activity. In sum, there is evidence that E-treated PCa patients tend to be more sexually active than castrated men who are not on E-therapy. However, a more thorough study needs to be conducted to assess how libido is affected in these patients and whether these patients continue non-coital sexual activity in the absence of erectile and/or orgasmic function, which can be indicative of sexual interest even when coital sex is unlikely.

There is evidence that E can raise sexual interest in other populations of androgen-deprived men. For example, in men castrated for other reasons than PCa, E elevates libido above castrate levels; in fact almost as well as when the subjects are taking testosterone (Davidson et al., 1983; Brett et al., 2007). Furthermore, many male-to-female transsexuals (MtFs) on E therapy remain sexually active (Klein and Gorzalka, 2009; Murad et al., 2010), but these studies are hard to compare as many MtFs concurrently take progesterone and/or antiandrogens.

Some relevant data are available on yet another group of men; those who have low E plasma levels as a result of an aromatase gene mutation. These men have a normal libido but in one case study, E treatment elevated the patient's sexual interest (Carani et al., 1999), while in three other case studies, E therapy alone did not change the patients' libidos (Bilezikian et al., 1998; Carani et al., 2005; Herrmann et al., 2005). However, this could be because testosterone is still endogenously produced in these patients.

Further evidence for E's role in maintaining libido comes from studies on PCa patients who are on antiandrogen monotherapy for ADT. Antiandrogens prevent the binding of testosterone to androgen receptors. The unbound testosterone is then converted to E by the enzyme aromatase. Therefore, PCa patients, who receive antiandrogen treatment, have an elevated plasma E2 level secondary to elevated serum testosterone levels. Some studies report that more patients on antiandrogen monotherapy retain their libido than those who are surgically castrated (Iversen et al., 1998; Tyrrell et al., 1998; Iversen et al., 2000). In one report, over 60% of patients taking antiandrogen monotherapy preserved their sexual interest as compared to ~30% of patients who were surgically castrated

(Iversen, 1999). Here, the preservation of libido by antiandrogens is understood to be due to elevated E2 levels, and it is notable that these men experience other estrogenic effects, such as gynecomastia and reduced hot flashes.

Similar to animal studies, the effectiveness of E in restoring libido for men may depend on multiple factors. Age is an important one as sexual performance declines naturally as men age (Corona et al., 2010). Pre-castration sexual behaviour should also be considered as some patients, even though they have a partner and normal erectile function, are not sexually active. Thus, they would remain sexually inactive after treatment. At the other extreme, some people are naturally hypersexual, thus, they retain high sexual activity after treatment. For example, in the Ellis and Grayhack study (1963), there was a man who claimed having intercourse 6 times per night pre-operatively. After castration and E treatment, he still reported having intercourse 15 times per week though it declined to twice per week 30 months later. Other factors, which may confound the effect of E on sexual interest, include stress levels and socio-cultural factors.

B.4 ORGASMIC FUNCTION

In both men and women, the pelvic floor muscles (PFM) are important for continence and orgasm. These muscles contract rhythmically during orgasm in both men and women (Bohlen et al., 1980; van Netten et al., 2008). Changes in the PFM after ADT have not been widely researched in humans, but in animal studies, castration causes atrophy of the PFM, suggesting their dependence on androgens. In fact, the PFM contain both androgen and estrogen receptors (Sengelaub and Forger, 2008). However, E cannot prevent castration-induced atrophy; although one study on male rats showed that E treatment partially maintains the morphology of the pubococcygeous muscle after castration (Alvarado et al., 2008).

In rats, one PFM (the bulbocavernosus) is innervated by the spinal nucleus of the bulbocavernosus (SNB) and the cell bodies of the motoneurons in the SNB atrophy after castration. E cannot reverse this shrinkage (Forger et al., 1992; Fraley and Ulibarri,

2002). However, E maintains the normal electrical activity of the PFM in castrated male rodents (Holmes and Sachs, 1992; Fargo et al., 2003; Foster and Sengelaub, 2004). These findings suggest that E may have some role in normal function of the PFM. In fact, high dose E treatment restores ejaculatory behaviour in castrated male rats (Södersten, 1973). Such findings may not translate into restoration of erectile function in androgen-deprived men, but they do not exclude the possibility that E has an effect on orgasmic function.

Unfortunately, there are few studies on how E may affect orgasm in men. Bergman et al. (1984) found that 11 out of 12 PCa patients lost their ability to reach orgasm following E therapy. Of note, the age of the men in that study ranged from 64 to 87 years, old enough to experience the natural decline in orgasmic capability that occurs with aging (Corona et al., 2010). As previously reported, ADT may not always lead to loss of orgasm (Wassersug, 2009; Wibowo et al., 2012b). In the report by Wassersug (Wassersug, 2009), the subject was able to reach orgasm using alternative sexual practices that did not depend on penile erections.

B.5 Skin Sensitivity

Women appear to have more sensitive skin than men (Galton, 1894; Weinstein and Sersen, 1961); at least on the hands (Gescheider et al., 1984), nipples, areolas and breasts (Robinson and Short, 1977). This sex difference only appears at puberty, suggesting that this is due to either the high testosterone in men or high E in women. In one study (Gescheider et al., 1984), a higher plasma E2 level in women was associated with increased sensitivity to vibrotactile stimulation on the hand. Therefore, E may have a direct influence on skin sensitivity.

In males, aromatase and ERs are expressed on the skin of the genitals, sensory corpuscles and penile nerves (Jesmin et al., 2002; Crescioli et al., 2003; Jesmin et al., 2004) suggesting E's involvement in afferent input and potentially in sexual arousal. As further support of this idea, ERs are present in the autonomic and sensory ganglionic neurons that are associated with male genitalia (Taleghany et al., 1999; Burke et al., 2000).

In castrated male rodents, the receptive field of the nerves that supply the perineal region is reduced (Enin et al., 1979) and there are changes in the physiology of the genital skin mechanoreceptor (Enin et al., 1979; Johnson and Murray, 1990). However, no changes in the genital sensory afferent activity after castration have been reported in the one other mammalian species examined to date; i.e., the cat (Cooper and Aronson, 1974). Currently, we do not know if E increases the size or sensitivity of the sensory field of the genital skin in castrated human males. However, two independent studies (Komisaruk et al., 1972; Kow and Pfaff, 1973) showed that E treatment to ovariectomized rats widens the sensory field of the genital skin, which may be beneficial in increasing sexual arousal by tactile stimulation. The effect of E on the female skin's receptive field is not restricted to genitalia, but has been replicated in facial (Bereiter and Barker, 1975) and trigeminal (Bereiter et al., 1980) neurons. Furthermore, the effect of E on trigeminal neurons was not exclusive to females and was also observed in castrated male rats (Bereiter and Barker, 1980). If E increases tactile sensitivity to genital skin, it may potentially help increase sexual arousal associated with tactile stimuli of an erotic nature.

B.6 Pros and Cons of Estrogen Therapy

B.6.1 Advantages

B.6.1.1 E Reduces Hot Flashes and may Improve Sleep

Hot flashes and night sweats are reported by 70-80% of men who are on ADT and the majority of these cases are severe enough to warrant intervention (Engstrom, 2008; Jones et al., 2012). In some studies, E has been proven to be one of the most effective agents to reduce the severity of hot flashes in men on ADT (Atala et al., 1992; Miller and Ahmann, 1992; Smith, 1994; Gerber et al., 2000).

Severe hot flashes often lead to sleep problems (Leibenluft et al., 1997; Hanisch et al., 2011). Sleep disturbance in patients can also interfere with the sleep of their partners, impacting the couple's quality of life. Whether E can improve sleep quality in PCa

patients on ADT is unknown. However, indirectly, sleep quality should improve if E reduces nocturnal hot flashes. In one study of MtFs (Kunzel et al., 2011), E in combination with antiandrogens prolonged one of the sleep stages, but the authors did not report whether the individuals subjectively reported better sleep quality.

We recently explored the effect of E2 administration on the sleep/wake behaviour of castrated male rats (Wibowo et al., 2012a). We found that E treatment promotes wakefulness and helps recover some sleep following sleep deprivation. If E can improve daytime alertness and help androgen-deprived men sleep better, it could indirectly also reduce the cognitive decline that has been reported with ADT in several studies (Nelson et al., 2008; Jamadar et al., 2012).

B.6.1.2 E Protects Bone

ADT induces bone resorption. This appears to be due to both androgen and estrogen deprivation, since receptors for both steroids are present in the skeletal system and are involved in bone mineral balance (Gielen et al., 2011). ADT reduces bone mineral density most rapidly shortly after ADT is started (Greenspan et al., 2005) and fracture incidence increases as well (Lattouf and Saad, 2010; Adler, 2011).

High dose E therapy, as a primary method for ADT, has been shown to maintain and improve bone mineral density in PCa patients (Ockrim et al., 2004; Morrison et al., 2011). This finding was not replicated in patients who had previously been treated with LHRH agonists (Kearns et al., 2010); however the E dose used in that study was low compared to the other two studies.

B.6.2 Critical Period Hypothesis

The interval from starting ADT to the beginning of E treatment may be crucial in maximizing the beneficial effects of E. This is based on the "critical period" (Sherwin, 2009), which is also called the "window of opportunity" (Rocca et al., 2011), hypothesis

on the cognitive performance of post-menopausal women on hormone replacement therapy. Based on this hypothesis, E treatment started in the perimenopausal period can be cognitively protective, but the benefits may be reduced or even lost with later administration. Data from ovariectomized rodents further support this hypothesis; i.e., female rats perform better on cognitive tasks when treated with E early rather than late after gonadectomy (Daniel and Bohacek, 2010). Brinton (2008) proposed a "healthy cell bias" hypothesis to explain this timing effect. In brief, neuronal function deteriorates naturally with age and/or after steroid deprivation, but E treatment can be neuroprotective when administered before there is substantial neuronal degeneration.

The effect of ADT on cognition has been controversial. While some studies found that cognition declines in certain domains after ADT (Cherrier et al., 2003; Cherrier et al., 2009), an improvement or no change in cognition following ADT has also been reported (Nelson et al., 2008; Jamadar et al., 2012). It has been previously suggested that these divergent results for men on ADT may be in part explained by the critical period hypothesis (Wassersug and Wadhwa, 2005).

A few studies have explored the effect of E on cognitive function in androgen-deprived genetic males, but with mixed results. One study showed that E treatment improved at least one cognitive domain in PCa patients on ADT (Beer et al., 2006). Similar data are available for MtFs (Miles et al., 1998; Miles et al., 2006). However, these findings were not supported in two other studies (Taxel et al., 2004; Matousek and Sherwin, 2010). It is important to note that the plasma E2 levels in those two studies were not as high as those in the Beer et al. (2006) study. Thus, the difference in results could be dose-related. Whether the timing onset of E treatment after ADT is important in maximizing its effect on cognition is not known, however, that has previously been suggested to account for these divergent results (Wassersug and Wadhwa, 2005).

We recently explored the critical period hypothesis as applied to the sexual behaviour of castrated male rats (Wibowo and Wassersug, 2013b). We found that late (i.e., 3 months delay in) E2 treatment after castration was as effective as early (i.e., no delay) E2

treatment in restoring sexual interest (indicated by mounting behaviour) in male rats. This finding is consistent with the study by Antliff and Young (1956) who compared the effect of early (1 week) versus late (10 weeks) initiation of estrone treatment after castration on the sexual behaviour of male guinea pigs. Therefore, the effects of E on male sexual behaviour may be relatively insensitive to when E treatment is started after castration.

B.6.3 DISADVANTAGES

B.6.3.1 Cardiovascular Morbidity

After Huggins and Hodges (1941) discovered that estrogen (E) treatment reduces the serum acid phosphatase level in PCa patients, oral E became the first drug treatment for PCa. Oral E, however, elevates thromboembolic risk and was subsequently replaced with LHRH agonists (Denmeade and Isaacs, 2002). Yet, LHRH agonists increase the risk of metabolic syndrome (Faris and Smith, 2010), which may carry its own cardiovascular morbidity risk (Keating et al., 2006; Keating et al., 2010).

Recent studies (Ockrim et al., 2006a; Hedlund et al., 2008) have determined that the thromboembolic risk of E can be reduced if the drug is administered parenterally. This is because when E is administered orally, the E is carried by the portal system directly to the liver and undergoes hepatic metabolism resulting in the up-regulation of clotting factors (von Schoultz et al., 1989). However, this surge to the liver can be avoided when E is administered parenterally; i.e., transdermally or through intramuscular injection.

A high plasma E2 level may increase cardiovascular morbidity. However, data to date suggest that this risk is not higher than that associated with LHRH agonists (Hedlund et al., 2008; Abel et al., 2011; Langley et al., 2011). One study suggests that this risk is only during the first two years of treatment and in the long term, E may reduce cardiovascular morbidity (Ockrim et al., 2006b). In addition, there is evidence that E can be cardioprotective through activation on its G-protein coupled receptors (Meyer et al., 2011). Furthermore, parenteral E administered to androgen-deprived PCa patients has

also been shown to improve their lipid profiles (Purnell et al., 2006). Recently, Scott et al. (2012) reviewed evidence showing that there is a critical period for E to be beneficial for cardiovascular function in post-menopausal women. Whether this is true for androgen-deprived men is uninvestigated.

B.6.3.2 Gynecomastia

E therapy in genetic males causes gynecomastia, which has both psychological and social implications (Wassersug and Oliffe, 2009; Wassersug and Gray, 2011). This is a desired effect for MtFs, but considered undesirable by most PCa patients. Interventions for gynecomastia are available and include subcutaneous mastectomy and the use of prophylactic breast radiation (Wassersug and Oliffe, 2009). In addition, selective estrogen receptor modulators, such as tamoxifen, have been recommended to counteract gynecomastia (Viani et al., 2012). Although tamoxifen can be effective in reducing this specific side effect of high E, those authors do not consider the positive benefits of E on other organs and tissues, most notably bone and the brain.

B.6.3.3 Breast Cancer Risk

Although men have a relatively lower risk than women, high dose E still elevates their risk of developing breast cancer (Karlsson et al., 2006). So far, there are only 6 reported cases of bilateral breast cancer in PCa patients on E (Kijima et al., 2009). Risk factors for men to develop E-sensitive breast cancer are not well known. Thus, regular breast cancer screening should be considered for genetic males on E therapy.

B.6.3.4 Prostate Cancer Risk

E appears to be active in a castration resistant state of PCa (Smith et al., 1998; Bland et al., 2005; Fizazi et al., 2007; Serrate et al., 2009; Montgomery et al., 2010; Clemons et al., 2011), although it is controversial as to whether E has a stimulatory or inhibitory effect on PCa cells (Risbridger et al., 2010). How E may potentially promote

carcinogenesis of PCa at the cellular level has recently been reviewed and the activation of ERα, but not ERβ, is thought to underlie this effect (Ho et al., 2011; Nelles et al., 2011). In addition, E may also activate mutated androgen receptors (Shi et al., 2002; Waltering et al., 2012); as has been observed with antiandrogens. If that happens, the use of E as a PCa treatment should be discontinued.

B.6.4 Treatment Regime

E exposure can induce the autoregulation of ERs by regulating their degradation (Valley et al., 2008; Bondar et al., 2009). For example, high plasma E2 levels lead to the degradation of ERs, which is presumed to be the mechanism that maintains an optimal cellular response to E2 in, for example, premenopausal women. Based on this, the effect of E is likely to be reduced under chronic E administration because the ERs will be down-regulated. For this reason, we hypothesize that cyclical administration of E would be a better option than continuous dosing. Consistent with this suggestion is the fact that intermittent LHRH agonist therapy attenuates the detrimental side effects of ADT (including some sexual recovery when the treatment is stopped) and therefore improving the patients' quality of life (Calais Da Silva et al., 2008; Calais da Silva et al., 2009). In a small retrospective report on intermittent diethylstilbestrol (DES) therapy, Klotz et al. (1986) reported that 10 out of 12 men became impotent while on DES therapy, however, 9 resumed sexual activity following the cessation of DES therapy. Based on these findings, intermittent or cyclic E therapy may be preferable to continuous therapy for maintaining sexual interest.

B.7 Implications for Future Research

Many questions remain about the effects of exogenous E on androgen-deprived men. For example, the critical period hypothesis has not been explored for men in terms of how the timing of administration may influence E's ability to preserve libido or cognitive function. Further research is needed in general to determine: 1) what factors influence the extent to which libido can be preserved by E in androgen-deprived males, 2) how E

maintains orgasm in some castrated men in the absence of erectile function, and 3) whether E can increase arousal by improving genital skin sensitivity.

What also remains to be explored is whether E can improve sleep quality and reduce fatigue in men on ADT as has been shown for rats in one laboratory study (Wibowo et al., 2012a). Furthermore, whether the beneficial effects of E in androgen-deprived males can be enhanced with cyclic or intermittent dosing is uninvestigated.

B.8 CONCLUSION

Estrogen plays a role in normal male physiology. Declining plasma E levels in men after ADT lead to adverse events, such as hot flashes, reduced libido, and osteoporosis. We suggest that PCa patients, who are prescribed androgen suppression to treat androgen dependant PCa, could be offered supplemental parenteral E2 (avoiding the thomboembolic risk of oral E) to attenuate the detrimental side effects of E deprivation. However, this offer should be made with caution and avoided if the patient has a family history of E sensitive breast cancer or a personal history of thromboembolic events.

Based on the studies reviewed above, we expect that exogenous E administration can raise libido above castrate levels in some men on ADT. This residual libido may be appreciated not only by the patients, but also their partners.

Both patients and their partners should be counseled about the pros and cons of parenteral E2 as either a supplement to LHRH agonists, or as a primary method of ADT. They need to understand, for example, the merits in preserving the patient's libido, even if erectile function is not recovered. Patients and their partners need to be informed that sexual intimacy is still possible with erectile dysfunction, and that there are options of rewarding sexual activity in the absence of penile-insertive intercourse (Warkentin et al., 2006; Wassersug, 2009). Patients further need to know that their partners may still appreciate physical contact in the absence of coital sex (Walker and Robinson, 2012). Having some

libido preservation in the male on ADT can thus help couples maintain intimacy of both a sexual and nonsexual nature.

Estrogens are currently prescribed to PCa patients as second line hormonal therapy (Shore et al., 2012). We suspect, however, that supplemental E2 will be most effective in preserving the quality of life of PCa patients, if provided earlier in treatment, concurrent with the initiation of ADT. Lastly we speculate that E's effectiveness may be enhanced if it is administered in a cyclic fashion that preserves ER density on target tissues.

Table B.1. The effect of estrogen therapy on the sexual behaviour of prostate cancer patients

Study	Sample size	E type and dose	Selected Results	Additional Notes
Ellis and Grayhack (1963)	20 (E alone) 41 (castration + E)	Stilbestrol at 3-500 mg per day and chlorotrianisene at 12.5-25 mg per day [both are synthetic E]	7 (E alone), 6 (castration+E), and 3 (castration only) patients retained potency after treatment. Among these patients; 4 (E alone), 2 (castration+E), 2 (castration only) remained sexually active—having intercourse more than once a month.	Before treatment, 9 (E alone), 22 (castration+E), and 7 (castration only) patients were sexually potent but only 26 were sexually active. The authors did not indicate how these 26 were assigned into treatment groups. Those who were impotent before treatment remained impotent after treatment.
Choi et al. (1998)	10 (E alone) 22 (castration + E)	2 mg of diethylstilbestrol per day	6 (E alone), 4 (castration+E), and 1 (castration only) patients retained potency after treatment. After treatment, those (regardless of treatment) that remained potent had a reduced frequency of sexual intercourse with a mean of 9.4 times in a year.	Before treatment 9 (E alone), 7 (castration+E), and 3 (castration only) patients were sexually potent. Before treatment those (regardless of treatment) that were potent had a mean sexual intercourse frequency of 16.4 times in a year.
Bergman et al. (1984)	12	Intramuscular injection of 80-160 mg polyestradiol phosphate per month and 150 µg ethinylestradiol per day	Among E-treated patients, 5 patients found their libidos were preserved, 2 had reduced libidos, and 5 completely lost their libidos (compared to 5, 4 and 3, respectively for orchiectomized men). 8/10 patients receiving E treatment remained sexually active with a partner compared to 3/10 for orchiectomized men.	Only men who had erections and were sexually active (either by intercourse or masturbation) before treatment were included in the study.

Petersen (1965) – this study included the data from Hauser (1952) and Petersen (1964) as well	50	45 patients were on intramuscular injection of 80 or 160 mg polyestradiol phosphate per month. 5 patients took high dose [total dose received was between 270-2000 mg] estradiol dipropionate; one of the 5 took additional ethynil estradiol.	8/36 retained some libido 6/41 were capable of erections 2/40 were capable of ejaculating 3/26 maintained coital activity	Only 41 patients had partial/full sexual function before treatment

Supplementary Table B.1. The effect of estrogen on sexual behaviour of castrated male rats

Study	Housing condition (Light:Dark cycle; animal(s) per cage)	E type and dose	Condition at castration (life stage; sexually experienced?)	Interval from castration to E treatment	Key Results	Additional Notes
Ball (1937)	Not stated	Daily injection of EB ranging from 5-100 RU at various durations for each dose. Each rat received a different treatment regime	Adult; not stated	Various intervals	50 or 100 RU of EB increased copulatory behaviour in 4 rats.	2 rats did not show elevated mounting but the maximum dose tested in these rats was 50 RU.
Ball (1939)	12:12; not stated	Sc implantation of crystalline E1 pellets; after 1-2.5 months changed to daily sc injection of EB (each rat received a total of 8600 RU in a 2 week period)	Before puberty; not stated	Immediate	2 rats copulated during EB only treatment; 2 more copulated during EB+P treatment. 2 rats never copulated under any treatments.	3 rats received EB; another 3 received P after 9 days of EB treatment. E1 did not increase sexual activity.
Beach (1942b)	Not stated	5 daily im injections of αED (dose not stated); 3 weeks after the last injection, 2 rats were implanted with 20 mg of crystalline E pellets for 1 month	Before puberty; not stated	208-210 days	In 88 tests before injections began, 5 castrates showed 256 sexual responses; in 134 tests during injection period, they showed 882 sexual responses.	Castrated male rats showed increased sexual activity with the E injections but not with E implants. Sexual responses included mounting (sexual clasp) and intromission (palpation & pelvic thrust).

Beach (1945)	Not stated	Daily injection of 100 RU EB for 5 days	Adult; yes	158 days	On the 2nd day, the male rat showed 7 sexual responses but no responses were observed in subsequent tests.	The rat received T injections between days 11-27 after castration.
Davidson (1969)	12:12; not stated	Daily sc injection of 70 µg EB for 19 days (immediate castrates) or 200 µg EB for 13 days (long-term castrates).	Adult; yes	Immediate or 53 days (long-term castrates)	Immediate castrates: On the 6th (final) test after E treatment was started, ~50% of E-treated rats ejaculated whereas <30% oil-treated rats did. Long-term castrates: 4/6 intromitted after 10 days and 2/6 ejaculated but none showed either behaviour afterwards.	Mounting was not assessed. EB treatment after castration preserved ejaculation longer than oil.
Pfaff (1970)	12:12; 1	Daily 10 µg of EB for 9-11 days	Adult; no	More than 4 weeks	Testing time = 8 min Total mounts: 53.0 (E) vs. 16.7 (Oil) Mounts with thrusts: 42.0 (E) vs. 0.1 (Oil)	
Pfaff and Zigmond (1971)	Not stated; 4	Daily 10 µg of EB for 9 days	Neonatal; no Adult; not stated	More than 100 days for neonatal castrates More than 25 days for adult castrates	Testing time = 8 min Total mounts: 20.4 (neonatal castrates) and 40.3 (adult castrates) Mounts with thrusts: 17.3 (neonatal castrates) and 32.0 (adult castrates)	Mounting frequency was lower in E-treated neonatal castrates than E-treated adult castrates. Neonatal castrates tend to mount less than control adult castrates.

Baum and Vreeburg (1973)	16:8; 4	Daily sc injection of 2 µg EB for 3 weeks	Adult; no	31 days	Testing time = 15 min but extended to 30 min if there was mounting with pelvic thrusting; extended to 1 hr if intromission occurs or until the rat ejaculated Intromission frequency before ejaculation: 30.0 (E), 14.5 (T), 14.75 (E+DHT), 13.0 (T+DHT)	3 out of 8 E-treated rats ejaculated
Södersten (1973)	14:10; not stated	Daily sc injection of 100 µg EB for 24 days for adult castrates 50 µg/day of EB for 28 days for prepubertal castrates	Adult; Yes Before puberty; not stated	6 weeks (adult castrates) 59 days (prepubertal castrates)	All adult castrates mounted; 10 out of 11 prepubertal castrates mounted (Test ended if IL or PEI > 15 min; or EL > 30 min) Mounting frequency of adult castrates: 21 (E), 11 (T)	
Larsson et al. (1973b)	Not stated	Daily sc injection of 5 µg EB for 26 days	Before puberty; not stated	60 days	<50% displayed mounting by day 26 of EB injection	
Larsson et al. (1973a)	14:10; 4 or 5	Daily sc injection of 0.05, 0.5, 5 or 50 µg EB for 26 days	Before puberty; not stated	60 days	Percentage of rats showing mounting with the lowest to highest E doses: 0, 10, 50, 78% (Test ended if IL or PEI > 15 min; or EL > 30 min) Mounting frequency with the lowest to highest E doses: 0, 46, 9.4, 14.6	

Christensen and Clemens (1974)	14:10; not stated	Intrahypothalamic implant of E2 [10 µg of crystalline E2 every 3 days for 12 days] Implants were in preoptic area or posterior hypothalamus.	Adult; yes	≥ 6 weeks	(Testing time = until 1st PEI or 20 min if no ejaculation occurs after the 1st intromission or if IL > 20 min) At the 3rd (final) test after E treatment was started, the total mounting frequency was: ~30 (E in preoptic area), ~10 (E in posterior hypothalamus) and 0 (Chol)	Implants in preoptic area were more effective in restoring mounting than those in posterior hypothalamus
Södersten (1975)	14:10; not stated	Exp. 2—Daily sc injection of 1 µg EB for 60 days	Before puberty; not stated	70 days	3 out of 8 rats showed mounting (Test ended if IL or PEI > 15 min; or EL > 30 min) Mounting frequency: 50.3 (E), 14.0 (T), 6.2 (E+T), 9.2 (DHT+T), 9.8 (DHT)	
Södersten and Larsson (1975)	14:10; not stated	Daily sc injection of 1 µg EB for 20 days	Adult; yes	7 weeks	At the 7th (final) test after E treatment was started, ~70% of NL (see additional note) and 100% of L groups displayed mounting (Test ended if IL or PEI > 15 min; or EL > 30 min) Mounting frequency for L castrates: 17.1 (before E treatment), 18.5 (after E treatment)	This study divided the analysis into two groups; i.e., male rats that did (L) or did not (NL) show lordosis before castration.

					Mounting frequency for NL castrates: 18.2 (before E treatment), 26.4 (after E treatment)	
Luttge et al. (1975)	12:12; 1 or 2	Exp. 1—Daily sc injection of 1 µg EB for 4 weeks	Adult; no	4 weeks	Cumulative % displaying mounting: 30 (E), 10 (Oil)	The rats were tested every 3-4 days during injection period.
Paup et al. (1975)	13:11; not stated	Daily sc injection of 25 µg EB for 40 days	Adult; no	6 weeks	Cumulative % displaying mounting: 100 (E), 0 (Oil)	The rats were tested 4 times during injection period.
Larsson et al. (1976)	14:10; 3 or 4	Daily sc injection of E1 (1 or 5 µg), E2 (1 or 5 µg), E3 (1, 5, or 25 µg) for 30 days	Before puberty; not stated	70 days	25 and 30 % of rats showed mounting with 1 and 5 µg E2 doses, respectively. <15% of rats mounted with any of the E1 or E3 doses.	
Lodder and Baum (1977)	10:14, 2	Exp. 1—Daily sc injection of 3 µg EB (6X per week) for 45 days Exp. 2—Daily sc injection of 0.5 or 5 µg EB (6X per week) for 23 days	Adult; yes	Exp. 1—Immediate Exp. 2—5 weeks	Exp. 1—% of tests with ejaculation after castration but before pudendectomy: 50% (E), 30% (Oil). Testing time = 15 min or sooner if EL < 15 min Mounts/min before pudendectomy: ~4 (E), 0 (Oil). Mounts/min after pudendectomy: ~1.5 (E), 0 (Oil).	The pudendal nerves of the rats were transected at either 4 weeks (Exp. 1) or 51 days (Exp. 2) after castration. Exp. 1—Mounting rate declined to ~1.5 mounts/min for E-treated rats after pudendectomy.

						Exp. 2—Mounts/min after pudendectomy: ~0.5 (5 µg E dose), 0 (0.5 µg E dose), 0 (Oil).	
	Davis and Barfield (1979)	12:12; not stated	Intrahypothalamic implant of crystalline EB [27 or 30 G cannulae filled to a depth of 1 mm] Exp. 1—Implants were located anterior to and within anterior hypothalamus; or posterior to anterior hypothalamus [primarily in ventromedial hypothalamus]. Exp. 2—Implants were in anterior hypothalamic preoptic region	Adult; yes	2 or 3 weeks	Testing time = till 1st intromission after 2nd ejaculation or if IL or PEI > 15 min; or EL > 30 min Exp. 1—Mounting frequency: 5.3 (after castration and pre-implantation), 9.2 (post-implantation). Exp. 2—Mounting frequency: 8.3 (E), 8.5 (blank).	Exp. 1—The data were only from rats that ejaculated. E reduced mounting, intromission and ejaculation latencies as well as PEI. Exp. 2—The E data were combined data from rats receiving E implant with/without systemic DHT treatment. Similarly, blank data were combined data from rats received blank implant with/ without systemic DHT treatment.
	Baum and Starr (1980)	12:12; 2	Slow-release implant (vol = 51 µL) containing crystalline E2 (diluted 10X in Chol)	Adult; yes	Immediate	(Test ended if IL > 30 min or EL > 60 min) Mounts/min for E group: ~1.7 (Exp. 1); ~1.6 (Exp. 2); ~1.7 (Exp. 3); ~1.1 (Exp. 4) Mounts/min for DHT group: ~0.7 (Exp. 1); ~0.8 (Exp. 2); ~0.7 (Exp. 3); ~0.2 (Exp. 4)	This study investigated how different neurotransmitters systems affect the restoration of sexual behaviour by E and/or DHT administration in castrated males. The selected results are from castrates which only

						received steroid alone. Rats were re-used in exp. 1-4.
Beyer et al. (1981)	14:10; not stated	Daily sc injection of 5 µg EB for 4-6 week; then increased to 50 µg of EB/day for another 3 weeks	Adult; yes	2 weeks after sexual behaviour disappeared post-castration	6 out of 7 castrated rats mounted after E treatment.	
Baum et al. (1982)	12:12, 2	Exp. 1—Slow-release implant (vol = 51 µL) containing crystalline E2 (diluted 10X in Chol) for 27 days	Adult; yes	27 days	Exp. 1—Percent ejaculating before intracranial implant: 25% (lateral septum); 33% (amygdala) Percent ejaculating after intracranial implant: 38% (lateral septum); 33% (amygdala)	The rats also received intracranial implant of Chol in lateral septum or amygdala on the 12th day after receiving slow-release implant.
Södersten et al. (1986)	12:12; not stated	Slow-release implant (vol = 58 µL) containing E2 (25, 50 or 100 µg/mL) for 2 days	Adult; not stated	17 days	None of the control rats or rats that received an implant containing 25 µg/mL E2 ejaculated. 25% of castrated rats ejaculated with 50 µg/mL E2 dose and ~60-70% with 100 µg/mL dose.	Mounting and intromission results were not shown.
Hawkins et al. (1988)	12:12, 1	Exp. 3—Daily sc injection of 5 µg EB for 8 weeks	Adult; yes	Immediate	Testing time = 15 min Mounting frequency at 8 weeks after starting E treatment: ~8 (E), 0 (Oil)	Mounting frequency peaked at 4th week after E treatment.

McGinnis and Dreifuss (1989)	12:12; 1	Slow-release implant (vol = 8.5 µL) containing 10% E2 for 2 weeks	Adult; yes	3-4 weeks	At the final test, ~90% of E-treated castrates showed mounting compared to none among rats with a blank implant. (Test ended if ML, IL, or PEI > 15 min; or if EL > 30 min) Mounting frequency at the final test: 11 (E), 0 (blank) Mounting latency (s) at the final test(s): 305 (E), 900 (blank)	
Rasia-Filho et al. (1991)	12:12; not stated	Intracerebral implant of crystalline E2 [50-60 µg] in medial amygdala for 12 days Implants were in medial amygdala.	Adult; yes	≥ 3 weeks (after significant reduction in mounting)	(Testing time = 10 min) Mounting frequency: 8.6 (intact), 1.2 (post-castration), 6.5 (E2–day 6) Mounting latency (s): 11.1 (intact), 431.2 (post-castration), 51.0 (E2– day 9)	After E2 was implanted, mounting frequency and latency reached intact level on day 6 and 9 respectively. Both parameters returned to pre-treatment level thereafter.
Matuszczyk and Larsson (1994)	12:12; 4-5	Slow-release implant (vol = 9.8 µL) containing crystalline E2 for 2 weeks	Adult; both sexually naïve and experienced rats were tested	2 weeks	Exp. 1—10 out of 14 castrates showed mounting after E2 administration Exp. 2—6 out of 12 castrates showed mounting after E2 administration	This study looked at the factor of sexual experience on sexual preference.

Vagell and McGinnis (1997)	12:12; 1	Slow-release implant (vol = 8.8 µL) containing crystalline 1% E2 for 2 weeks	Adult; yes	2 weeks	(Test ended if IL, EL or PEI >15min) Mounting frequency: 20 (E), 7 (T)	
Cross and Roselli (1999)	12:12; 2	Single ip injection of either 20 or 100 µg/kg E2 15 minutes before test.	Adult; yes	3 weeks	Testing time = 20 min Total mounting frequency: ~30 (both E doses) and ~10 (control). Genital sniffing frequency: ~58 (100 µg/kg E2), ~42 (20 µg/kg E), ~35 (control)	The animals received a single ip injection of testosterone a week before testing.
Roselli and Chambers (1999)	12:12; ≤4	Slow-release implants containing crystalline E2 (vol = 19 µL) for 6 weeks	Adult; no	Immediate	Testing time = 20 min Control rats showed low genital sniffing frequency and no mounting with thrust (data not shown). At the last test, genital sniffing and mounting with thrust frequencies of E-treated rats were ~17 and ~21 respectively.	The rats' genitals were anesthetized to eliminate intromission behaviour.
Bialy and Sachs (2002)	12:12; 3	Intracranial implant [30G cannulae] in medial amygdala or sc implant containing crystalline E2 [dose not stated]	Adult; yes	Received implant before castration	9 out of 10 rats receiving intracranial implant mounted whereas 5 out of 6 rats receiving sc implant mounted.	

Putnam et al. (2003)	14:10; 1	Daily sc injection of 20 µg EB for 3 weeks	Adult; yes	1 day	Testing time = 30 min Mounting frequency: 0.3 (Oil), 17 (E) Intromission frequency: 0 (Oil), 6.9 (E)	
Putnam et al. (2005)	14:10; 1	Daily sc injection of 20 µg EB for 3 weeks	Adult; yes	1 day	All E2-treated rats mounted. Testing time = 30 min Total mounting frequency: 21.4 (Oil), 21.4 (E) Total intromission frequency: 3.5 (Oil), 6.5 (E)	
Attila et al. (2010)	12:12, 2	Slow-release implant containing 10% (Exp. 1) or 5% (Exp. 3) E2 (vol = 9.9 µL)	Adult; yes	37 days	Exp. 1—By day 21, ~80% of the E-treated rats mounted whereas only <40% of the control rats mounted. Exp. 3—By day 21, ~60% of the E-treated rats mounted whereas none of the control rats mounted.	Exp. 1—The rats received extensive sexual experience before castration. By day 11 of E treatment, 80% rats displayed mounting. Exp. 3—The rats only had 3 pre-castration sexual experiences. By day 16, ~ 60% of rats showed mounting.

Chol = cholesterol; DHT = dihydrotestosterone; EB = estradiol benzoate; E1 = estrone; E2 = estradiol; E3 = estriol; EC = estradiol cypionate; ED = estradiol dipropionate; EL = ejaculation latency; im = intramuscular; IL = intromission latency; ip = intraperitoneal; ML = mounting latency; P = progesterone; PEI = post-ejaculatory interval; RU = rat unit; sc = subcutaneous; T = Testosterone

Under the results column, E, T, DHT, Chol, or Oil means that the results were from a group treated with estrogen, testosterone, dihydrostestosterone, cholesterol, or oil respectively.

Cholesterol and Oil are common [though not in all case] vehicles for delivering steroid compounds to animals in *in vivo* studies. Alone they several as controls.

"Exp." refers to the experiment number in that particular paper. Some paper reports several experimental studies.

"~" in the 6th column is an approximate value obtained by observing the figure in that particular study.

The frequency of behaviour(s) mentioned in the 6th column is the mean value unless specified.

Supplementary Table B.2. The effect of estrogen on sexual behaviour of castrated male tetrapods, excluding studies on the genus *Rattus*

Animals	Housing Condition (Light:Dark cycle; animal(s) per cage)	E type and dose	Condition at castration (life stage; sexually experienced?)	Interval from castration to E treatment	Did E increase sexual interest?	Additional Notes
Mammals						
Mus musculus						
Edwards and Burge (1971)	14:10; 1 (from 75 days old onwards)	Daily sc injection of 1 µg EB in the first week, raised to 10, 50 and 100 µg/day in the subsequent weeks. Each rat was treated with EB for 5 weeks.	Before adulthood; not stated	52 days	Yes	There was a dose-dependent increase in the percentage of mice showing mounting.
Wallis and Luttge (1975)	12:12, 1	Daily injection of 1 µg EB for 6 weeks then reduced to 0.5 µg/day for 3 weeks and returned back to 1 µg/day for 2 weeks	Adult; yes	Immediate	Yes	E-treatment slowed down the decline in mounting and intromission over time.
Dalterio et al. (1979)	14:10; group housing of the same treatment	Exp. 3—Single sc injection of 1 µg of E2 either 5 minutes or 5 hours before testing Exp. 4—Single sc injection of 0.2 µg of E2 or 1 µg of EB either 5 minutes or 1 hour before testing respectively	Adult; yes	2 weeks	Exp. 3—Yes Exp. 4—Yes (with E2); No (with EB)	One E-treated group in exp. 3 and all mice in exp. 4 were treated daily with T between castration and E administration.
Wee et al. (1988)	14:10; 1	Daily im injection of 5 µg EB for 4 weeks	Adult; yes	35 weeks	Yes	All mice showed mounting after 2 weeks of treatment.

Nyby et al. (1992)	12:12; 1	Exp. 2—Intracranial implant [27G cannulae] filled with E2 (to a depth of ~1 mm) for 28 days	Adult; not stated	3 weeks	No	Implants were placed in either the medial preoptic area or medial septum.
Peromyscus maniculatus bairdi						
Clemens and Pomerantz (1981)	16:8; 1	Exp. 2—Daily sc injection 2 µg EB for 6 weeks	Adult; yes	After 3 weeks without showing sexual behaviour	Yes	Sexual behaviour increased after 4 weeks of treatment.
Pomerantz et al. (1983)	16:8; 1	Daily sc injection of 1, 2, or 3 µg EB for 2 weeks	Adult; yes	6 weeks	No	
Cavia porcellus						
Antliff and Young (1956)	Not stated; group housing based on treatment groups	Daily ip injection of 100 IU/100 g BW of E1 or α-EB for 16 weeks	Adult; yes	8 days or 10 weeks	Yes (with E1); No (with α-EB)	The results between those treated 8 days and 10 weeks after castration were similar.
Alsum and Goy (1974)	Not stated; 5-8	Daily sc injection of 2 µg EB/100 g BW for 43 days	Before puberty; no	75-100 days	No	
Mesocricetus auratus						
Tiefer (1970)	14:10; 1	Single sc injection of 6 µg EB per week for two weeks	Adult; yes	79 days	Yes	Hamsters received 3 E+P injections between 51-72 days after castration
Johnson (1975)	14:10; 2	Daily sc injection of 6 µg EB for 16 days then increased to 200 µg for another 16 days	One group as neonates; another group as adults No previous	90-100 days for neonate castrates; and 20-40 days for	Yes for the adult castrates with high dose	With E-treatment, adult castrates showed more mounting than neonate castrates.

			sexual experience	adult castrates		
Noble and Alsum (1975)	14:10; 3-5	Daily injection of 6 µg EB for 5 weeks	Adult; yes	7 weeks	Yes	Mounting increased after 17 daily EB treatments.
DeBold and Clemens (1978)	14:10; same-sex group housing based on treatment groups	Daily sc injection of 5 µg EB for 10 weeks	Adult; not stated	8 weeks	Yes	≥75% hamsters mounted after 5 weeks of EB treatment.
DeBold et al. (1978)	14:10; 6	Exp. 1—Daily sc injection of 5 or 50 µg EB for 14 weeks	Adult; yes	Immediate	Yes with high dose	EB maintained mounting and intromission but the effect declined over time.
Lisk and Bezier (1980)	14:10; 1	Intrahypothalamic implant [using 20G cannulae] of E2 for 28 days	Adult; yes	>7 or 9 weeks	Yes	By day 21 post-implantation, 88% of hamsters mounted. Implants were in anterior hypothalamic area.
Lisk and Greenwald (1983)	14:10; 1	Intrahypothalamic implant [using 23G cannulae] of EB for 21 days	Adult; yes	12 weeks	Yes	Implants were located in different sites in the hypothalamus; those in preoptic and parolfactory areas stimulated highest sexual activity.
Wood (1996)	14:10; 3-6	Intracranial implant [using 23G cannulae] of E2	Adult; yes	>12 weeks	Yes	Implants were in postero-medial amygdala.
Romeo et al. (2002)	14:10; 1	Implants of EB pellets at 0.05, 0.1, 0.25 mg doses for 1 week	Before puberty for 1 group and after puberty for another group; no	Immediate	Yes (adult castrates only)	0.05 mg dose stimulated highest sexual activity.

Arteaga-Silva et al. (2005)	14:10; 6	Daily sc injection of 50 µg E1 or E2 for 3 weeks	Adult; yes	5 weeks	Yes (in 38% of hamsters)	All E-treated hamsters showed high level of anogenital sniffing.
Oryctolagus cuniculus						
Agmo and Södersten (1975)	12:12; 1	Daily sc injection of 0.33 or 1 mg EB for 90 days	Adult; yes	3 months	No (with 0.33 mg dose); moderately (with 1 mg dose)	20% and 75% of E-treated rabbits mounted with 0.33 mg and 1 mg E dose, respectively.
Beyer et al. (1975)	Not stated	Daily sc injection of 5 µg EB for 30 days	Before puberty; no	At least 3 months	No	
Foote et al. (1977)	12:12; 1	Slow-release implant containing E1 (vol = 44 µL) with release rate of 2-5 µg/day for 8 weeks	Adult; no	Immediate	Yes	Mounting and ejaculating latencies were low in the first few weeks after E1 treatment but increased over time.
Sus scrofa						
Dinusson et al. (1951)	Not stated	Sc implants of 12 or 24 mg stilbestrol pellets	Not stated	Not stated	Yes	Sexual behaviour was not quantitatively measured. The authors only reported increased libido after E administration.
Booth (1983)	Not stated; 2/3 (after 22 weeks old)	Twice weekly sc injection of 1 mg E1/5kg for 22 weeks	Before puberty; not stated	8 weeks	Yes (3)/ No (2)	E induced some courtship behaviours.
Parrott and Booth (1984)	Not stated	Twice weekly sc injection of 0.1 mg ED/kg for 12 weeks	Before puberty; not stated	13 weeks	Yes (3)/ No (2)	E induced some courtship behaviours.

Levis and Ford (1989)	Lighting condition was not stated Exp. 1&2—Single housing Exp 3—Not stated	Weekly injection of: Exp. 1A—75 µg of EC/kg BW for 4 weeks Exp. 2—100 µg of EC/kg BW for 8 weeks Exp. 3—100 µg of EC/kg BW for 5 weeks	Adult; yes	Exp. 1—30 or 60 days (depending on the breed) Exp. 2—90 days Exp. 3—77 or 98 days	Yes for all experiments	Exp. 2—the effect of E declined over time.
Bos taurus						
Sawyer and Fulkerson (1981)	Animals were kept on a pasture	Exp. 1—Weekly sc injection of 10 mg EB/ 250 kg for 16 weeks Exp. 2—Weekly sc injection of 2, 4, 8, or 16 mg EB/ 250 kg for 15 weeks	Exp. 1—At birth for one group and before adulthood for another group; Exp. 2—At birth All had no sexual experience before castration.	Exp. 1—8-16 months Exp. 2—16-20 months	Yes for both experiments	Exp. 2—showed dose-dependent increase in mounting frequency with E treatment.
Dykeman et al. (1982)	Not stated; 1 (from midnight-6am and noon-6pm), in group paddock at other times	Im injection of 200 µg E2 every 2 days for 10 days.	Before puberty; no	6-8 months	Yes	
Ovis aries						
D'Occhio and Brooks (1976)	Not stated	Slow-release implant [releasing 100 or 200 µg E2 per day] for 15 days; Or daily im injection of 1 mg DES or either 0.2 or 1	Before puberty; no	Not stated	Yes (partially – see note)	Increased sex behaviour was observed in: 2/6 rams with slow-release implant; 1/1 with DES; 1/3 with 0.2 mg E2; 2/3 with 1 mg E2

		mg E2 for 2 weeks				
Mattner (1976)	Not stated	Daily im injection of 100 µg E2 for 14 weeks	Adult; yes	Not stated	Yes	
Parrott (1978)	Lighting was not controlled; group housing separated by treatment group	5 daily sc injection of 2 mg ED per week for 6 weeks	Neonates; no	15-22 months	Yes (2)/ No (2)	E induced some courtship behaviours.
D'Occhio and Brooks (1980)	Maintained under field condition	Exp. 1—Two sc slow-release implants containing crystalline 17β-E2 releasing 50 or 100 µg hormone per day for 87 weeks. Exp. 2—Daily im injection of 0.2 or 1 mg 17β-E2 for 6 weeks. Exp. 3—Daily im injection of 0.5 or 1 mg DES; 0.5 or 1 mg E1; 0.5 mg 17α-E2; 0.5 mg E3; or 1 mg hexoestrol for 6 weeks Exp. 4—Daily im injection of 1 mg 17β-E2 for 12 weeks	Castrated either before (exp. 1-3) or after puberty (exp. 4) Adult rams were sexually experienced but the prepubertal rams were not	Exp. 1-3—Not stated Exp. 4—2 years	Yes for all experiments (see note for exp. 1-3)	Exp. 1—Mounting was activated in all rams after prolonged E treatment (85 weeks). Exp. 2&3—Each E compound was effective in restoring sexual behaviour to varying degrees, except for E3 and hexoestrol, which were not effective.
Parrott and Baldwin (1984)	Not stated	5 daily sc injection of 2 mg ED per week for 4 weeks	Neonates; no	Approx. 15 months	Yes (2)/ No (3)	E induced some courtship behaviours.

Pinckard et al. (2000)	Natural lighting; 1	Two slow-release implants, each containing 309 mg of E2 for 6 weeks	Adult; yes	6 weeks	No	E induced some mounting behaviour in female-oriented rams but not in male-oriented rams.
Cervus elaphus						
Fletcher and Short (1974)	The deer lived in the wild	Sc implant of 100 mg E2 for 3.5 months (1 deer) or more than 3 weeks (2 deer).	Adults; [possibly sexually experienced because they were wild deer]	11 months for one deer, the other 2 were unspecified	Yes	
Equus ferus						
Thompson et al. (1980)	Not stated	Daily sc injection of 44 µg EB every two days for 18 days then increased to 88 µg for another 20 days	Adult; not stated	30 days	Yes (when the dose was 88 µg)	
Mustela furo						
Baum (1976)	16:8 (from the age of 5 months onwards); 2-3	Daily sc injection of 10 µg EB/kg for 17 days	Before adulthood; no	23-24 months	Yes	The ferrets received T injections for 3 weeks at 20 months after castration.
Felis catus						
Green et al. (1957)	Not stated; 1	E2 (6000 RU) or stilbestrol (1-5 mg); method of administration was not stated	Adult; not stated	Not stated	Yes	
Macaca mulatta						
Phoenix and Chambers (1982)	Not stated; 1	Daily im injection of 20 µg EB for 51 days	Adult; yes	Not stated	No	E treatment reduced mounting latency. Mounting was observed in more tests with E-treated monkeys than with untreated

						castrated monkeys.
Michael et al. (1990)	14:10; 1	For immediate castrates: 2 µg EB/kg for 4 weeks then re-treated again 30 weeks later with the same treatment For 8-weeks castrates: 2 µg EB/kg for 4 weeks, followed by 5 µg EB/kg for 2 weeks. Eight weeks later re-treated with 2 µg EB/kg for 4 weeks	Adult; yes	Immediate or 8 weeks	No	The 5 µg EB/kg caused penile edema.
Avians						
Cortunix japonica						
Adkins and Adler (1972)	8:16 (while on E treatment); 1	Exp. 3—Daily im injection of 50 µg EB for 8-10 days	Adult, yes	At least 3 weeks	Yes	Castrated by exposing the birds to a short photoperiod. [*Short photoperiod reduces gonadotrophin secretion and causes gonadal regressions in quails.*]
Adkins (1975)	8:16; 1	Daily im injection of 50 µg EB for 9-11 days	Adult; yes	3 weeks	Yes	Castrated by exposing the birds to a short photoperiod.
Adkins and Nock (1976)	16:8; 1	Exp. 2—Daily im injection of 50 µg EB for 16 days	Adult, yes	10 days	Yes	
Adkins and Pniewski (1978)	8:16; 1	Exp. 2—Daily im injection of 100 or 50 µg EB for 26 days	Adult; yes	3 weeks	Yes	Castrated by exposing the birds to a short photoperiod.
Hutchison (1978)	12:12; group-housed (10-12) until 3 months old	Part III. Exp. 2—Daily im injection of 0.1 mg EB for 5 days then increased to 0.5	At hatching; no	120 days	No	Exp. 2 was done when the birds were 120 days old.

	then housed singly	mg EB per day for another 5 days				
Adkins et al. (1980)	8:16; not stated	Exp.1—Three ip slow-release implants (vol = 19 µL each) containing EB for 3 weeks Exp. 2—Daily injection of 50 µg EB for 2 weeks	Adult; yes	At least 3 weeks	Yes	Castrated by exposing the birds to a short photoperiod. Birds were previously treated with TP for 2 weeks but TP was stopped 2 weeks before the first EB injection.
Balthazart et al. (1980)	16:8 (during treatment); 1	Daily injection of 0.2 µg EB for 6 days then raised to 2 µg/day for another 8 days	Adult; yes	6 weeks	No	
Wada (Wada, 1982)	16:8, 1	Two slow-release implants (total content = 50 mg E2) for 2 weeks	Adult; not stated	4 weeks	Yes (3)/ No (3)	The birds received T implant on the third and fourth weeks after castration.
Van Krey et al. (1983)	Exp. 1—5:19, 1	Daily im injection of 5 mg EB	Adult; yes	3 weeks	Moderate increase in high, but not low, mating line birds	The birds were castrated using short photoperiod. The birds were bred from high and low mating lines, which show high and low sexual behaviour respectively. Of note, all birds showed high mating behaviour when tested with freshly killed quails.
Schumacher and Balthazart (1983)	16:8 (during treatment); 1 (after castration)	Slow-release implant (vol = 39 µL) containing E2 for 3 weeks then received an additional identical implant	Adult; not stated	Not stated	Yes (2)/ No (3) with the first implant	None copulated after the second implant.

		for another 2 weeks				
Balthazart and Schumacher (1984)	Not stated; 1 (starting at 34 days old)	Exp. 2—Neonates received a slow release implant containing E2 for 2 weeks then an additional identical implant for another 2 weeks. When they reach adulthood, they received another E2 implant for 46 days with 4X the dose of the first implant.	Neonates; no	2 days	Yes	Adult birds received T implant for 54 days immediately before the E-treatment.
Balthazart et al. (1985)	Not stated; 1 (after castration)	Exp. 2—Daily injection of 0.01, 0.1, or 1 mg E2 for 19 days Exp. 3—Slow-release implant (vol = 39 µL) containing E2 for 2 weeks then received a second, identical, implant for another 6 weeks Exp. 4—Slow-release implant (vol = 9.7 µL) containing E2 for 18 days then a second, identical, implant for another 21 days	Exp. 2,3,4—Before adulthood Pre-castration sexual experience was not stated.	Exp. 2—1 week Exp. 3—4 weeks Exp. 4—2 weeks	Exp. 2—Yes (dose-dependent) Exp. 3—Yes (weak response) Exp 4—No	Daily injection in exp. 2 was more effective in restoring copulatory behaviour than the use of E2 implant in Exp.3. The dose in Exp. 4 was lower than in Exp. 3.
Adkins-Regan and Garcia (1986)	8:16; 1	Exp. 2—Daily im injection of either 50 µg EB, or 25, 50, or 100 µg of DES for 22 days Exp. 3—Slow-release implant containing E2 or	Adult, not stated	2-8 weeks	Exp. 2—Moderately (with EB); weakly (with DES) Exp. 3—Yes	Exp. 2—EB induced copulation in 57% of birds whereas less than 30% b copulated after DES treatment. Exp.3—All DES-implanted

		DES (vol = 38 µL) for 24 days			(DES); weakly (E2)	birds copulated whereas only 33% of E2-implanted birds copulated.
Alexandre and Balthazart (1986)	Not stated; 1 (from the age of 6 weeks onwards)	Exp. 1—Slow-release implant (vol = 19.3 µL) containing E2 for 15 days Exp. 6—Daily injection of 200 µg of DES for 17 days	Exp. 1&6—Before adulthood Pre-castration sexual experience was not stated.	Exp. 1—2 weeks Exp. 6—Not stated	Exp. 1— No Exp. 6—Yes	Exp. 6—In the DES-treated group, ~75% of birds attempted mounting at least once during the mating test but the mounting frequency during the test was not significantly elevated.
Schumacher et al. (1987)	Not stated; 1 (after castration)	Daily im injection of 200 µg DES for 25 days	Not stated	Not stated	Yes	
Watson and Adkins-Regan (1989)	8:16; 1	Intracranial implant containing 300 µg EB for 2 weeks	Adult, yes	2-7 days	Yes (with implants in preoptic area)	Implants were in different sites in forebrain and diencephalon; implants in the preoptic area restored the most mounting.
Balthazart and Surlemont (1990)	Not stated; 1	Exp. 2—Intrahypothalamic implant [27G needle] filled with DES (up to 1 mm depth) for 2-3 weeks	Before adulthood; not stated	2-3 weeks	Yes	The implants were in the preoptic area.
Watson et al. (Watson et al., 1990)	8:16; 1 (after 2 months old)	Exp. 2—Slow-release implant (vol = 4.8, 9.7, 19, 39, 58.1 µL) containing crystalline E2 for 26 days	Adult; yes	Immediate	Yes	There was a dose-dependent increase in copulatory behaviour.
Balthazart et al. (1995)	16:8; 1	Exp. 3—Daily im injection of 200 µg DES for 26 days then increased to 1 mg per day for 17 days	Exp. 3,4—Before adulthood Pre-castration sexual experience	Exp. 3—3 weeks Exp. 4—2 weeks	Exp. 3—With 1 mg dose: Yes (3); No (3) Exp. 4—Yes	In exp. 4 DES was more effective than E2 in restoring copulatory behaviour.

		Exp. 4—Two slow-release implant (vol = 39 µL each) containing E2 or DES for 3 weeks	was not stated.			
Cornil et al. (2006)	16:8; 1	Exp. 1—Single ip injection of 500 µg E2/kg at 5, 15, or 30 minutes before test Exp. 2—Single ip injection of 500 µg E2/kg at 15 minutes before test	Before adulthood; no	5 weeks	Yes	The birds each received a small T-implant at 2 weeks post-castration to stimulate minimal copulation. Highest improvement in sexual behaviours when E2 was injected 15 minutes before test.
Seredynski et al. (2011)	16:8; 1	Daily im injection of 250 µg of DES, ERα-specific agonist, or ERβ-specific agonist for 13 days	Before adulthood; not stated	At least 3 weeks	Yes	More E-treated birds attempted mounting than control. DES activated cloacal contact movement in ~40% of birds (no birds showed this behaviour with ER-specific agonists).
Streptopelia risoria						
Cheng and Lehrman (1975)	Not stated	Daily im injection of 50, 100, or 200 µg EB for 10 days	Adult; yes	2-3 weeks	Yes (courtship)	Only courtship, not copulatory, behaviours were analyzed.
Hutchison (1970b)	8.5:15.5 (after castration); 1	Daily im injection of 300 µg EB for 15 days	Adult; yes	30 days	Yes	Only courtship, not copulatory, behaviours were analyzed.
Hutchison (1970a)	Not stated	Intrahypothalamic implant containing 47 µg EB	Not stated; not stated	Not stated	Yes	Only courtship, not copulatory, behaviours were

						analyzed. Implants were in anterior hypothalamus.
Hutchison (1971)	13:11 (before castration) 8.5:15.5 (after castration); 1	Intrahypothalamic implant containing crystalline EB [weight = 47 µg] for 14 days	Adult; yes	30 days	Yes	Only courtship, not copulatory, behaviours were analyzed.
Martinez-Vargas (1974)	14:10, 1	Daily im injection of 0.2 mg EB for 21 days	Adult; yes	21 days	Yes	Only courtship, not copulatory, behaviours were analyzed.
Adkins-Regan (1981)	14:10; 1	Exp. 1—Daily im injection of 100 µg EB for 13-15 days	Adult; yes	3 weeks	Yes (courtship)/ No (copulatory)	Only 2 out of 9 birds attempted to copulate, however, all of the birds displayed wing flipping (one type of courtship behaviours). The birds were TP-treated in the first two weeks after castration.
Hutchison et al. (1981)	14:10; 1	Exp. 1&2—Daily im injection of 300 µg EB for 10 days Exp. 3—Daily im injection of 30 µg EB for 10 days followed by another 300 µg/day for 5 days	Adult, yes	200 days	Yes	Only courtship, not copulatory, behaviours were analyzed.
Cohen and Cheng (1982)	14:10; 1	Implant in mid-brain areas using 30G needle	Adult, yes	3 weeks	Yes (only in inter-	Implants were in inter-collicularis region and

		(containing 7 μg E2)			collicularis area)	various other midbrain areas. Only courtship, not copulatory, behaviours were analyzed.
Gallus gallus						
Davis and Domm (1941)	Not stated	Daily injection of either 0.5-2 mg E2 or 1-2 mg stilbestrol for various durations.	Not stated; not stated	Not stated	Yes	
Guhl (1949)	12:12; group housing with both sexes	Injection of 4.5 mg DES once every 2 days for a 67-day period	Castrated at 10 weeks of age; not stated	12 weeks	Yes	The sexual response declined over time.
Van Krey et al. (1983)	Exp. 2—14:10; 1	Exp. 2—Daily im injection of 5 mg EB for 13 days	Exp 2—Castrated at 7 weeks of age; not stated	Exp. 2—12 weeks	No (with live hens); moderately (with dead hens)	The birds showed high mating behaviours when tested with a freshly killed hen.
Taeniopygia guttata						
Harding et al. (1983)	14:10; same-sex group housing	Slow-release implant (vol = 2.3 μL) packed with crystalline E2 for 7 weeks	Adult; not stated	3 weeks	No	Only 1 out of 9 male birds attempted to mount a female; courtship activity was low in E-treated birds.
Melopsittacus undulates						
Brockway (1974)	12:12; 1	Im injection of 0.2 or 0.4 mg EC every 2 days for 12 days	Before adulthood; not stated	1-7.5 months	Yes	The male birds were visually but not acoustically isolated from female birds. The birds showed courtship and precopulatory behaviours

						after E treatment.
Reptiles						
Anolis sagrei						
Tokarz (1986)	14:10; 1	50 µg E2 pellets for 2 weeks	Adults; not stated [possibly sexually experienced because they were captured in the wild]	1 week	No	Only 2 out of 12 animals displayed copulatory behaviours though ~50% showed courtship behaviours.
Anolis carolinensis						
Mason and Adkins (1976)	14:10; 1 (when injections began)	Daily sc injection of 2 µg EB for 10 days	Adults; not stated	At least 1 week	Yes (2)/ No (4)	Only courtship, not copulatory, behaviours were assessed.
Crews et al. (1978)	14:10; not stated	Slow-release implant (volume = 2.5 µL) containing E2 for 12 days The plasma E2 levels of E-treated animals reached 8 ng/mL [non-detectable in intact (<0.51 ng/mL)]	Adult; yes	15 days	No	Only courtship, not copulatory, behaviours were assessed.
Crews and Morgentaler (1979)	14:10; 1	Intrahypothalamic implant containing 20 µg 17β-E2	Adult; yes	3 weeks	Yes	Only courtship, not copulatory, behaviours w assessed. Implants were in different brain areas; only those in anterior hypothalamus-preoptic area can restore sexual behaviour.
Winkler and	14:10; 1	Slow-release implant (vol =	Adult; not stated	Immediate	No	

Wade (1998)		2.7 µL) containing EB				
Latham and Wade (2010)	14:10 or 10:14; 1	Two slow-release implants each containing 2 mg E2	Adult, not stated [possibly sexually experienced because they were captured from the wild]	Immediate	Yes	Two lighting conditions were used to simulate Spring and Fall. E only activated copulatory behaviour under the 14:10 (Spring) condition.
Eublepharis macularius						
Rhen and Crews (1999)	Not stated; 1	Slow release implant (vol = 17 µL) containing E2 [reaching plasma level of 8 ng/mL] for 4 weeks	Adult; yes	Immediate	Yes (courtship)/ No (copulatory)	E increased courtship behaviour but not copulatory behaviour
Amphibians						
Taricha granulosa						
Deviche and Moore (1988)	Not stated; 150 newts in a single pond	Slow release implant [1 capsule (vol = 12 µL) for low dose or 3 capsules for high dose] packed with E2 for 75 days	Adult; not stated [possibly sexually experienced because they were sexually mature at the time of capture]	Immediate	No	< 10% displayed clasping behaviour with low E dose; none with high E dose.
Triturus cristatus carnifex						
Andreoletti et al. (1983)	Seasonal photoperiod; Same-sex group housing	Exp. 3. Slow release implant containing 3.5 mg E2 for 21 days	Adult; yes	Immediate	No	
Xenopus laevis						
Kelley and	14:10; 5-6	Exp. 2 & 3. Slow release	Adult, yes	Not stated	No	Some males received human

Pfaff (1976)	implants containing either 10 or 5 mg E2 (pellets or via Silastic tubes) into the dorsal lymph sac	(after no clasping behaviour was displayed)	chorionic gonadotropin injection on the testing day. Mortality was observed in 85% of frogs receiving 10 mg E2 pellets, 57% and 8% receiving 10 mg, and 5 mg E2 pellets respectively via Silastic tubes.

BW = body weight; DES = diethylstilbestrol; DHT = dihydrotestosterone; EB = estradiol benzoate; E1 = estrone; E2 = estradiol; E3 = estriol; EC = estradiol cypionate; ED = estradiol dipropionate; im = intramuscular; ip = intraperitoneal; RU = rat unit; sc = subcutaneous; T = testosterone; TP = testosterone propionate

The number in parentheses in the 6th column indicates the number of animal(s) with particular response.

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