INVOLVEMENT OF ERALPHA AND ERBETA IN ESTRADIOL-INDUCED ENHANCEMENT OF HIPPOCAMPAL NEUROGENESIS AND SEXUAL BEHAVIOUR IN THE FEMALE RAT

by

CHRISTINE ANNE MAZZUCCO

BSc(Hons), The University of Toronto, June 2001

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

(Neuroscience Program)

THE UNIVERSITY OF BRITISH COLUMBIA

April 2005

©Christine Mazzucco, 2005

ABSTRACT

In this study we investigated the involvement of estrogen receptors alpha and beta in both sexual behaviour and estradiol-induced enhancement of hippocampal neurogenesis in the adult female rat. Our study utilized recently available subtype selective alpha and beta estrogen receptor (ER) agonists, propyl-pyrazole triol (PPT) and diarylpropionitrile (DPN) to examine each receptor's contribution, individual and co-operative, for these primarily estradiol-mediated processes. To evaluate sexual behaviour and cell proliferation, rats were bi-laterally ovariectomized (OVX) and received subcutaneous injections of estradiol (10 µg), PPT and DPN alone (1.25, 2.5, 5.0 mg /0.1 ml DMSO) or in combination (2.5 mg PPT + 2.5 mg DPN/ 0.1 ml DMSO). For experiment 1, our findings demonstrate that ERalpha is involved in eliciting both proceptive and receptive behaviour. Our laboratory has previously found that estradiol increases cell proliferation within 4 hrs in the dentate gyrus of adult female rats, therefore, for experiment 2 we administered estradiol or the agonist(s) 4 hrs prior to the cell synthesis marker bromodeoxyuridine (BrdU; 200 mg/kg). DPN enhanced cell proliferation by approximately 40% at all three administered doses (1.25 mg, p<0.008; 2.5 mg, p<0.003; 5 mg, p<0.005), whereas PPT showed increased cell proliferation (by approximately 50%, p< 0.0002) at a specific dose of 2.5 mg. Our results demonstrate both ERalpha and ERbeta are individually involved in estradiol-enhanced cell proliferation. Dual receptor activation resulted in decreased levels of proceptivity, receptivity and cell proliferation, compared to either agonist alone, supporting previous studies suggesting that ERalpha and ERbeta may modulate each other's activity. Our results demonstrate the exclusive involvement of each ER subtype in two distinct physiological processes, sexual behaviour and hippocampal cell proliferation and suggest that a component of ER regulated cell proliferation may take place through alternative ligand and/or cell-signaling mechanisms.

ii

TABLE OF CONTENTS

Abstractii
Гable of Contentsiii
List of Tablesiv
List of Figuresv
Acknowledgementsvi
Introduction1
Materials and Methods5 Results
Discussion
Regulation of proceptive and receptive female rat sexual behaviour is through
ERalpha28
Both ERalpha and ERbeta are involved in estradiol-induced upregulation of cell
proliferation
ERalpha and ERbeta demonstrate opposing roles to regulate transcriptional
activity
Estradiol-induced enhancement of cell proliferation may also be modulated
through a ligand independent mechanism32
Conclusion34
References

iii

LIST OF TABLES

<u>Table 1.</u> Mean (\pm S.E.M.) dentate gyrus volume of vehicle, estradiol and agonist-
treated rats in Experiment 226
Table 2. Mean (± S.E.M.) Percentage of BrdU-ir cells expressing an immature neuronal
(Doublecortin-ir) or glial (GFAP-Ir) phenotype26

LIST OF FIGURES

Figure 1A & B. Frequency of ear wiggling as a function of treatment in female rats18
Figure 2A & B. Frequency of hopping and darting as a function of treatment in
female rats19
Figure 3A & B. Lordosis quotient as a function of treatment in female rats21
Figure 4A & B. Lordosis rating as a function of treatment in female rats
Figure 5A & B. Rejection quotient as a function of treatment in female rats23
Figure 6A & B. Mean (± S.E.M.) number of new cells in the dentate gyrus of adult
female rats given BrdU 4 hrs following a treatment injection25
Figure 7. Two newly synthesized BrdU-labeled cells located in the subgranular zone of
an adult female rat administered 10 µg of estradiol27
Figure 8. Confocal image of a BrdU-labeled cell expressing immature neuronal protein
in the subgranular zone of an adult female rat administered 2.5 mg of PPT27

v

ACKNOWLEDGEMENTS

I would like to express my gratitude to Dr Liisa Galea for this incredible research and academic opportunity. Through her guidance and support I have grown as an individual and researcher. I am grateful for the countless memorable and life-changing academic experiences that have enhanced my existence, both professionally and personally.

I would like to thank my family and friends for their love and support during some of my most challenging moments.

A special thanks to my lab mates, Katia, Jas, Jodi, & Hope for their technical assistance, words of encouragement and company.

I would like to thank Stephanie Lieblich and Lucille Hoover for their technical assistance and their incredible resourcefulness in the lab.

This research was supported by a CIHR operating grant to LAMG.

INTRODUCTION

Estradiol can have profound effects on the brain and behaviour, from involvement in sexual behaviour (Pfaff et al., 1994) to the modulation of neuronal proliferation, survival and plasticity (McEwen et al., 2001; Behl, 2002). Examining the mechanisms that induce neurogenesis in the adult mammalian brain may provide a basis for neuronal replacement therapy in neurodegenerative diseases such as Alzheimer's Disease (AD).

The majority of estradiol's identified transcriptional actions in mammals are mediated by the classical estrogen receptor (ER) alpha (White et al., 1987) and the more recently cloned ERbeta, (Kuiper et al., 1996; Mosselman et al., 1996; Enmark et al., 1997; Tremblay et al., 1997). Since the discovery of ERbeta, studying and resolving the mechanisms of action behind estrogens and related synthetic drugs has become increasingly complex. For example, the synthetic estrogenic agent tamoxifen has both agonist/antagonist properties depending on the tissue in which it is examined; it serves as an estrogen-receptor antagonist in breast tissue (Jordan, 1995) and the brain (Hilton et al., 2004) however, acts as an estrogen agonist in bone (Turner et al., 1987; Moon et al., 1991). Furthermore, the mode of interaction of estrogenic agents also depends on the ER subtype involved; for instance, tamoxifen is a mixed agonist/antagonist of estrogen receptor alpha (ERalpha) and a pure antagonist of ERbeta (McInerney and Katzenellenbogen, 1996; Barkhem et al., 1998; Zou et al., 1999; Buteau-Lozano et al., 2002). Therefore, understanding the differential effects of estradiol requires the consideration of cell context along with receptor subtype involvement.

ERalpha and ERbeta belong to the steroid hormone superfamily of nuclear receptors that act as ligand-inducible transcription factors. ERalpha and ERbeta appear to be complementary but not redundant and are genetically and functionally distinct (Simonian and Herbison, 1997; Kuiper et al., 1998). Investigation into how estradiol is mediating genomic effects involves

determining which receptor(s) is initiating transcription of estrogen-responsive elements (ERE) and non-ERE sites within the target cell genome. The use of ER subtype-selective ligands provides an alternative, complementary approach to the use of receptor knock out mice (Ogawa et al., 1998; Ogawa et al., 1999; Rissman et al., 1999; Kudwa and Rissman, 2003). There are several experimental caveats to consider when working with gene knock out animals including, pleotropic effects, developmental failures and the production of truncated proteins that have uncertain activity *in vivo*. Recently available ER subtype agonists, propyl-pyrazole triol (PPT), an ERalpha agonist and diarylpropionitrile (DPN) an ERbeta agonist are used in the present study to examine the individual physiological roles of each of the two receptors and their potential co-modulatory effects in mediating two estradiol-dependent processes, sexual behaviour and enhanced cell proliferation in the dentate gyrus of adult female rats.

PPT and DPN are non-steriodal ligands and valuable tools for elucidating the biological activity of ERs by selectively targeting each receptor subtype. PPT is a potent ERalpha agonist, which does not activate ERbeta. PPT binds to ERalpha with high affinity (50% that of estradiol). It has a 410-fold binding selectivity preference for ERalpha and demonstrates almost no binding to ERbeta (Kraichely et al., 2000; Stauffer et al., 2000). Additionally, PPT only activates gene transcription through ERalpha and the potency selectivity may be greater than 10,000 (Stauffer et al., 2000). PPT increased progesterone receptor mRNA expression and suppressed experimentally induced hot flashes, both of which are known to be estrogen regulated, suggesting its estrogenic effects in the brain induced by ERalpha activation (Harris et al., 2002). In contrast, the estrogen receptor agonist DPN has a 70-fold higher binding selectivity for ERbeta over ERalpha and is a full ERbeta agonist (Meyers et al., 2001). DPN exhibits a 170-fold higher relative potency for ERbeta over ERalpha in transcriptional activation studies (Meyers et al., 2001). Additionally, DPN significantly reduced anxiogenic behaviour (Lund et

al., 2005; Walf and Frye, 2005), which is mediated by ERbeta (Krezel et al., 2001; Imwalle et al., 2005; Lund et al., 2005; Walf and Frye, 2005) demonstrating it's effects in the brain mediated through this subtype. The availability of receptor specific agonists provides a promising alternative strategy for unraveling ERs role in a multitude of functions.

Estradiol is the primary chemical communicator for the expression of female sexual behaviour in all vertebrates (Pfaff et al., 1994). Female rat sexual behaviour has two active components, proceptivity (the female's appetitive activities directed at the male) and receptivity (lordosis posture). Both ERalpha and ERbeta are expressed differentially in the neural sites for mating behaviour (Shughrue et al., 1997; Shughrue and Merchenthaler, 2001; Shima et al., 2003) and single neurons in these areas have been shown to contain both ERs (Shughrue et al., 1998), specifically, the ventromedial hypothalamus (VMH), which facilitates lordosis (Kennedy, 1964; Dorner, 1969; Carrer et al., 1973; Rubin and Barfield, 1983; Meisel et al., 1987; Pfaff, 1989) and the medial preoptic area (mPOA), which has been implicated in both appetitive and consummatory behaviours (Aou et al., 1988; Hoshina et al., 1994; Sakuma, 1995; Kato and Sakuma, 2000). Studies using knock-out mice have found that ERalpha is essential for the expression of lordosis (Rissman et al., 1997a; Ogawa et al., 1998; Kudwa and Rissman, 2003) and demonstrated the survival of reproductive behaviors in ERbeta gene-deficient mice (Ogawa et al., 1999; Kudwa and Rissman, 2003). Knockout studies suggest the exclusive involvement of ERalpha for female rodent receptivity, however, the role of ERbeta in sexual behaviour warrants further investigation. ERbeta is found in the ER-containing POA projections suggested to elicit proceptive behaviour and neurons within this area display distinct firing patterns in response to proceptive behaviour (Kato and Sakuma, 2000). Additionally, ERbeta has been detected along with Fos expression in response to mating stimuli, suggesting involvement in the hormonal integration of sensory information (Greco et al., 2003). The aim of experiment 1 was

to determine whether the two novel estrogen receptor (ER) agonists, PPT, and DPN alone or in combination could elicit either or both aspects of female rat sexual behaviour and if so, at what dose(s).

In recent years, estradiol has also been shown to dynamically influence neurogenesis (both cell proliferation and survival) in the adult rodent hippocampus (Tanapat et al., 1999; Ormerod and Galea, 2001; Ormerod et al., 2003b; Tanapat et al., 2005). Studies from our laboratory have demonstrated that estradiol exposure for 4 hours enhances whereas estradiol exposure for 48 hours suppresses cell proliferation in the dentate gyrus of adult female meadow voles and rats (Ormerod and Galea, 2001; Ormerod et al., 2003b). Estradiol appears to increase cell proliferation directly through a serotonin mechanism (Banasr et al., 2001) and suppress cell proliferation by stimulating adrenal activity (Ormerod et al., 2003b). ERalpha and ERbeta mRNA have been detected both isolated (Shughrue et al., 1997; Shughrue and Merchenthaler, 2001) and co-localized (Su et al., 2001) in the adult rat hippocampus. The aim of experiment 2 was to determine the involvement of each receptor, via ER agonists PPT and DPN, in estradiolinduced enhancement of cell proliferation within the 4 hour time period. Understanding the involvement of each receptor subtype in hippocampal cell proliferation is a primary investigative step into determining the mediators involved in the neuroprotective effects of estradiol.

<u>4</u> ·

MATERIALS AND METHODS

All experiments were conducted in accordance with the policies established by the University of British Columbia and the Canadian Council on Animal Care regarding the ethical treatment of animals used for research. Every effort was made to minimize the number of animals used per group and their suffering.

Subjects.

Seventy-six adult female Sprague-Dawley (250-300 grams) rats were obtained from Charles Rivers Laboratories (Quebec, Canada). All rats were housed singly in polyurethane cages with access to food (Purina Lab Diet 5012, Richmond, Indiana, USA) and water ad libitum. Experiment 1 female animals (n=24) were maintained on a reversed 12:12 hr light dark cycle with lights off at 0900 hours. The purpose of the reverse cycle was to schedule testing during their active cycle. Twelve sexually experienced Long-Evan male rats (450-500 grams) served as stud males for sexual behavior testing. Experiment 2 female rats (n=52) were maintained on a 12:12 hour light dark cycle with lights on at 0700 hours to correspond with previous literature investigating estradiol's effects on neurogenesis (Tanapat et al., 2001; Ormerod et al., 2003b).

Surgery.

Approximately 1 to 2 weeks after arrival, all females were bi-laterally ovariectomized. First animals were placed in a chamber to which halothane was delivered at an induction flow rate of 3% (flow rate of O_2 was 2%) and maintained on a flow rate of 1-3% to maintain a stable respiratory rate. Animals were given 7 days to recover prior to the commencement of experimental manipulation.

Experiment 1. ERalpha and ERbeta Involvement in Female Rat Sexual Behaviour.

Experiment 1 was conducted to determine a physiological relevant dose for two novel estrogen receptor (ER) agonists, propyl-pyrazole triol (PPT), an ERalpha specific agonist and diarylpropionitrile (DPN), an ERbeta specific agonist to elicit and investigate female rat sexual behaviour. Previous work has shown that estradiol acts within the VMH to facilitate receptivity, defined as the lordosis response, the posture adopted by a receptive female rat in response to a mounting male during mating (Kennedy, 1964; Dorner and Staudt, 1969; Carrer et al., 1973; Rubin and Barfield, 1980; Meisel et al., 1987; Pfaff, 1989). ERalpha is the predominant estrogen receptor in the VMH and is found in neurons throughout the rostrocaudal extent of this brain region (Pfaff and Keiner, 1973; Simerly et al., 1990; Shughrue et al., 1997; Shughrue and Merchenthaler, 2001). The importance of this receptor subtype for lordosis was demonstrated in studies utilizing ERalpha and ERbeta knock out mice where by the lordosis reflex was either eradicated or maintained respectively (Ogawa et al., 1998; Ogawa et al., 1999; Rissman et al., 1999; Kudwa and Rissman, 2003). However, knock out models have some limitations and ER subtype specific agonists provide a complementary method to corroborate ERalpha's exclusive involvement for sexual receptivity. Therefore, the first aim of this experiment was to determine an effective dose of the ERalpha agonist PPT that could elicit sexual receptivity and proceptivity in order to confirm ERalpha versus ERbeta involvement in sexual receptivity and second, to further investigate each ER's involvement, individual and/or co-operative, in receptive and proceptive behaviour in ovariectomized female rats. More recently, studies have localized a second ER subtype, ERbeta, in the VMH (Shughrue and Merchenthaler, 2001; Shima et al., 2003). Although previous studies have suggested no involvement of ERbeta in sexual behaviour with the use of estrogen receptor knock out (ERKO) mice (Ogawa et al., 1999; Kudwa and Rissman, 2003), it is necessary to confirm these findings using selective agonists due to the

known caveats when working with gene knock out animals. Therefore, we also investigated sexual receptivity with the novel ERbeta agonist, DPN. Unlike receptive behaviour, being solely defined as the lordosis response, proceptive behaviour includes unique solicitory actions such as ear wiggling and hops and darts (Beach, 1976; Hlinak and Madlafousek, 1977). The neural circuitry for proceptive behaviour is not yet known, however, the major neural sites implicated are the VMH (Aou et al., 1988) and medial preoptic area (mPOA; (Aou et al., 1988; Hoshina et al., 1994; Sakuma, 1995; Kato and Sakuma, 2000), which contain both ERalpha and ERbeta (Shughrue et al., 1997; Shughrue and Merchenthaler, 2001; Shima et al., 2003), therefore agonists specific to each receptor subtype were used to examine proceptive behaviour. To our knowledge, sexual behaviour has yet to be examined using ER specific agonists, therefore our investigation includes an exclusive approach by employing novel ER agonists individually, and in combination, to determine a physiological relevant dose to demonstrate their relative contributions towards proceptive and receptive behaviour. A plausible hypothesis is that estradiol is mediating sexual behaviour through ERalpha and ERbeta, individually, and/or in combination, to elicit proceptive and receptive behaviour in adult female rats. Thus the aim of experiment 1 was to determine the relative contribution of each receptor subtype in the two active components of female rat sexual behaviour.

Drug treatment.

Twenty-four ovariectomized female rats were randomly assigned to 1 of 12 groups (n=6/8 per group) and received subcutaneous (s.c.) 0.1 ml injections of either: sesame oil (OIL), dimethylsulfoxide (DMSO), estradiol benzoate (EB; 10 μ g/0.1 ml OIL), one of 5 doses of the ER alpha agonist propyl-pyrazole triol (PPT; 0.3125 mg, 0.625 mg , 1.25 mg, 2.5 mg or 5.0 mg/ 0.1 ml DMSO), one of 3 doses of the ER beta agonist diarylpropionitrile (DPN; 1.25 mg, 2.5 mg or 5.0 mg/0.1 ml DMSO) or a combination dose of PPT and DPN (2.5 mg/0.1 ml DMSO) for two

consecutive days, 48 hours and 24 hours prior to testing followed by a progesterone injection (P = $500 \ \mu g \ P/0.1 \ ml \ OIL$) which was administered 4 hours prior to testing in accordance with (Hansen et al., 1991). Female rats were tested 3 times to account for all treatment groups and groups were counterbalanced with re-testing occurring at least 10 days apart in order for the treatment to fully dissipate before the next treatment condition.

Drug preparation.

The ER alpha agonist, propyl-pyrazole triol (PPT; Tocris Chemicals) and the ER beta agonist, diarylpropionitrile (DPN; Tocris Chemicals) were dissolved in dimethylsulfoxide (DMSO; Sigma Chemicals). 17beta-estradiol benzoate (Sigma Aldrich Chemicals) was combined with sesame oil (Sigma Aldrich Chemicals) over low heat to obtain a concentration of 10 µg EB per 0.1ml sesame oil (Ormerod et al., 2003a). The solution was mixed and stored in a light insensitive container. Progesterone (Sigma Chemicals) was combined with sesame oil (Sigma Chemicals) to obtain a concentration of 500 µg progesterone per 0.1 ml sesame oil and stored in a light insensitive container.

Sexual behaviour handling and habituation.

Following post-operative recovery (7 days), each animal was handled for 5 minutes on 2 occasions. Following handling, all animals were habituated to the sex testing apparatus, a bilevel chamber (described below), for 10 minutes on 2 occasions.

Testing Procedure and Sexual Behaviour Measurements.

Female rats were tested for sexual behaviour in a bi-level testing chamber. These chambers are narrow in width (width x length; 7 x 24 inches), which maintains an optimal sideway orientation of the animal to the experimenter and consist of two levels (height; 30 inches) connected by a set of ramps on either side. These chambers have been used previously to examine the relationship of appetitive and consummatory sexual behaviours in male and female

rats (Pfaus et al., 1999). Testing was done within 4-6 hours after progesterone administration, with the female rat placed on the top level of the bi-level chamber and a sexually vigorous male rat placed on the bottom level. Behaviours were video recorded for 10 min for further analysis. The female behaviour measurements evaluated included: 1) level changes, 2) ear wiggling 3) hops and darts 4) lordosis quotient, 5) lordosis rating and 6) rejection quotient. In addition, the number of mounts and mount attempts by the male rat was recorded to evaluate their sexual aggressiveness and mount latency was recorded to examine attractivity of the female rats. Distinct proceptive behaviours of female rats were ear wiggling (a rapid oscillatory movement of her ears) and hops and darts (jump and scatter within close proximity directly in front of the male). The receptive behaviour was the lordosis response; a postural reflex with a dorsiflexion of the vertebral column (Hardy and Debold, 1971). Two sexual receptivity measurements were determined by examining the lordosis response; the percentage of times the female rat exhibited lordosis in response to a sexual contact (lordosis quotient; LQ = # of lordosis response scores of 2 or 3/# of mounts) and the intensity of lordosis responses (lordosis rating; LR = sum of 0, 1, 2, or 3 response scores/# mounts) (Hardy and Debold, 1971; Brandling-Bennett et al., 1999). Defensive behaviours were measured by addition of three rejection responses (fending, kicking and rolling) with each response receiving one point (rejection quotient; RQ = total number of rejection scores/#mounts).

Statistical Analysis.

The dependent variables (level changes, ear wiggling, hopping and darting, lordosis quotient, lordosis rating, rejection quotient, mount attempts and mount latency) were each analyzed using an analysis of variance (ANOVA) with treatment group (OIL, EB, DMSO, PPT0.3125, PPT 0.625, PPT 1.25, PPT 2.5, PPT 5, DPN 1.25, DPN 2.5, DPN 5 and PPT&DPN 2.5) as the independent variable. *A priori* comparisons used Dunnett's procedure while post-hoc

comparisons used Neuman-Keul's method unless otherwise specified. All statistical procedures set $\alpha = 0.05$.

Experiment 2. ERalpha and ERbeta Involvement in Estradiol-Induced Enhancement of Cell Proliferation in the Dentate Gyrus of Adult OVX Female Rats.

Experiment 2 was conducted to determine whether estrogen receptors, alpha and beta, were involved in estradiol-induced enhancement of neurogenesis in the dentate gyrus of adult female rats. Neurogenesis (both cell proliferation and survival) in the dentate gyrus is regulated by estradiol in a complex manner. A high level of estradiol exposure for 4 h enhances whereas a high level of estradiol exposure for 48 h suppresses cell proliferation in the dentate gyrus of both adult female meadow voles and rats (Ormerod and Galea, 2001; Ormerod et al., 2003b). These findings suggest that estradiol initially enhances and subsequently suppresses cell proliferation. The aim of this experiment was to determine which estrogen receptor subtype (if any) mediates the enhancement in cell proliferation at the 4 h time interval. Both estrogen receptor subtypes (ERalpha and ERbeta) are found either in isolation (Shughrue et al., 1997; Shughrue and Merchenthaler, 2001) or coexpressed (Su et al., 2001) in the hippocampus. These two subtypes have a differential distribution and also vary quantitatively, specifically with ERbeta being more abundant in the hippocampus (Shughrue et al., 1997). With regard to regional differences of receptor expression in the rat hippocampus, ERalpha (mRNA and protein expression) has been identified in the CA1 pyramidal layer, CA3 pyramidal layer and in the hilus of the dentate gyrus (Weiland et al., 1997; Orikasa et al., 2000; Blurton-Jones et al., 2004), whereas ERbeta protein expression has been identified in the CA2 pyramidal layer, CA3 pyramidal layer, dentate gyrus granule cell layer and subiculum (Blurton-Jones et al., 2004). Additionally, non-nuclear ERalpha and beta protein expression has been identified in the same corresponding areas as their nuclear counterparts in the hippocampus of the adult female rat (Kalita et al., 2005). Thus, both

ERalpha and ERbeta are located in areas that could influence estradiol's effects on cell proliferation. Therefore, the aim of Experiment 2 was to investigate, with novel ER subtype specific ligands, which subtypes were mediating estradiol-induced enhancement of cell proliferation.

Drug Treatment.

Ovariectomized female Sprague-Dawley rats were randomly assigned to 1 of 10 treatment groups (n=4/5 per group) and received a single s.c. injection of either: OIL, DMSO, EB (10 µg/0.1 ml OIL), one of 3 doses of the ERalpha agonist PPT (1.25 mg, 2.5 mg or 5.0 mg/ 0.1 ml DMSO), one of 3 doses of the ERbeta agonist DPN (1.25 mg, 2.5 mg or 5.0 mg/0.1 ml DMSO) or a PPT and DPN combination dose (2.5 mg/0.1 ml DMSO). Four hours following the treatment injection, each animal received an i.p. injection of the cell synthesis marker BrdU in a volume of 1.0 ml/100g body weight (200 mg BrdU/kg). Changes in blood-brain barrier permeability may alter BrdU availability; however, blood-brain barrier permeability is only altered by estradiol after at least 3 weeks of exposure in rats (Ziylan et al., 1990). The animals were perfused 24 hours after BrdU administration in order to examine cell proliferation, as 24 h is sufficient time for one complete mitotic cycle (Cameron and McKay, 2001).

Drug Preparation.

The ER alpha agonist, propyl-pyrazole triol (PPT), the ER beta agonist, diarylpropionitrile (DPN), 17beta-estradiol benzoate and progesterone were prepared as mentioned above in Experiment 1. The cell synthesis marker bromodeoxyuridine (BrdU; Sigma Aldrich Chemicals), a marker of dividing cells (Nowakowski et al., 1989), was prepared just prior to injection. BrdU was dissolved in a concentration of 20mg/ml warm freshly prepared 0.9% saline (buffered with 7 µl 2N NaOH/ml saline).

·11

Histology.

Rats were anaesthetized with sodium pentobarbital and then perfused with 4% paraformaldehyde within 24 hours (to assess cell proliferation) or 4 days (to assess cell phenotype) after injection of BrdU. A 24-hour survival time post-BrdU injection was followed to allow for one mitotic division. The phenotype of BrdU-ir cells was determined in the 4 day survival group using confocal microscopy and counting the number of BrdU cells co-labeled with doublecortin (DCX is a microtubule-associated protein expressed by migrating and differentiating granule neurons (Francis et al., 1999; Gleeson et al., 1999; Jin et al., 2001) or glial fibrillary acidic protein (GFAP; an astroglia marker) immunoreactivity-ir (Cameron et al., 1993; Gould et al., 1999; Smith et al., 2001). Following extraction, brains were stored in 4% paraformaldehyde for 48 hrs, before transfer into a solution of tris-buffered saline (TBS; Trizma HCl, Trizma Base + NaCl) for a minimum 24 hrs (all at 4 degrees Celsius). Brains were sliced through the entire extent of the dentate gyrus (18-20 sections per rat) in a bath of TBS (PH 7.5) using an oscillating tissue slicer (Leica VT1000S) in a bath of TBS. The 40 µm sections were stored in sterile culture plates filled with TBS prior to BrdU immunohistochemistry processing. BrdU-labeled cells were counted on peroxidase treated tissue and the phenotype of new cells was determined on fluorescent probe-treated tissue.

BrdU Immunohistochemistry.

Tissue was processed to reveal BrdU labeling by applying solutions to the free-floating tissue sections. The sections were rinsed repeatedly between steps in TBS (0.1 M tris-phosphate buffer in 0.9 % saline; pH 7.4) unless stated otherwise. DNA was denatured by applying 2 N HCl for 30 min at 37°C. Sections were blocked with 3.0% normal horse serum (NHS) for 30 min and then incubated overnight in mouse monoclonal antibody against BrdU (1:200+3% NHS+10% Triton-X; Boehringer Mannheim, Laval, Quebec, Canada) at room temperature. The

following day, sections were incubated in mouse secondary antisera (1:129+3% normal horse serum; Vector Laboratories, Burlington, ON, Canada) for 4 h. Sections were incubated in avidin-biotin horseradish peroxidase complex (ABC Elite Kit; 1:50; Vector Laboratories) for 120 min. Sections were reacted for approximately 10 min in 0.02% diaminobenzidine (DAB; Sigma Aldrich Chemicals) with 0.0003% H₂O₂. The sections were mounted on slides treated with 3% 3-aminopropyltriethoxy-silane in acetone (Sigma Chemicals) to enhance slide adherence and dried overnight. The sections were counterstained with cresyl violet acetate, dehydrated and coverslipped with Permount (Fisher Scientific, Nepeon, CDA).

Fluorescent Microscopy

Immunofluorescent labeling was done on free floating tissue to verify the phenotype of BrdU-labeled cells by applying solutions directly into culture-wells containing separate sets of sections. Each set was triple-stained with fluorescent probes to assess BrdU-, DCX-, and GFAPir. Unless otherwise stated, all sections were rinsed several times with tris-buffered saline (TBS; pH 7.5) between steps. Sections were blocked in a TBS Plus solution (3% normal donkey serum (Jackson Immunoresearch), 3% triton-X 10%) for 20 minutes. Sections were incubated over night in a cocktail of TBS Plus (3% normal donkey serum, 1% triton-X 10%) containing goat polyclonal anti-DCX (1:200; Santa Cruz) and mouse monoclonal anti-GFAP (1:200; Novocastra) at 4°C. Sections were incubated in a cocktail of donkey antigoat Cy3 (1:200; Jackson Immunoresearch) to visualize DCX and donkey antimouse Alexa647 (1:200; Invitrogen Canada) to visualize GFAP, for 4 hrs. Sections were fixed in 4% paraformaldehyde for 10 minutes. Sections were rinsed twice with 0.9% saline. DNA was denatured by applying 2 N HCl for 30 minutes at 37°C. Sections were blocked in a TBS Plus solution (3% normal donkey serum, 3% triton-X 10%) for 20 minutes. Sections were incubated in donkey antirat fluorescein (1:500;

Jackson Immunoresearch) to visualize anti-BrdU. Sections were then rinsed and coverslipped with an antifading agent (Molecular Probes) and stored at 4°C.

Data Analysis.

The slides were coded prior to analysis so that at the time of counting the experimenter was blind to the conditions. Total BrdU-ir cell through the granule cell layer and subgranular zone (defined as approximately the 50 µm band between the granule cell layer and the hilus (Palmer et al., 2000) were stereologically estimated using peroxidase-treated tissue. To stereologically estimate cell numbers, total BrdU-labeled cells were counted on every 5th section (one side per section) throughout the dentate gyrus per rat. Cells were considered BrdU-labeled and counted if they exhibited oval or medium round cell bodies (Cameron et al., 1993; Ormerod and Galea, 2001). BrdU-ir cells were counted with a 100X objective on a Nikon Elipse (E600) light microscope, and the total number of cells was estimated using a procedure by Tanapat et al., 1999. Dentate gyrus area estimates were measured using the software program Simple PCI and dentate gyrus volume estimates were made using Cavalieri's principle (Gundersen et al., 1988).

BrdU-ir cell phenotypes for 3 treatment groups (PPT 2.5 mg/0.1 ml DMSO, DPN 2.5 mg/0.1 ml DMSO and DMSO) were analyzed on fluorescent probe-treated tissue. Twenty-five BrdU-labeled cells on five sections per brain (40 µm apart; n=3 per group) were counted where the infrapyramidal and suprapyramidal blades join at the crest and their phenotype confirmed on a confocal microscope (BioRad 2000) using a 63X objective. The percentage of BrdU-ir cells that expressed a neuronal (DCX-ir) or glial (GFAP-ir) phenotype was determined. Z-sections at 0.4 µm intervals were taken and optical stacks of 10 images were created with NIH Image for PC (<u>http://www.scioncorp/com/pages/menu.htm</u>) (Scion Corporation, Frederick, Maryland) and imported into Adobe Photoshop (Adobe Systems, Inc., San Jose, California) for channel

merging. Digital manipulations were restricted to contrast enhancements and color level adjustments.

Statistical Analysis.

For experiment 2 the dependent variables (total BrdU-ir cells, dentate gyrus volume, percentage BrdU/DCX-ir cells or BrdU/GFAP-ir cells) were analyzed using an analysis of variance (ANOVA) with group (OIL, EB, DMSO, PPT 1.25, PPT 2.5, PPT 5.0, DPN 1.25, DPN 2.5, DPN 5.0 and PPT&DPN 2.5), respectively as the independent variable. *A priori* comparisons used Dunnett's procedure while post-hoc comparisons used Neuman-Keul's method unless otherwise specified. All statistical procedures were set at $\alpha = 0.05$.

RESULTS

Experiment 1. ERalpha and ERbeta Involvement in Female Rat Sexual Behaviour.

There was no significant effect of treatment on the number of mounts $[F(11,64) = 1.27, p \ge 0.26]$, demonstrating that all males were *initially* equally sexually vigorous irrespective of female treatment. During the mating tests, there was no effect of treatment on mount latency $[F(11,64) = 1.17, p \ge 0.32]$, indicating that female attractivity to the males was not effected. *Treatment with agonists did not affect general motor ability.*

The number of level changes did not significantly differ between treatments $[F(11,64) = 0.46, p \ge 0.92]$, demonstrating that regardless of treatment gross overall motor activity was not effected.

ERalpha agonist treatment induces Proceptive Behaviour.

There was a significant main effect of treatment on ear wiggling $[F(11,64) = 10.23, p \le 0.0001;$ Fig. 1A & B]. Post-hoc analysis revealed that EB and PPT 5 females showed significantly more ear wiggling relative to their respective vehicles ($p \le .0001; p \le 0.0007$, respectively). There were no other significant differences between treatment groups. The concomitant administration of PPT and DPN dose produced very little effect on ear wiggling, specifically, only one female (1/6) from this treatment group exhibited ear wiggling.

There was a significant effect of treatment on hopping and darting between treatments $[F(11,64) = 3.20, p \le 0.002; Fig. 2A \& B)$. Post-hoc analysis revealed that both the EB and PPT 2.5 treated groups demonstrated significantly more hopping and darting relative to their respective vehicles ($p \le 0.03; p \le 0.01$, respectively). PPT 2.5 treated animals exhibited more hopping and darting (by approximately 30%) than the EB treated animals, however this difference was not statistically significant ($p \ge 0.29$). Treatment groups PPT 1.25, PPT 5 and the combination dose (PPT and DPN) demonstrated lower frequencies of hopping and darting. The

two lower doses of PPT and the three DPN dose groups did not display any significant

hopping and darting and there was no significant difference relative to vehicle (all p's > 0.8).







Fig.2 A. & B. Number of hops and darts as a function of treatment in female rats. For Figure 2A and 2B, the OIL, EB and DMSO treated rats are the same groups and have been included in each graph for comparative purposes against each subtype agonist, PPT and DPN, respectively. (A) EB and PPT 2.5 demonstrated significantly more hopping and darting relative to their respective vehicles ($p \le .03$; $p \le 0.01$, respectively). The combination agonist treatment (PPT and DPN 2.5 mg/0.1 ml DMSO) did not statistically differ from vehicle. (B) DPN treated females did not exhibit any hopping and darting suggesting no involvement of ERbeta in this proceptive behaviour. Error bars indicate standard error of the mean (n = 6 - 8). * Significantly different from control group (OIL or DMSO) (p < 0.05).

ERalpha agonist, PPT elicits Receptive Behaviour in a dose-dependent manner.

There was a significant effect of treatment on lordosis quotient $[F(11,64) = 10.70, p \le 0.0001;$ Fig. 3A & B]. Post-hoc analysis demonstrated that EB, PPT 2.5 and PPT 5 treated rats demonstrated a significantly higher lordosis quotient relative to their respective vehicle (p $\le 0.0001, p \le 0.001, p \le 0.0001, respectively$). There were no other significant differences between groups (all p's > 0.1).

There was a significant effect of treatment on lordosis rating $[F(11,64) = 10.68, p \le 0.0001;$ Fig. 4A & B). Post-hoc analysis demonstrated that EB-treated and PPT 5 treated females displayed significantly and PPT 2.5 treated females tended to have a higher lordosis rating (LR) relative to their vehicles (EB p $\le 0.0001;$ PPT 2.5, p $\le 0.06;$ PPT 5, p $\le 0.0001)$. There were no other significant differences between groups (all p's > 0.1).

There was an effect of treatment on rejection quotient $[F(11,64) = 2.20, p \le 0.025;$ Fig. 5A & B]. Post-hoc analyses revealed that EB treated animals demonstrated significantly less rejection behaviour relative to vehicle ($p \le 0.016$). There were no other significant differences between groups (all p's > 0.5).



Fig.3 A. & B. Lordosis quotient (LQ) as a function of treatment in female rats. For Figure 3A and 3B, the OIL, EB and DMSO treated rats are the same groups and have been included in each graph for comparative purposes against each subtype agonist, PPT and DPN, respectively. (A) EB, PPT 2.5 and PPT 5 treated rats demonstrated a significantly higher LQ relative to their respective vehicle ($p \le 0.0001$, $p \le 0.01$, $p \le 0.0001$, respectively) in a dose-dependent manner. P&D2.5 treated females did not statistically differ from vehicle. (B) DPN treated females did not display the lordosis posture. Error bars indicate standard error of the mean (n = 6 - 8). * Significantly different from control group (OIL or DMSO) (p < 0.05).



Fig.4 A. & B. Lordosis rating (LR) as a function of treatment in female rats. For Figure 4A and 4B, the OIL, EB and DMSO treated rats are the same groups and have been included in each graph for comparative purposes against each subtype agonist, PPT and DPN, respectively. (A) EB and PPT 5 treated females displayed significantly and PPT 2.5 tended to have a higher LR relative to their vehicles (EB $p \le 0.0001$; PPT 2.5, $p \le 0.06$; PPT 5, $p \le 0.0001$). (B) DPN treated females did not statistically differ from vehicle. Error bars indicate standard error of the mean (n = 6-8). * Significantly different from control group (OIL or DMSO) (p < 0.05).



Fig.5 A. & B. Rejection quotient as a function of treatment in female rats. For Figure 5A and 5B, the OIL, EB and DMSO treated rats are the same groups and have been included in each graph for comparative purposes against each subtype agonist, PPT and DPN, respectively. (A) EB treated animals demonstrated significantly less rejection behaviour relative to vehicle ($p \le 0.016$). All PPT doses (PPT1.25, PPT2.5 and PPT5) and combined agonist (P&D2.5) treated rats demonstrated no significant differences relative to vehicle (all p's > 0.5). (B) There was no significant differences in rejection quotient for all DPN treated rats relative to vehicle (all p's > 0.5). Error bars indicate standard error of the mean (n = 6 - 8). * Significantly different from control group (OIL or DMSO) (p < 0.05).

ERalpha and ERbeta in the Dentate Gyrus of Adult OVX Female Rats.

The number of BrdU-labeled cells significantly differed between groups [F(9, 42) = 6.42;p < 0.00001]. As expected, we replicated the finding that estradiol increases cell proliferation at the 4 hr time period, analyses demonstrated that EB females had significantly more labeled cells (approximately 50%), relative to vehicle (OIL) ($p \le 0.002$; Fig. 6A). There was no statistical difference between the two vehicles groups, OIL and DMSO ($p \ge 0.14$). The ERalpha agonist, PPT significantly increased cell proliferation (approximately 50%), relative to vehicle only at a dose of 2.5 mg ($p \le 0.0002$); Fig 6A). However, analysis demonstrated that the ERbeta agonist, DPN, significantly increased cell proliferation (approximately 40%), at all three doses relative to vehicle (DPN 1.25 mg, $p \le 0.008$; DPN 2.5 mg, p < 0.003; DPN 5 mg, p < 0.005; Fig 6B). The results indicate that both ER subtypes are mediating estradiol enhanced cell proliferation. The agonists in combination demonstrated a non-significant trend for an increase in cell proliferation relative to vehicle (p < 0.06; Fig 6A.) compared to each agonist alone. This finding is consistent with previous studies indicating that ERbeta may modulate, by suppression, ERalpha transcriptional activity, and therefore influence the relative expression level of the two subtypes and alter the cellular response to an agonist (Hall and McDonnell, 1999; Frasor et al., 2003).



Fig. 6 A) Mean (\pm S.E.M.) number of new cells in the dentate gyrus of adult female rats given BrdU 4 hrs after a treatment injection. The vehicle groups and estradiol group are the same for both figures and have been included on both graphs for comparison purposes. Estradiol significantly increased the number of BrdU-ir cells relative to the vehicle group, OIL ($p \le 0.002$). PPT at a dose of 2.5 mg significantly increased the number of BrdU-ir cells relative to the vehicle ($p \le 0.0002$). B) DPN at all 3 doses significantly increased the number of BrdU-ir cells relative to the vehicle (DPN 1.25, $p \le 0.008$; DPN 2.5, $p \le 0.003$; DPN 5, $p \le 0.005$). * Significantly different from control group (OIL or DMSO) (p < 0.05).

Total area of the granule cell layer and subgranular zone did not statistically differ between groups indicating that treatment did not affect dentate gyrus volume [F(9,42) = 0.57; p \geq 0.82]. Each group had 20 sections analyzed with one side of each section counted.

Table 1. Mean (± S.E.M.) dentate gyrus volume of vehicle, estradiol and agonist-treated rats in

Condition	Volume (mm ³)
OIL	1.26 ± 0.08
EB	1.15 ± 0.07
DMSO	1.25 ± 0.05
PPT 1.25	1.28 ± 0.07
PPT 2.5	1.19 ± 0.07
PPT 5	1.27 ± 0.07
DPN 1.25	1.27 ± 0.07
DPN 2.5	1.31 ± 0.08
DPN 5	1.34 ± 0.07
PPT & DPN 2.5	1.25 ± 0.07

Experiment 2.

Most 4-day-old BrdU labeled cells expressed the neuronal protein DCX (approximately 64%) and this percentage was consistent across all treatment groups (F(3,6) = 0.87; $p \ge 0.50$). Fewer BrdU-labeled cells expressed the glial marker GFAP (approximately 29%) and this percentage did not statistically differ between groups (F(3,6) = 0.34, $p \ge 0.79$).

<u>Table 2.</u> Mean (± S.E.M.) Percentage of BrdU-ir cells expressing a neuronal (Doublecortin-ir) or glial (GFAP-ir) phenotype.

Condition	% Doublecortin-ir	% GFAP-ir
PPT 2.5 (n = 3)	65.3 ± 4.8	29.3 ± 5.3
DPN 2.5 $(n = 3)$	62.6 ± 3.5	30.6 ± 1.3
DMSO $(n = 3)$	58.6 ± 1.3	30.6 ± 2.6



Fig. 7. Two newly synthesized BrdU-labeled cells located in the subgranular zone of an adult female rat administered 10 μ g of estradiol.



Figure 8. A newly synthesized cell that has incorporated BrdU (immunoreactive for anti-BrdU conjugated with FITC) is shown in green. Cells expressing the immature neuronal protein double cortin are shown in red (immunoreactive for anti-DCX conjugated with CY3) and cells expressing the glial protein GFAP are shown in blue (immunoreactive for anti-GFAP conjugated with Alexa647). The white arrow points to a newly synthesized cell that has incorporated BrdU (green label) and is expressing the immature neuronal protein double cortin (surrounding red label) in the subgranular granular zone of an OVX female rat treated with 2.5 mg PPT. No significant differences were observed between groups in the number of new cells that expressed DCX or GFAP ($p \ge 0.50$ and $p \ge 0.79$, respectively).

DISCUSSION

The results from the present experiment clearly show a dissociation in function between ERalpha and ERbeta using the newly available ERalpha and beta selective agonists, propylpyrazole triol (PPT) and diarylpropionitrile (DPN). Both ERs appear to be involved in estradiolinduced enhancement in adult hippocampal neurogenesis. Intriguingly, both agonists in combination did not stimulate cell proliferation to the same degree as estradiol, which suggests each receptor is working independently in upregulating neurogenesis in the hippocampus. The ERalpha agonist, PPT, elicited sexual proceptivity and receptivity when administered a minimal treatment dose of 2.5 mg of PPT, however the ERbeta agonist, DPN, and the combined agonist treatment (2.5 mg) did not elicit any proceptive or receptive behaviour. Therefore, our findings indicate ERalpha but not ERbeta is involved in both proceptive and receptive female sexual behaviour, which is consistent and extends prior findings in knockout animal studies (Ogawa et al., 1998; Ogawa et al., 1999; Rissman et al., 1999; Kudwa and Rissman, 2003).

Regulation of proceptive and receptive female rat sexual behaviour is through ERalpha

Our results for Experiment 1 show that ERbeta is not essential to elicit proceptive and receptive behaviour, although this subtype is present in both neural areas (VMH and mPOA) implicated in proceptive and receptive behaviour (Aou et al., 1988; Hoshina et al., 1994; Sakuma, 1995; Shughrue et al., 1997; Kato and Sakuma, 2000; Shughrue and Merchenthaler, 2001; Greco et al., 2003; Shima et al., 2003). Our results demonstrating exclusive involvement of ERalpha in sexual behaviour is in complete agreement with knockout studies that found no reproductive behaviour in female mice lacking a functional estrogen receptor alpha gene (Rissman et al., 1997b; Wersinger et al., 1997; Ogawa et al., 1998) and the survival of reproductive behaviour in estrogen receptor beta-deficient mice (Ogawa et al., 1999; Ogawa et al., 2000; Kudwa and Rissman, 2003). Our findings are also consistent with studies

demonstrating that each ER subtype has the ability to regulate different genes and have separate or shared functional roles when both expressed in the same neural area. For instance, ERalpha in the hippocampus has been suggested to influence neuronal morphology by stimulating dentritic branching (Audesirk et al., 2003) whereas ERalpha (Fugger et al., 1998) and ERbeta (Rissman et al., 2002) have been suggested to play a role in spatial learning. Previous knock out studies and our observations using an ERbeta selective agonist provides additional evidence that ERbeta is not essential for female rodent sexual behaviour. Alternatively, ERbeta may possess a more subtle role in the regulation of sexual behaviour by acting as a modulator of ERalpha's transcriptional activity, as in our study the combined dose of PPT and DPN did not enhance female sexual behavior. Previous studies examining ER dependent processes have suggested a regulatory role for ERbeta; findings have revealed an extended period of receptivity in ERbeta knock out mice (Ogawa et al., 1999), a decrease in cellular response to estrogen (Hall and McDonnell, 1999; Frasor et al., 2003) and a decrease in uterine weight gain (Frasor et al.,2003). Studies demonstrating ERbeta's ability to modulate ERalpha activity are beginning to elucidate how estradiol can regulate a plethora of distinct effects in a variety of cellular and physiological contexts.

Both ERalpha and ERbeta are involved in estradiol-induced upregulation of cell proliferation

Consistent with previous literature we found that exposure to a high dose of estradiol for 4 h increased the number of BrdU-labeled cells (assessed 24 h post-BrdU) in adult female rats (Ormerod et al., 2003b). The ERalpha agonist, PPT, demonstrated a significant increase in cell proliferation at a dose of 2.5 mg, while the ER beta agonist, diarylpropionitrile (DPN), showed a significant increase in cell proliferation at all 3 administered doses (1.25, 2.5 and 5 mg) relative to vehicle-treated rats. Administration of the agonists in combination demonstrated a weaker increase in cell proliferation, which may be an indication of ERbeta modulating ERalpha activity

in a response-specific and dose-dependent manner (Hall and McDonnell, 1999; Frasor et al., 2003). Moreover, given that the agonists when administered alone or in combination did not generate an equivalent response in cell proliferation as estradiol alone suggests several alternative possibilities for estradiol enhanced cell proliferation including; a ligand-independent mechanism (Perez-Martin et al., 2003), and/or a novel estradiol receptor (Toran-Allerand et al., 2002), or the agonists are limited in their ability to transactivate each of their respective estrogen responsive gene elements and subsequent molecular cascades. This is the first demonstration that estradiol-induced enhancement of hippocampal neurogenesis is partially mediated by both estrogen receptors (ERs), alpha and beta.

The significant increase in cell proliferation by the ERbeta agonist DPN for all administered doses may be consistent with the evidence suggesting that ERbeta has a predominant presence in the hippocampus over ERalpha (Shughrue et al., 1997; Shughrue and Merchenthaler, 2001). The abundance of ERbeta is partially due to it's presence on astrocytes (Azcoitia et al., 1999). Intriguingly, astrocytes and/or cells that express GFAP may possess the ability to divide and generate new neurons in the subgranular zone of the hippocampus (Seri et al., 2001; Seri et al., 2004). Although speculative, this suggests there may be a direct effect of estrogen or estrogen-like agents on these cells. Indeed, although ER expression has not been observed on dividing hippocampal progenitors, progenitors harvested from the subventricular zone of fetal and adult rats express both ERalpha and ERbeta (Brannvall et al., 2002), suggesting that estradiol could mediate its effects on cell proliferation directly. Although ERalpha is the stronger transcriptional activator of the two ER subtypes, our current results using ER subtypeselective ligands and the qualitative difference between each subtype indicate that ERbeta may be a more potent regulator of the estradiol-mediated increase in cell production in the hippocampus at this particular time frame of estradiol administration.

PPT administration resulted in a significant increase in cell proliferation at only one dose, suggesting that ERalpha has lesser involvement in estradiol-induced enhancement of hippocampal cell proliferation. This finding may be due to the lower quantitative expression of ERalpha (Shughrue et al., 1997) or due to down-regulation of this subtype after acute estradiol administration (Cheng et al., 2004). ER subtype expression has been shown to fluctuate in proliferating epithelial cells after acute estradiol administration in a time-dependent manner analogous to our study, specifically, 4 h following acute estradiol administration ERalpha was down-regulated while ERbeta was unchanged (Cheng et al., 2004). In order to further establish the level of involvement of ER subtypes for mediating changes in estradiol-induced hippocampal cell proliferation, future studies will need to identify and quantify their presence as well as colocalize with proliferation markers.

ERalpha and ERbeta demonstrate opposing roles to regulate transcriptional activity

The agonists in combination did not demonstrate an additive effect to mimic the number of cells generated with estradiol exposure, nor did it increase the level of proceptivity or receptivity relative to the ERalpha agonist administration for sexual behaviour. Instead, a weak increase in cell proliferation was observed and sexual behaviour elicited was reduced. Our results suggest that ERalpha has a predominant role in sexual behaviour and the notable decrease for both proceptive and receptive behaviour when both agonists were administered may be due to ERbeta's ability to modulate the transcriptional activity of ERalpha (Weihua et al., 2000). In vitro studies have shown opposing action and dominance of ERbeta over ERalpha in activation of cyclin D1 gene expression, (Liu et al., 2002) as well as differential ligand activation, where ERalpha activates and ERbeta inhibits transcription (Paech et al., 1997). These results are supported by further work in mammalian cells (Hall and McDonnell, 1999; Pettersson et al., 2000) and in ER knockout mice (Couse et al., 2001; Pettersson and Gustafsson, 2001) which also

found that ERalpha mediates the proliferative effects, while ERbeta favours suppression of gene expression. Lastly, although controversial, a novel estrogen receptor may be involved with evidence suggesting that an indirect genomic pathway, by activation of MAPK (an important signaling pathway for cell division) along with family member kinases (ERK1 and ERK2) mediates through a novel, putative estradiol receptor, termed ER-X (Toran-Allerand et al., 2002). *Estradiol-induced enhancement of cell proliferation may also be modulated through a ligand independent mechanism*

In addition to hormone-mediated activation, the estradiol-induced enhancement of cell proliferation may also involve a ligand-independent mechanism. For instance, estradiol regulates a variety of growth factors including the expression of brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) (Simpkins et al., 1997), trkA (Gibbs, 1994) and insulin growth factor (IGF) (Toran-Allerand et al., 1988). Estradiol's influence on cell proliferation may be partially mediated through the regulation of BDNF, which affects neuronal survival, differentiation and synaptic plasticity (Lu and Chow, 1999; Thoenen, 2000). Evidence suggests the involvement of a ligand independent signaling pathway between BDNF and estradiol for any molecular cascade mediated through estradiol's classical signaling pathway, including estradiol-enhanced hippocampal neurogenesis. Studies have identified an estrogen response element (ERE)-like sequence within the BDNF gene (Sohrabji et al., 1995) and ERalpha and BDNF have been found co-localized in the hippocampus (Solum and Handa, 2002; Blurton-Jones et al., 2004). Studies examining the effect of short-term estradiol treatment on BDNF expression levels in female rats have given conflicting results, exhibiting decreased BDNF protein levels (Murphy et al., 1998; Gibbs, 1999), no change (Gibbs, 1998) or increased BDNF mRNA levels (Gibbs, 1999). Future studies are required to provide further insight into

the relationship between estradiol and BDNF levels and how this may be contributing towards estradiol enhanced hippocampal neurogenesis.

Another potential ligand interacting with estradiol and influencing new neuron production is IGF-I that has demonstrated neural effects including proliferation, migration and differentiation on progenitor cells (Drago et al., 1991; Dicicco-Bloom et al., 1998; Aberg et al., 2000; Learish et al., 2000; Arsenijevic et al., 2001). Studies have shown that the expression of IGF-I receptors in the hippocampus is up-regulated by estrogen receptors (Cardona-Gomez et al., 2001). Moreover, a recent study has shown an interaction between IGF-I and estrogen receptors for the induction of increased cell proliferation in the dentate gyrus of adult ovariectomized rats (Perez-Martin et al., 2003). An interesting observation was that ICI 182,780, an estrogen receptor antagonist, had an inhibitory effect on IGF-I induced enhancement of cell proliferation suggesting that estrogen receptors are directly involved in IGF-I's modulation of adult hippocampal neurogenesis. Additionally, the inhibitory effect of ICI 182,780 was observed in the absence of estradiol treatment, demonstrating ER activity by IGF-I in the absence of estradiol. Future studies examining the relationship between IGF-I signaling and estrogen receptors will further elucidate the ligand-independent mechanisms potentially contributing to estradiol-induced enhancement of hippocampal neurogenesis.

CONCLUSION

In conclusion, we have shown that both estrogen receptors, alpha and beta, are involved in estradiol-induced enhancement of hippocampal neurogenesis in the female rat and that ERalpha, but not ER beta, is primarily involved in eliciting both proceptive and receptive female sexual behavior. Our previous studies have demonstrated that estradiol has the ability to increase proliferation in a time-dependent manner and future studies will determine whether this increase in granule cells has a functional outcome on behaviour. Certainly, estradiol has been shown to regulate cognitive function in a complex manner (Hampson, 1990; Galea et al., 1996; Galea et al., 2001; Holmes et al., 2002). Understanding how estradiol dynamically regulates neurogenesis is of vital importance to further evaluate the potential therapeutic role of estradiol to promote proliferation and survival of endogenous neural stem cells. The present study adds to our understanding of how estradiol mediates an increase of hippocampal neurogenesis by demonstrating that although both ER subtypes are involved, each cognate receptor suggests to have an individual rather than a co-operative level of involvement that may be dependent on a variety of mechanisms as well as an assortment of molecules whose interactions with estradiol will be the focus in future studies. The quantity of ER subtypes and their respective activity is altered in neurodegenerative diseases, specifically, ERbeta is upregulated in brains of AD patients (Savaskan et al., 2001) and inhibition of ERbeta's pathway is crucial in the development of neurodegenerative diseases (Zhang et al., 2004). Therefore, research indicates the importance of understanding the activity of these subtypes, how they modulate neurogenesis and their therapeutic potential for advancing neurodegenerative treatments.

REFERENCES

- Aberg MA, Aberg ND, Hedbacker H, Oscarsson J, Eriksson PS (2000) Peripheral infusion of IGF-I selectively induces neurogenesis in the adult rat hippocampus. J Neurosci 20:2896-2903.
- Aou S, Oomura Y, Yoshimatsu H (1988) Neuron activity of the ventromedial hypothalamus and the medial preoptic area of the female monkey during sexual behavior. Brain Res 455:65-71.
- Arsenijevic Y, Weiss S, Schneider B, Aebischer P (2001) Insulin-like growth factor-I is necessary for neural stem cell proliferation and demonstrates distinct actions of epidermal growth factor and fibroblast growth factor-2. J Neurosci 21:7194-7202.
- Audesirk T, Cabell L, Kern M, Audesirk G (2003) beta-estradiol influences differentiation of hippocampal neurons in vitro through an estrogen receptor-mediated process. Neuroscience 121:927-934.
- Azcoitia I, Sierra A, Garcia-Segura LM (1999) Localization of estrogen receptor betaimmunoreactivity in astrocytes of the adult rat brain. Glia 26:260-267.
- Banasr M, Hery M, Brezun JM, Daszuta A (2001) Serotonin mediatés oestrogen stimulation of cell proliferation in the adult dentate gyrus. Eur J Neurosci 14:1417-1424.
- Barkhem T, Carlsson B, Nilsson Y, Enmark E, Gustafsson J, Nilsson S (1998) Differential response of estrogen receptor alpha and estrogen receptor beta to partial estrogen agonists/antagonists. Mol Pharmacol 54:105-112.
- Beach FA (1976) Sexual attractivity, proceptivity, and receptivity in female mammals. Horm Behav 7:105-138.

Behl C (2002) Sex hormones, neuroprotection and cognition. Prog Brain Res 138:135-142.

- Blurton-Jones M, Kuan PN, Tuszynski MH (2004) Anatomical evidence for transsynaptic influences of estrogen on brain-derived neurotrophic factor expression. J Comp Neurol 468:347-360.
- Brandling-Bennett EM, Blasberg ME, Clark AS (1999) Paced mating behavior in female rats in response to different hormone priming regimens. Horm Behav 35:144-154.
- Brannvall K, Korhonen L, Lindholm D (2002) Estrogen-receptor-dependent regulation of neural stem cell proliferation and differentiation. Mol Cell Neurosci 21:512-520.
- Buteau-Lozano H, Ancelin M, Lardeux B, Milanini J, Perrot-Applanat M (2002) Transcriptional regulation of vascular endothelial growth factor by estradiol and tamoxifen in breast cancer cells: a complex interplay between estrogen receptors alpha and beta. Cancer Res 62:4977-4984.
- Cameron HA, McKay RD (2001) Adult neurogenesis produces a large pool of new granule cells in the dentate gyrus. J Comp Neurol 435:406-417.
- Cameron HA, Woolley CS, McEwen BS, Gould E (1993) Differentiation of newly born neurons and glia in the dentate gyrus of the adult rat. Neuroscience 56:337-344.

Cardona-Gomez GP, Mendez P, DonCarlos LL, Azcoitia I, Garcia-Segura LM (2001) Interactions of estrogens and insulin-like growth factor-I in the brain: implications for neuroprotection. Brain Res Brain Res Rev 37:320-334.

- Carrer H, Asch G, Aron C (1973) New facts concerning the role played by the ventromedial nucleus in the control of estrous cycle duration and sexual receptivity in the rat. Neuroendocrinology 13:129-138.
- Cheng G, Weihua Z, Warner M, Gustafsson JA (2004) Estrogen receptors ER alpha and ER beta in proliferation in the rodent mammary gland. Proc Natl Acad Sci U S A 101:3739-3746.

Couse JF, Dixon D, Yates M, Moore AB, Ma L, Maas R, Korach KS (2001) Estrogen receptor-alpha knockout mice exhibit resistance to the developmental effects of neonatal diethylstilbestrol exposure on the female reproductive tract. Dev Biol 238:224-238.

Dicicco-Bloom E, Lu N, Pintar JE, Zhang J (1998) The PACAP ligand/receptor system regulates cerebral cortical neurogenesis. Ann N Y Acad Sci 865:274-289.

Dorner G (1969) [The significance of sex hormone dependent differentiation of the

hypothalamus for gonadal function and sex behavior]. Acta Biol Med Ger 23:709-712.

- Dorner G, Staudt J (1969) Structural changes in the hypothalamic ventromedial nucleus of the male rat, following neonatal castration and androgen treatment. Neuroendocrinology 4:278-281.
- Drago J, Murphy M, Carroll SM, Harvey RP, Bartlett PF (1991) Fibroblast growth factormediated proliferation of central nervous system precursors depends on endogenous production of insulin-like growth factor I. Proc Natl Acad Sci U S A 88:2199-2203.
- Enmark E, Pelto-Huikko M, Grandien K, Lagercrantz S, Lagercrantz J, Fried G, Nordenskjold M, Gustafsson JA (1997) Human estrogen receptor beta-gene structure, chromosomal localization, and expression pattern. J Clin Endocrinol Metab 82:4258-4265.
- Francis F, Koulakoff A, Boucher D, Chafey P, Schaar B, Vinet MC, Friocourt G, McDonnell N,
 Reiner O, Kahn A, McConnell SK, Berwald-Netter Y, Denoulet P, Chelly J (1999)
 Doublecortin is a developmentally regulated, microtubule-associated protein expressed in migrating and differentiating neurons. Neuron 23:247-256.
- Frasor J, Barnett DH, Danes JM, Hess R, Parlow AF, Katzenellenbogen BS (2003) Responsespecific and ligand dose-dependent modulation of estrogen receptor (ER) alpha activity by ERbeta in the uterus. Endocrinology 144:3159-3166.

Fugger HN, Cunningham SG, Rissman EF, Foster TC (1998) Sex differences in the activational effect of ERalpha on spatial learning. Horm Behav 34:163-170.

- Galea LA, Kavaliers M, Ossenkopp KP (1996) Sexually dimorphic spatial learning in meadow voles Microtus pennsylvanicus and deer mice Peromyscus maniculatus. J Exp Biol 199 (Pt 1):195-200.
- Galea LA, Wide JK, Paine TA, Holmes MM, Ormerod BK, Floresco SB (2001) High levels of estradiol disrupt conditioned place preference learning, stimulus response learning and reference memory but have limited effects on working memory. Behav Brain Res 126:115-126.
- Gibbs RB (1994) Estrogen and nerve growth factor-related systems in brain. Effects on basal forebrain cholinergic neurons and implications for learning and memory processes and aging. Ann N Y Acad Sci 743:165-196; discussion 197-169.
- Gibbs RB (1998) Levels of trkA and BDNF mRNA, but not NGF mRNA, fluctuate across the estrous cycle and increase in response to acute hormone replacement. Brain Res 810:294.
- Gibbs RB (1999) Treatment with estrogen and progesterone affects relative levels of brainderived neurotrophic factor mRNA and protein in different regions of the adult rat brain. Brain Res 844:20-27.
- Gleeson JG, Lin PT, Flanagan LA, Walsh CA (1999) Doublecortin is a microtubule-associated protein and is expressed widely by migrating neurons. Neuron 23:257-271.
- Gould E, Beylin A, Tanapat P, Reeves A, Shors TJ (1999) Learning enhances adult neurogenesis in the hippocampal formation. Nat Neurosci 2:260-265.
- Greco B, Blasberg ME, Kosinski EC, Blaustein JD (2003) Response of ERalpha-IR and ERbeta-IR cells in the forebrain of female rats to mating stimuli. Horm Behav 43:444-453.

Gundersen HJ, Bendtsen TF, Korbo L, Marcussen N, Moller A, Nielsen K, Nyengaard JR, Pakkenberg B, Sorensen FB, Vesterby A, et al. (1988) Some new, simple and efficient stereological methods and their use in pathological research and diagnosis. Apmis 96:379-394.

Hall JM, McDonnell DP (1999) The estrogen receptor beta-isoform (ERbeta) of the human estrogen receptor modulates ERalpha transcriptional activity and is a key regulator of the cellular response to estrogens and antiestrogens. Endocrinology 140:5566-5578.

Hampson E (1990) Estrogen-related variations in human spatial and articulatory-motor skills. Psychoneuroendocrinology 15:97-111.

Hansen S, Harthon C, Wallin E, Lofberg L, Svensson K (1991) Mesotelencephalic dopamine system and reproductive behavior in the female rat: effects of ventral tegmental 6-hydroxydopamine lesions on maternal and sexual responsiveness. Behav Neurosci 105:588-598.

Hardy DF, Debold JF (1971) Effects of mounts without intromission upon the behavior of female rats during the onset of estrogen-induced heat. Physiol Behav 7:643-645.

Harris HA, Katzenellenbogen JA, Katzenellenbogen BS (2002) Characterization of the biological roles of the estrogen receptors, ERalpha and ERbeta, in estrogen target tissues in vivo through the use of an ERalpha-selective ligand. Endocrinology 143:4172-4177.

Hilton GD, Ndubuizu AN, McCarthy MM (2004) Neuroprotective effects of estradiol in newborn female rat hippocampus. Brain Res Dev Brain Res 150:191-198.

Hlinak Z, Madlafousek J (1977) Female precopulatory behaviour as a determinant of sexual activity in male rats [proceedings]. Act Nerv Super (Praha) 19:242-243.

<u>39</u>

- Holmes MM, Wide JK, Galea LA (2002) Low levels of estradiol facilitate, whereas high levels of estradiol impair, working memory performance on the radial arm maze. Behav Neurosci 116:928-934.
- Hoshina Y, Takeo T, Nakano K, Sato T, Sakuma Y (1994) Axon-sparing lesion of the preoptic area enhances receptivity and diminishes proceptivity among components of female rat sexual behavior. Behav Brain Res 61:197-204.
- Imwalle DB, Gustafsson JA, Rissman EF (2005) Lack of functional estrogen receptor beta influences anxiety behavior and serotonin content in female mice. Physiol Behav 84:157-163.
- Jin K, Minami M, Lan JQ, Mao XO, Batteur S, Simon RP, Greenberg DA (2001) Neurogenesis in dentate subgranular zone and rostral subventricular zone after focal cerebral ischemia in the rat. Proc Natl Acad Sci U S A 98:4710-4715.
- Jordan VC (1995) Alternate antiestrogens and approaches to the prevention of breast cancer. J Cell Biochem Suppl 22:51-57.
- Kato A, Sakuma Y (2000) Neuronal activity in female rat preoptic area associated with sexually motivated behavior. Brain Res 862:90-102.
- Kennedy GC (1964) Hypothalamic Control of the Endocrine and Behavioural Changes Associated with Oestrus in the Rat. J Physiol 172:383-392.
- Kraichely DM, Sun J, Katzenellenbogen JA, Katzenellenbogen BS (2000) Conformational changes and coactivator recruitment by novel ligands for estrogen receptor-alpha and estrogen receptor-beta: correlations with biological character and distinct differences among SRC coactivator family members. Endocrinology 141:3534-3545.

- Krezel W, Dupont S, Krust A, Chambon P, Chapman PF (2001) Increased anxiety and synaptic plasticity in estrogen receptor beta -deficient mice. Proc Natl Acad Sci U S A 98:12278-12282.
- Kudwa AE, Rissman EF (2003) Double oestrogen receptor alpha and beta knockout mice reveal differences in neural oestrogen-mediated progestin receptor induction and female sexual behaviour. J Neuroendocrinol 15:978-983.
- Kuiper GG, Shughrue PJ, Merchenthaler I, Gustafsson JA (1998) The estrogen receptor beta subtype: a novel mediator of estrogen action in neuroendocrine systems. Front Neuroendocrinol 19:253-286.
- Kuiper GG, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson JA (1996) Cloning of a novel receptor expressed in rat prostate and ovary. Proc Natl Acad Sci U S A 93:5925-5930.
- Learish RD, Bruss MD, Haak-Frendscho M (2000) Inhibition of mitogen-activated protein kinase kinase blocks proliferation of neural progenitor cells. Brain Res Dev Brain Res 122:97-109.
- Liu MM, Albanese C, Anderson CM, Hilty K, Webb P, Uht RM, Price RH, Jr., Pestell RG, Kushner PJ (2002) Opposing action of estrogen receptors alpha and beta on cyclin D1 gene expression. J Biol Chem 277:24353-24360.
- Lu B, Chow A (1999) Neurotrophins and hippocampal synaptic transmission and plasticity. J Neurosci Res 58:76-87.
- Lund TD, Rovis T, Chung WC, Handa RJ (2005) Novel actions of estrogen receptor-beta on anxiety-related behaviors. Endocrinology 146:797-807.
- McEwen B, Akama K, Alves S, Brake WG, Bulloch K, Lee S, Li C, Yuen G, Milner TA (2001) Tracking the estrogen receptor in neurons: implications for estrogen-induced synapse formation. Proc Natl Acad Sci U S A 98:7093-7100.

- McInerney EM, Katzenellenbogen BS (1996) Different regions in activation function-1 of the human estrogen receptor required for antiestrogen- and estradiol-dependent transcription activation. J Biol Chem 271:24172-24178.
- Meisel RL, Dohanich GP, McEwen BS, Pfaff DW (1987) Antagonism of sexual behavior in female rats by ventromedial hypothalamic implants of antiestrogen. Neuroendocrinology 45:201-207.
- Meyers MJ, Sun J, Carlson KE, Marriner GA, Katzenellenbogen BS, Katzenellenbogen JA (2001) Estrogen receptor-beta potency-selective ligands: structure-activity relationship studies of diarylpropionitriles and their acetylene and polar analogues. J Med Chem 44:4230-4251.
- Moon LY, Wakley GK, Turner RT (1991) Dose-dependent effects of tamoxifen on long bones in growing rats: influence of ovarian status. Endocrinology 129:1568-1574.
- Mosselman S, Polman J, Dijkema R (1996) ER beta: identification and characterization of a novel human estrogen receptor. FEBS Lett 392:49-53.
- Murphy DD, Cole NB, Segal M (1998) Brain-derived neurotrophic factor mediates estradiolinduced dendritic spine formation in hippocampal neurons. Proc Natl Acad Sci U S A 95:11412-11417.
- Nowakowski RS, Lewin SB, Miller MW (1989) Bromodeoxyuridine immunohistochemical determination of the lengths of the cell cycle and the DNA-synthetic phase for an anatomically defined population. J Neurocytol 18:311-318.
- Ogawa S, Eng V, Taylor J, Lubahn DB, Korach KS, Pfaff DW (1998) Roles of estrogen receptor-alpha gene expression in reproduction-related behaviors in female mice. Endocrinology 139:5070-5081.

- Ogawa S, Chan J, Chester AE, Gustafsson JA, Korach KS, Pfaff DW (1999) Survival of reproductive behaviors in estrogen receptor beta gene-deficient (betaERKO) male and female mice. Proc Natl Acad Sci U S A 96:12887-12892.
- Ogawa S, Chester AE, Hewitt SC, Walker VR, Gustafsson JA, Smithies O, Korach KS, Pfaff DW (2000) Abolition of male sexual behaviors in mice lacking estrogen receptors alpha and beta (alpha beta ERKO). Proc Natl Acad Sci U S A 97:14737-14741.
- Orikasa C, McEwen BS, Hayashi H, Sakuma Y, Hayashi S (2000) Estrogen receptor alpha, but not beta, is expressed in the interneurons of the hippocampus in prepubertal rats: an in situ hybridization study. Brain Res Dev Brain Res 120:245-254.
- Ormerod BK, Galea LA (2001) Reproductive status influences cell proliferation and cell survival in the dentate gyrus of adult female meadow voles: a possible regulatory role for estradiol. Neuroscience 102:369-379.
- Ormerod BK, Falconer EM, Galea LA (2003a) N-methyl-D-aspartate receptor activity and estradiol: separate regulation of cell proliferation in the dentate gyrus of adult female meadow vole. J Endocrinol 179:155-163.
- Ormerod BK, Lee TT, Galea LA (2003b) Estradiol initially enhances but subsequently suppresses (via adrenal steroids) granule cell proliferation in the dentate gyrus of adult female rats. J Neurobiol 55:247-260.
- Paech K, Webb P, Kuiper GG, Nilsson S, Gustafsson J, Kushner PJ, Scanlan TS (1997)
 Differential ligand activation of estrogen receptors ERalpha and ERbeta at AP1 sites.
 Science 277:1508-1510.
- Palmer TD, Willhoite AR, Gage FH (2000) Vascular niche for adult hippocampal neurogenesis. J Comp Neurol 425:479-494.

Perez-Martin M, Azcoitia I, Trejo JL, Sierra A, Garcia-Segura LM (2003) An antagonist of estrogen receptors blocks the induction of adult neurogenesis by insulin-like growth factor-I in the dentate gyrus of adult female rat. Eur J Neurosci 18:923-930.

- Pettersson K, Gustafsson JA (2001) Role of estrogen receptor beta in estrogen action. Annu Rev Physiol 63:165-192.
- Pettersson K, Delaunay F, Gustafsson JA (2000) Estrogen receptor beta acts as a dominant regulator of estrogen signaling. Oncogene 19:4970-4978.
- Pfaff D, Keiner M (1973) Atlas of estradiol-concentrating cells in the central nervous system of the female rat. J Comp Neurol 151:121-158.
- Pfaff DW (1989) Features of a hormone-driven defined neural circuit for a mammalian behavior.
 Principles illustrated, neuroendocrine syllogisms, and multiplicative steroid effects. Ann
 N Y Acad Sci 563:131-147.
- Pfaff DW, Freidin MM, Wu-Peng XS, Yin J, Zhu YS (1994) Competition for DNA steroid response elements as a possible mechanism for neuroendocrine integration. J Steroid Biochem Mol Biol 49:373-379.
- Pfaus JG, Smith WJ, Coopersmith CB (1999) Appetitive and consummatory sexual behaviors of female rats in bilevel chambers. I. A correlational and factor analysis and the effects of ovarian hormones. Horm Behav 35:224-240.
- Rissman EF, Wersinger SR, Taylor JA, Lubahn DB (1997a) Estrogen receptor function as revealed by knockout studies: neuroendocrine and behavioral aspects. Horm Behav 31:232-243.
- Rissman EF, Wersinger SR, Fugger HN, Foster TC (1999) Sex with knockout models: behavioral studies of estrogen receptor alpha. Brain Res 835:80-90.

Rissman EF, Early AH, Taylor JA, Korach KS, Lubahn DB (1997b) Estrogen receptors are essential for female sexual receptivity. Endocrinology 138:507-510.

Rissman EF, Heck AL, Leonard JE, Shupnik MA, Gustafsson JA (2002) Disruption of estrogen receptor beta gene impairs spatial learning in female mice. Proc Natl Acad Sci U S A 99:3996-4001.

Rubin BS, Barfield RJ (1980) Priming of estrous responsiveness by implants of 17 beta-estradiol in the ventromedial hypothalamic nucleus of female rats. Endocrinology 106:504-509.

Rubin BS, Barfield RJ (1983) Progesterone in the ventromedial hypothalamus facilitates estrous behavior in ovariectomized, estrogen-primed rats. Endocrinology 113:797-804.

- Sakuma Y (1995) Differential control of proceptive and receptive components of female rat sexual behavior by the preoptic area. Jpn J Physiol 45:211-228.
- Seri B, Garcia-Verdugo JM, McEwen BS, Alvarez-Buylla A (2001) Astrocytes give rise to new neurons in the adult mammalian hippocampus. J Neurosci 21:7153-7160.
- Seri B, Garcia-Verdugo JM, Collado-Morente L, McEwen BS, Alvarez-Buylla A (2004) Cell types, lineage, and architecture of the germinal zone in the adult dentate gyrus. J Comp Neurol 478:359-378.
- Shima N, Yamaguchi Y, Yuri K (2003) Distribution of estrogen receptor beta mRNA-containing cells in ovariectomized and estrogen-treated female rat brain. Anat Sci Int 78:85-97.
- Shughrue PJ, Merchenthaler I (2001) Distribution of estrogen receptor beta immunoreactivity in the rat central nervous system. J Comp Neurol 436:64-81.
- Shughrue PJ, Lane MV, Merchenthaler I (1997) Comparative distribution of estrogen receptoralpha and -beta mRNA in the rat central nervous system. J Comp Neurol 388:507-525.

- Shughrue PJ, Scrimo PJ, Merchenthaler I (1998) Evidence for the colocalization of estrogen receptor-beta mRNA and estrogen receptor-alpha immunoreactivity in neurons of the rat forebrain. Endocrinology 139:5267-5270.
- Simerly RB, Chang C, Muramatsu M, Swanson LW (1990) Distribution of androgen and estrogen receptor mRNA-containing cells in the rat brain: an in situ hybridization study. J Comp Neurol 294:76-95.
- Simonian SX, Herbison AE (1997) Differential expression of estrogen receptor alpha and beta immunoreactivity by oxytocin neurons of rat paraventricular nucleus. J Neuroendocrinol 9:803-806.
- Simpkins JW, Green PS, Gridley KE, Singh M, de Fiebre NC, Rajakumar G (1997) Role of estrogen replacement therapy in memory enhancement and the prevention of neuronal loss associated with Alzheimer's disease. Am J Med 103:19S-25S.
- Smith MT, Pencea V, Wang Z, Luskin MB, Insel TR (2001) Increased number of BrdU-labeled neurons in the rostral migratory stream of the estrous prairie vole. Horm Behav 39:11-21.
- Sohrabji F, Miranda RC, Toran-Allerand CD (1995) Identification of a putative estrogen response element in the gene encoding brain-derived neurotrophic factor. Proc Natl Acad Sci U S A 92:11110-11114.
- Solum DT, Handa RJ (2002) Estrogen regulates the development of brain-derived neurotrophic factor mRNA and protein in the rat hippocampus. J Neurosci 22:2650-2659.
- Stauffer SR, Coletta CJ, Tedesco R, Nishiguchi G, Carlson K, Sun J, Katzenellenbogen BS, Katzenellenbogen JA (2000) Pyrazole ligands: structure-affinity/activity relationships and estrogen receptor-alpha-selective agonists. J Med Chem 43:4934-4947.

- Su JD, Qiu J, Zhong YP, Li XY, Wang JW, Chen YZ (2001) Expression of estrogen receptor (ER)-alpha and -beta immunoreactivity in hippocampal cell cultures with special attention to GABAergic neurons. J Neurosci Res 65:396-402.
- Tanapat P, Hastings NB, Gould E (2005) Ovarian steroids influence cell proliferation in the dentate gyrus of the adult female rat in a dose- and time-dependent manner. J Comp Neurol 481:252-265.
- Tanapat P, Hastings NB, Reeves AJ, Gould E (1999) Estrogen stimulates a transient increase in the number of new neurons in the dentate gyrus of the adult female rat. J Neurosci 19:5792-5801.
- Tanapat P, Hastings NB, Rydel TA, Galea LA, Gould E (2001) Exposure to fox odor inhibits cell proliferation in the hippocampus of adult rats via an adrenal hormone-dependent mechanism. J Comp Neurol 437:496-504.

Thoenen H (2000) Neurotrophins and activity-dependent plasticity. Prog Brain Res 128:183-191.

- Toran-Allerand CD, Ellis L, Pfenninger KH (1988) Estrogen and insulin synergism in neurite growth enhancement in vitro: mediation of steroid effects by interactions with growth factors? Brain Res 469:87-100.
- Toran-Allerand CD, Guan X, MacLusky NJ, Horvath TL, Diano S, Singh M, Connolly ES, Jr., Nethrapalli IS, Tinnikov AA (2002) ER-X: a novel, plasma membrane-associated, putative estrogen receptor that is regulated during development and after ischemic brain injury. J Neurosci 22:8391-8401.
- Tremblay GB, Tremblay A, Copeland NG, Gilbert DJ, Jenkins NA, Labrie F, Giguere V (1997) Cloning, chromosomal localization, and functional analysis of the murine estrogen receptor beta. Mol Endocrinol 11:353-365.

- Turner RT, Wakley GK, Hannon KS, Bell NH (1987) Tamoxifen prevents the skeletal effects of ovarian hormone deficiency in rats. J Bone Miner Res 2:449-456.
- Walf AA, Frye CA (2005) ERbeta-Selective Estrogen Receptor Modulators Produce Antianxiety Behavior when Administered Systemically to Ovariectomized Rats. Neuropsychopharmacology.
- Weihua Z, Saji S, Makinen S, Cheng G, Jensen EV, Warner M, Gustafsson JA (2000) Estrogen receptor (ER) beta, a modulator of ERalpha in the uterus. Proc Natl Acad Sci U S A 97:5936-5941.
- Weiland NG, Orikasa C, Hayashi S, McEwen BS (1997) Distribution and hormone regulation of estrogen receptor immunoreactive cells in the hippocampus of male and female rats. J Comp Neurol 388:603-612.
- Wersinger SR, Sannen K, Villalba C, Lubahn DB, Rissman EF, De Vries GJ (1997) Masculine sexual behavior is disrupted in male and female mice lacking a functional estrogen receptor alpha gene. Horm Behav 32:176-183.
- Ziylan YZ, Lefauconnier JM, Bernard G, Bourre JM (1990) Blood-brain barrier permeability: regional alterations after acute and chronic administration of ethinyl estradiol. Neurosci Lett 118:181-184.
- Zou A, Marschke KB, Arnold KE, Berger EM, Fitzgerald P, Mais DE, Allegretto EA (1999) Estrogen receptor beta activates the human retinoic acid receptor alpha-1 promoter in response to tamoxifen and other estrogen receptor antagonists, but not in response to estrogen. Mol Endocrinol 13:418-430.