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Estrogen Promotes Neurite Outgrowth In Olfactory Epithelial Explant Cultures Through The Estrogen Receptor

Apryl E. Pooley
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Estrogen Promotes Neurite Outgrowth in Olfactory Epithelial Explant Cultures Through the Estrogen Receptor

BY

Apryl E. Pooley

THESIS

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FOR THE DEGREE OF

MASTER IN BIOLOGICAL SCIENCES

IN THE GRADUATE SCHOOL, EASTERN ILLINOIS UNIVERSITY
CHARLESTON, ILLINOIS

2011
YEAR

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Dr. Britto Nathan — Date: 6/27/11

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Department of Biological Sciences
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Summer 2011

ABSTRACT

Estrogen is well known as a hormone that regulates reproduction and sexual differentiation, but recent findings have shown estrogen to be important in maintaining a healthy environment in the brain by promoting neural plasticity, protection, regeneration, and repair. Estrogen has been shown to promote neurite outgrowth, which may have implications in the treatment or prevention of neurodegenerative diseases such as Alzheimer's disease. Alzheimer's disease is three times more prevalent in postmenopausal women than in men, which may suggest a role of the loss of estrogen in neurodegeneration. The mechanism by which estrogen promotes neurite outgrowth is not clearly understood, and the goal of the present study is to investigate the effects of estrogen on neurite outgrowth in olfactory receptor neurons.

We treated olfactory epithelial explant cultures from postnatal mice with various concentrations of cholesterol, testosterone, progesterone, and estradiol. Estradiol treatment (100 nM) significantly increased neurite outgrowth of olfactory receptor neurons. As an initial step toward understanding the mechanisms underlying the neurite outgrowth-promoting effect of estradiol, we used immunocytochemistry to determine that the two nuclear estrogen receptors, ERα and ERβ are expressed in olfactory receptor neurons. ERα was found in the cell body of olfactory receptor neurons, and treatment with estradiol increased the expression of ERα in the cell body as well as in the neurites. Immunoreactivity for ERα in the neurites of estradiol-treated cultures was three-fold higher than that in water-treated cultures.

We performed an estradiol treatment time course study that showed ERα expression increased in the cell body and neurites within 2 hours of estradiol treatment.

Within 24 hours of estradiol treatment ERα is expressed in the nucleus, suggesting gene activation. After 48 hours of estradiol treatment, ERα expression was not observed in the nucleus, and after 72 hours, ERα expression decreased in the neurites, which may have caused by downregulation of ERα due to prolonged estradiol treatment.

Because estradiol treatment increased ERα expression in the neurites, we hypothesized that ERα is the receptor subtype that facilitates the neurite outgrowth-promoting effect of estradiol. We used selective estrogen receptor modulators, compounds that selectively activate either ERα or ERβ, to test this hypothesis in olfactory epithelial explant cultures. Treatment with PPT, an ERα agonist, produced neurons with significantly longer neurites compared to cultures treated with DPN, an ERβ agonist, or vehicle alone. We also used cultures derived from ER knockout mice to examine the effect of estradiol on neurite outgrowth through ERα and ERβ. Treatment with estradiol in cultures derived from ERβ KO mice had significantly longer neurites than water-treated cultures or estradiol-treated cultures derived from ERα KO mice. These results suggest that ERα is the receptor subtype that facilitates estradiol-mediated neurite outgrowth.

DEDICATION

I would like to thank my mentor and friend, Dr. Britto Nathan, for giving me the opportunity to work in his lab doing such interesting and important research, and for his teaching, support, patience, and encouragement.

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INTRODUCTION

Estrogens are 18-carbon steroidal hormones that exist in three naturally occurring isoforms: estrone, estriol, and estradiol (2, 3). While estrogens are primarily produced in the ovaries, they are synthesized in both males and females. Aside from estrogens produced by developing follicles and the corpus luteum in the ovaries, they are also produced in the liver, adrenal glands, bones, skin, colon, and breasts (8, 11). Estrogens are also synthesized in men by the aromatization of circulating testosterone in various tissues, including the brain (8). Estrogens, like all steroid hormones, are derived from cholesterol. Cholesterol is transported into the mitochondria of steroidogenic cells where it is cleaved by a cytochrome P450 enzyme to form pregnenolone (3). Through a number of enzymatic reactions, pregnenolone is converted into progesterone, progesterone into androgens, and androgens into estrogens (3). The rate-limiting step in estrogen biosynthesis is the aromatization of testosterone and androstendione to 17β-estradiol and estrone, respectively, by P450 aromatase (3, 13). The dominant and most biologically-active estrogen in humans is 17β-estradiol (9). While estrogens are primarily synthesized by the aromatization of androgens in the ovaries of premenopausal women, most 17β-estradiol synthesis occurs in extragondal tissues by men and postmenopausal women (3, 13).

Aromatase, which converts androgens to estrogens, is highly expressed in areas of the mouse brain, including the hypothalamus, thalamus, preoptic area, olfactory bulb, hippocampus, cerebral cortex, pons, and midbrain (3). Because the formation of estrogens is the sole function of aromatase, the abundance of the enzyme in the brain suggests a role for estrogens in brain functioning. Interestingly, the expression and

activity of aromatase is increased following brain injury, and inhibiting aromatase results in increased neurodegeneration (5). These findings implicate the role of estrogen in neuronal repair and maintenance mechanisms.

Traditionally, estrogen has been considered to be a hormone that regulates sexual differentiation and reproduction, but many studies have illustrated the role of estrogen in neuronal plasticity, regeneration, protection, and cognitive functions (2,7). Estrogen has been shown to promote neurite outgrowth (any projection from the cell body of a neuron) in various regions of the developing brain, which is typically not seen in the adult brain (2, 11). However, after estrogen loss, treatment with estrogen promotes the growth of neurites, suggesting a neuroprotective effect of estrogen (2). Because of the dramatic decrease in estrogen levels after menopause, post-menopausal women serve as a natural model for the effects of estrogen on the brain.

With the cessation of ovarian function after menopause, women experience a dramatic decline in circulating estrogen levels. Estradiol levels in the blood of post-menopausal women are typically only 1% of normally menstruating women (19). Many symptoms associated with menopause, including memory loss, depression, and anxiety are neurological in nature, and could be attributed to the decrease of estrogen (4). This decrease in estrogen has been implicated in the increased occurrence of osteoporosis, cardiovascular disease, stroke, and Alzheimer's disease in post-menopausal women (7, 19). Estrogen replacement was proposed to treat or prevent these neurological changes after menopause. Hormone therapy that consists of either estrogen treatment alone or combined estrogen and progestin treatment has been shown to have beneficial effects on

cognitive function in human and animal models that have been naturally or surgically depleted of estrogen (2, 4, 5).

Decreased estrogen levels in these models have been accompanied by neurological changes that have been implicated in the progression of Alzheimer's disease, which may suggest a link between estrogen and the development of neurodegenerative diseases such as Alzheimer's disease (2). Alzheimer's disease is three times more prevalent in postmenopausal women than in men, which may also suggest a role of the loss of estrogen in neurodegeneration (2, 6, 13, 32).

Alzheimer's disease (AD) primarily occurs late in life and is characterized by the dramatic and progressive decline in cognitive function, mostly involving memory and language (6). The trademark physical indications of AD are the accumulation of β-amyloid plaques around neurons and neurofibrillary tangles inside neurons, which lead to cell death and brain atropy (6, 13). Alzheimer's disease is most common in post-menopausal women, and decreased estrogen is a risk factor for AD (2, 6). Estradiol levels in the frontal cortex of postmortem AD patients were found to be significantly lower than those of non-AD patients (13). Epidemiological studies have shown that a history of estrogen replacement therapy reduces the likelihood of developing Alzheimer's disease and Parkinson's disease with dementia later in life (33, 34, 35). Although a subject of much debate, many studies suggest that estrogen treatment may prevent or delay AD in post-menopausal women, but the mechanism by which estrogen exerts its effects is still unclear (6).

Estrogen acts through two genomic receptors, estrogen receptor α (ERα) and estrogen receptor β (ERβ). ERα and ERβ belong to the steroid nuclear receptor family

that modulates gene transcription in an estrogen-dependent manner (1, 9, 13). In humans, ERα and ERβ are encoded by genes on chromosomes 6 and 14, respectively (13). The classic estrogen activation pathway involves the ligand-activated dimerization of nuclear estrogen receptors, which then act as transcription factors to activate their target genes (3, 9, 12). ERα and ERβ form heterodimers or homodimers that bind to estrogen response elements on their targets genes to modulate transcription (13). While the hypothalamus is the traditional site of estrogen action in the brain with regard to reproduction, estrogen receptors are abundantly expressed in various areas of the brain, including the cerebral cortex, olfactory bulb, amygdala, thalamus, substansia nigra, and cerebellum (1, 6). The cortex and hypothalamus, regions of the brain highly associated with neurodegenerative diseases such as Alzheimer's disease, have been shown to express both ERα and ERβ (6). Estrogen receptors have previously been found to be primarily localized in the nucleus of neurons, but some extranuclear expression has been reported in select areas, including the olfactory bulb (1). Some areas of the brain express ERα and ERβ equally, while other regions demonstrate a dominant ER subtype (1, 13). Both ERs have been shown to potentially be expressed in the same cell, suggesting the formation of a heterodimer receptor (1, 12).

Recent evidence has shown that estrogen may also act through other signaling mechanisms aside from the classical estrogen activation pathway (9). Estradiol has been shown to have some effects of rapid onset, within seconds to minutes, such as the intracellular release of Ca^{2+} and activation of mitogen-activated protein kinases (13). While estrogens can exert some of their effects through modulating gene expression, many of the rapid actions of estrogens can be explained through non-genomic pathways

involving membrane-associated estrogen receptors (9, 12, 13). More recently, immunoreactivity for ERα and ERβ has been found in neurites of the hippocampus and in glial cells, suggesting an alternative pathway of estrogen action (12). In addition to the extranuclear localization of ERα and ERβ , a recently identified protein that was reactive with ERα antibodies but was unique and functionally distinct based on its molecular weight, ligand specificity, cellular localization, and apparent response characteristics, has been identified as estrogen receptor X (ER-X) (10, 12). ER-X is a plasma membrane-associated estrogen receptor that could be responsible for the rapid, non-genomic actions of estrogen (10). Understanding the regional expression of estrogen receptors in the brain, as well as the cellular localization of these receptors, can provide insight into the mechanism by which estrogen exerts its effects on the brain.

The present study examined the function of estrogen and its receptors on olfactory neuronal growth in olfactory epithelial explant cultures derived from postnatal mice. The olfactory epithelium is comprised of olfactory receptor neurons that continuously regenerate from stem cells, called basal cells, throughout the life of an organism (22). The ability of olfactory receptor neurons to regenerate makes them an invaluable model to demonstrate the mechanisms of neuronal growth, plasticity, repair, and regeneration. Olfactory epithelium derived from ERα and ERβ knockout mice can be used to examine the estrogen receptor by which estrogen exerts its effects on olfactory receptor neurons; thus, making the olfactory epithelium an appropriate model for the present study.

The effects of various hormones, most importantly estradiol, on the growth of olfactory receptor neurons and the cellular localization and expression of ERα and ERβ were studied. The possible implications for Alzheimer's disease and other

neurodegenerative diseases make understanding the mechanisms involved in the beneficial effects of estrogen on neuronal growth a relevant goal for this study.

GOALS AND HYPOTHESES

Determine the effects of steroidal hormones on the neurite outgrowth of olfactory receptor neurons in wild type olfactory epithelial cultures:

Estrogen has been shown to promote neurite outgrowth, but since it is derived from cholesterol, progesterone, and testosterone, it was important to determine whether these precursor molecules have an effect on neurite outgrowth. Because of the abundance of aromatase and estrogen receptors in the brain, we hypothesized that estrogen has a unique effect on neurite outgrowth.

The supply of estrogen receptors in the normal brain indicates an important role of estrogen in brain functioning. 17β-estradiol is the most abundant and biologically active form of estrogen in humans, and based on previous studies, we hypothesized that treatment with estradiol would increase the neurite outgrowth in wild type olfactory epithelial cultures.

Determine the effect of estradiol on the cellular localization, distribution, and expression of ERα and ERβ:

As an initial step toward understanding the mechanisms by which estrogen promotes neurite outgrowth, we wanted to determine whether ERα and ERβ are

expressed in olfactory receptor neurons. Based on previous studies, we hypothesized that both receptor subtypes will be expressed in the olfactory receptor neurons.

It has been found that estradiol influences the neurite outgrowth of olfactory receptor neurons through its receptors, and in order to understand the mechanism by which estradiol produces these effects, it is important to understand whether estradiol affects the expression or cellular localization of these receptors.

Identify which ER subtype mediates the neurite outgrowth-promoting effect of estradiol in the olfactory epithelial culture

Because estrogen can act through ERα and ERβ, it was important to determine which receptor subtype facilitates the effects of estrogen on neurite outgrowth. Using ER knockout mice, the differential responses of each receptor subtype to estrogen can be examined. The neuroprotective effect of estradiol on cultures derived from ERβ knockout mice, but not ERα knockout cultures, led to our hypothesis that estradiol-induced neurite outgrowth promotion is facilitated via ERα.

Compounds that have been synthesized to selectively activate either ERα or ERβ can also be used to confirm by which receptor subtype estradiol exerts its effects. Observing the effects of ERα and ERβ agonists on the neurite outgrowth of olfactory receptor neurons can provide more insight into the estrogen receptor-specific actions of estradiol. Because it has been previously found through ER knockout cultures that estradiol increases neurite outgrowth via ERα, we hypothesized that an ERα agonist would have a similar effect.

MATERIALS AND METHODS

Animals:

WT (C57BL/6J, Cat. #000664) mice were purchased from Jackson Laboratory (Bar Harbor, ME). Heterozygous ERβKO (ERβ+/-) female and homozygous ERβKO (ERβ-/-) male mice (B6.129P2-*Esr2*^{tm1Unc}/J, Cat. #004745) that were backcrossed onto the C57BL/6J background for eight generations were purchased from Jackson Laboratory (Bar Harbor, ME). The line of ERβKO mice was maintained by mating ERβ+/- females with ERβ-/- males and identifying offspring genotype by PCR. Heterozygous ERαKO (ERα+/-) male and female mice (B6.129P2-*Esr1*^{tm1Ksk}/J, Cat. #004744) that were backcrossed onto the C57BL/6J background for ten generations were purchased from Jackson Laboratory (Bar Harbor, ME). The line of ERαKO mice was maintained by mating ERα+/- males and females and identifying offspring genotype by PCR.

Olfactory epithelial explant culture:

Three to six postnatal pups (2-3 days old) were used for each experiment. Twelve hours prior to the experiment, 24 glass coverslips (Fisher Scientific, Pittsburgh, PA, Cat. #12-545-80) were placed in a 24-well plate and coated in a 50μg/ml fibronectin solution prepared by dissolving fibronectin (Sigma Aldrich, St. Louis, MO, Cat #F1141) in Neurobasal-A (NBA) media (Invitrogen, Carslbad, CA, Cat. #10888-022). The slips were coated in just enough solution to cover the surface of the glass and placed in a 37°C incubator for 12 hours to allow the fibronectin to coat the slips. The pups were then decapitated using sterile surgical scissors, and the olfactory epithelium was exposed by making a sagital incision through the nasal cavity with a sterile razor blade. The

olfactory epithelium was dissected and placed in 0°C 10 ml Hanks' Balanced Salt Solution (Invitrogen, Carlsbad, CA, Cat. #14175-095) containing 50 μg/ml gentamycin (Invitrogen, Carlsbad, CA, Cat. #15710-064) and 6 mg/ml glucose. The olfactory epithelium was then washed twice with cold (4°C) growth media: NBA solution containing 20 μg/ml B27 supplement (Invitrogen, Carlsbad, CA, Cat. #17504-044), 0.5 mM L-glutamine (Sigma Aldrich, St. Louis, MO, Cat. #G8540), 13.0 mM NaCl (Sigma Aldrich, St. Louis, MO, Cat. #S5886-500G), and 50 μg/ml gentamycin. The tissue was sliced into explants of about 200 μm thickness with a sterile razor blade. One explant was placed on each of the 24 fibronectin-coated coverslips and incubated at 37°C and 5% CO_2 without media for 30 minutes. Following incubation, 500 μl of warm (37°C) growth media (as prepared above) supplemented with 5 ng/ml FGF2 (Invitrogen, Carlsbad, CA, Cat. #13256-029) was added to each well, and the plate was returned to the incubator. Growth media was changed every 2 days. At 8 DIV (days in vitro), neurite outgrowth assays were performed as described below.

Treatment of cultures and measurement of neurite outgrowth:

Hormones used in this study purchased from Sigma Aldrich (St. Louis, MO) include 17β-estradiol (Cat. #E4389), cholesterol (Cat. # C4951), progesterone (Cat. #P7556), and testosterone (Cat. #T1500). 100 pM, 100 nM, 100 μM, and 100 mM of estradiol, cholesterol, and progesterone were prepared by dissolving in water, and the same concentrations of testosterone were prepared by dissolving in 95% ethanol. DPN (Cat. #1494) and PPT (Cat. #1426) were purchased from Tocris (Ellisville, MO) and were used at concentrations of 0.3 nM and 0.5 nM, respectively. Olfactory epithelial

explant cultures were treated at 2 DIV with the test reagents when the growth media was changed, and test reagents were added again with each media change thereafter. At 8 DIV, the cultures were rinsed in warm (37°C) PBS and fixed with 4% paraformaldehyde for 10 minutes (Sigma Aldrich, St. Louis, MO, Cat. #441244). Pictures were taken of various fields of view of each culture well using a digital microscope camera (AmScope, Irvine, CA Cat. #MD900-CK) attached to an inverted microscope. Neurons were identified by their bipolar morphology and the neurite outgrowth was measured using image processing software (ImageJ, Bethseda, MD).

Immunocytochemistry:

Cells from 8 DIV cultures were rinsed with warm PBS and fixed with 4% paraformaldehyde for 10 minutes at room temperature. After rinsing with PBS, cells were permeabilized and blocked with 0.2% triton (Sigma Aldrich, St. Louis, MO, Cat. #T-9284) and 2% donkey serum (Jackson ImmunoResearch, West Grove, PA, Cat. #017-000-121) in PBS for 1 hour at room temperature. Cells were then incubated in primary antibody with 1% donkey serum and 0.1% triton in PBS for 48 hours at 4°C in a fluid-filled chamber. Primary antibodies used in this study were 1:100 anti-estrogen receptor α C1355 (Millipore, Billerica, MA, Cat. #06-935) and 1:100 anti-estrogen receptor ß 1531 (Santa Cruz Biotechnology, Santa Cruz, CA, Cat. #sc-53494). After primary antibody incubation, cells were rinsed with PBS and permeabilized with 0.1% triton in PBS for 15 minutes at room temperature. Cells were then incubated in secondary fluorescent antibody with 1% donkey serum in PBS for 1 hour. Seconary antibodies used in this study were 1:500 Alexa Fluor 488 donkey anti-mouse IgG (Invitrogen, Carlsbad, CA, Cat. #A21202) and 1:500 Alexa Fluor 488 donkey anti-rabbit IgG (Invitrogen, Carlsbad,

CA, Cat. #A-21206). After secondary antibody incubation, cells were rinsed with PBS, then rinsed with 0.1% triton in PBS to reduce background noise, then rinsed with PBS again. Cells were then mounted on a microscope slide with Vectasheild Mounting Medium (Vector Labs, Burlingame, CA, Cat. #H-1000) and sealed with Cytoseal (Fisher Scientific, Pittsburg, PA, Cat. #23-244-256). Immunofluorescence was evaluated by fluorescent microscopy (Olympus BX-50) with a digital microscope camera (Penguin Pixera, San Jose, CA).

Statistical Analyses:

All experiments were repeated 2-3 times using different preparations of olfactory epithelial explant cultures and reagents. The data in individual experiments were presented as the mean ± standard error, and statistical analyses (ANOVA, repeated measures ANOVA, post-hoc Bonferroni adjustment) were performed using SYSTAT.

RESULTS

Dose response of steroid hormones on neurite outgrowth in olfactory epithelial explant cultures:

The neurite outgrowth of OEC treated with 0.1 pM, 100 pM, and 100 μM estradiol was not different from water-treated OEC, but OEC treated with 100 nM estradiol had neurons with significantly ($p < 0.05$) longer neurite outgrowth compared to vehicle-treated OEC (Figure 1). Neurite outgrowth in cultures treated with cholesterol or progesterone was similar to that in cultures treated with water alone (Figure 1). Testosterone at low concentrations (1 pM and 100 pM) produced ORN with longer neurite outgrowth compared to ethanol-treated OEC, but high concentrations of testosterone (100 nM and 100 mM) produced ORN with significantly shorter neurite outgrowth than vehicle-treated OEC ($p < 0.05$) (Figure 1). Quantification of the results described above is shown in Figure 2.

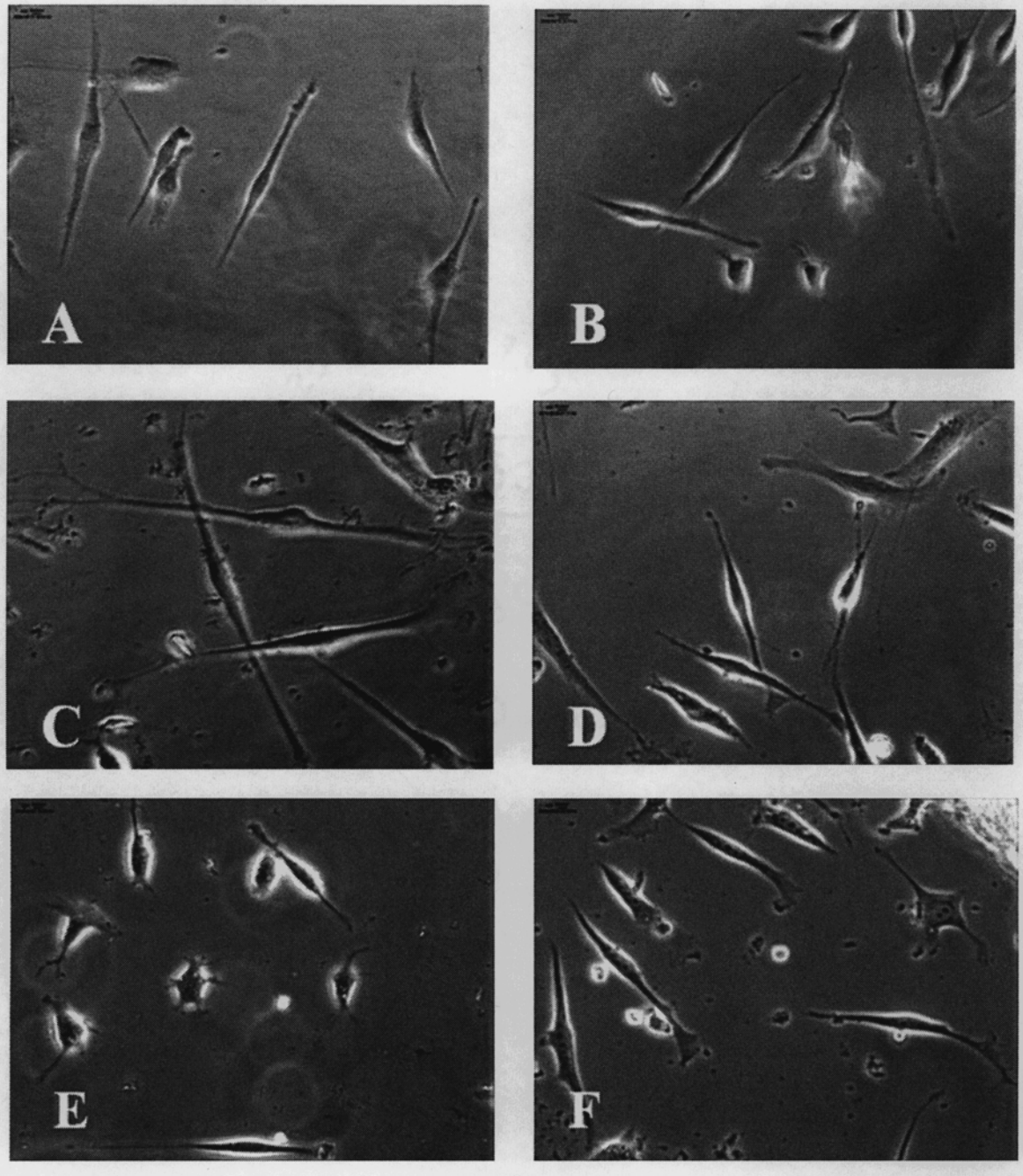

Figure 1. Representative phase contrast photomicrographs (400x magnification) of olfactory epithelial neuronal cultures treated with (A) cholesterol 100 nM, (B) Progesterone 100 nM, (C) estradiol 100 nM, (D) water alone, (E) testosterone 100 μM, and (F) ethanol alone.

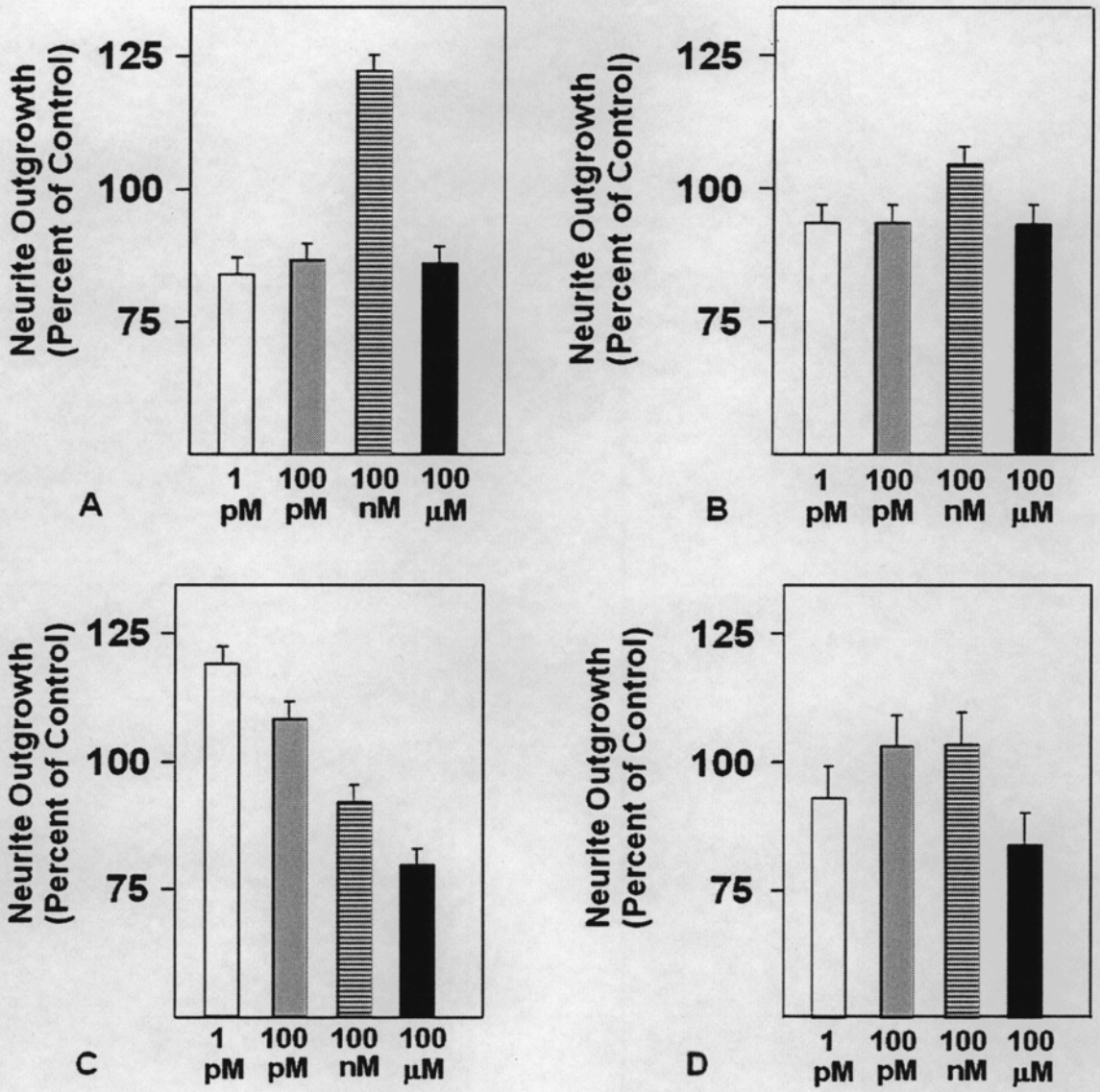

Figure 2. Quantification of the effects of various concentrations of (A) estradiol, (B) cholesterol, (C) testosterone, and (D) progesterone on the neurite outgrowth of olfactory receptor neurons from wild type olfactory epithelial cultures expressed as percentage of control (mean ± SE).

Effect of estradiol on ERα and ERβ expression and localization in olfactory epithelial explant cultures:

To determine whether ERα and ERβ are expressed in OEC, immunocytochemistry was performed in OEC treated with estradiol or vehicle alone. Both ERα and ERβ were expressed in ORN (Figure 3). ERα was found in the cell body of ORN, and treatment with estradiol increased the expression of ERα in the cell body as well as in the neurites (Figure 3). ERβ was found only in the nucleus of ORN, and treatment with estradiol increased its expression (Figure 3). As shown in Figure 4, the percentage of ORN expressing ERα in the neurites was three-fold higher in estradiol-treated cultures compared to vehicle-treated cultures.

To determine the effect of estradiol on the cellular localization and expression of ERα, OEC were treated with estradiol for 2, 24, 48, and 72 hours or vehicle alone. These cultures were fixed at 5 DIV, and immunocytochemistry was performed to visualize the expression of ERα. The expression of ERα in the cell body and neurites of ORN increased after 2 hours of estradiol (100 nM) treatment, and the expression of ERα in the cell body, neurites, and nucleus of ORN increased after 24 hours of estradiol treatment (Figure 5). The expression of ERα in the nucleus decreased after 48 hours of estradiol treatment and decreased in the neurites after 72 hours of estradiol treatment; however, ERα expression was still higher than that in water-treated ORN (Figure 5).

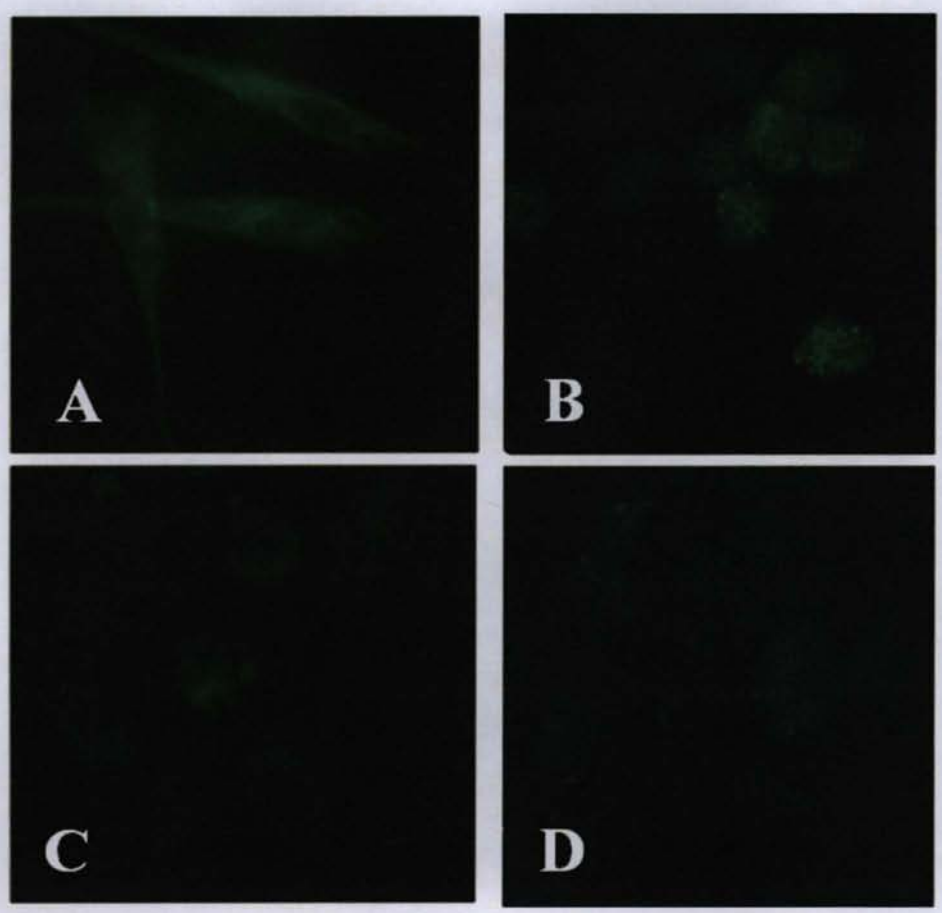

Figure 3. Immunostaining of (A) ERα in olfactory epithelial cultures treated with estradiol 100 nM, (B) ERβ in OEC treated with estradiol 100 nM, (C) ERα in OEC treated with water, and (D) ERβ in OEC treated with water.

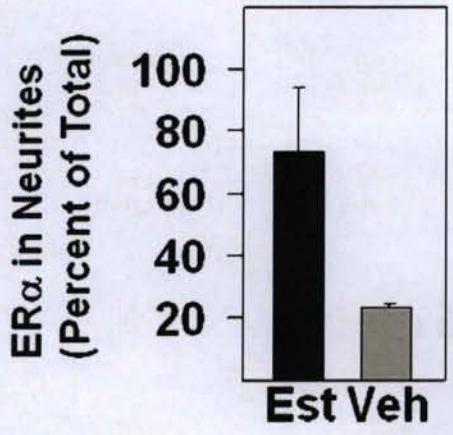

Figure 4. Immunoreactivity of ERα in neurites of olfactory epithelial cultures treated with estradiol (Est) or vehicle (Veh) expressed as percentage of total cells in culture (mean ± SE).

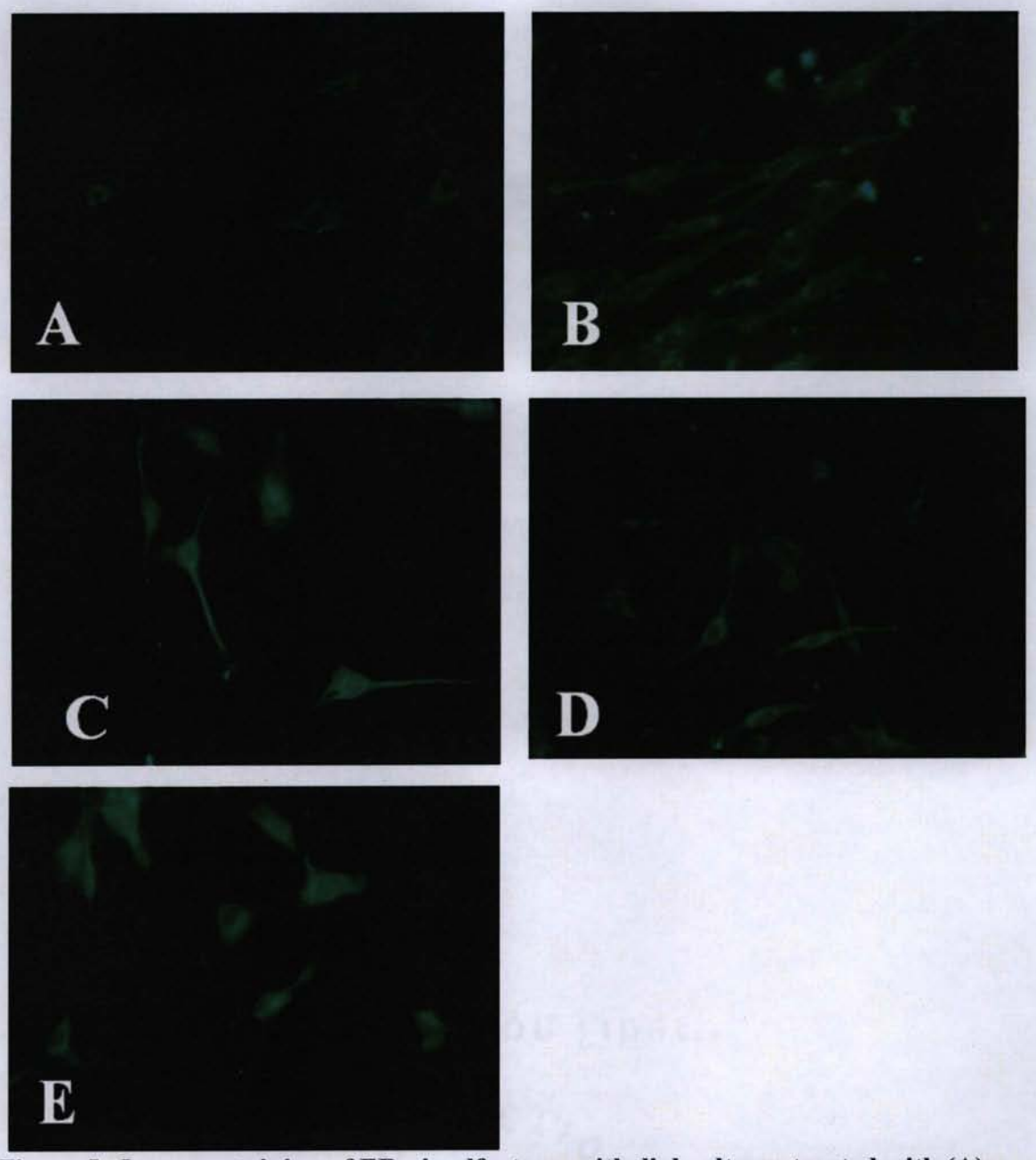

Figure 5. Immunostaining of ERα in olfactory epithelial cultures treated with (A) water, (B) estradiol 100 nM for 2 hours, (C) estradiol (100 nM) for 24 hours, (D) estradiol for 48 hours, and (E) estradiol for 72 hours.

Effect of selective estrogen receptor modulators (SERMs) on neurite outgrowth of olfactory receptor neurons in wild type olfactory epithelial cultures:

To determine the estrogen receptor subtype by which estradiol promotes neurite outgrowth, olfactory epithelial cultures from wild type mice were treated with the estrogen receptor alpha agonist propylpyrazoletriol (PPT) 0.5 nM, the estrogen receptor beta agonist diarylpropionitrile (DPN) 0.3 nM, or estradiol (100 nM) starting at 2 DIV. Reagents were added again every 2 days when the growth media was changed. At 8 DIV, the cultures were fixed with 4% paraformaldehyde, and the neurite outgrowth of ORN were quantified. Olfactory epithelial cultures treated with PPT or estradiol had ORN with significantly ($p < 0.05$) longer neurite outgrowth compared to cultures treated with DPN or water alone (Figure 6). Quantification of the results described above is shown in Figure 7.

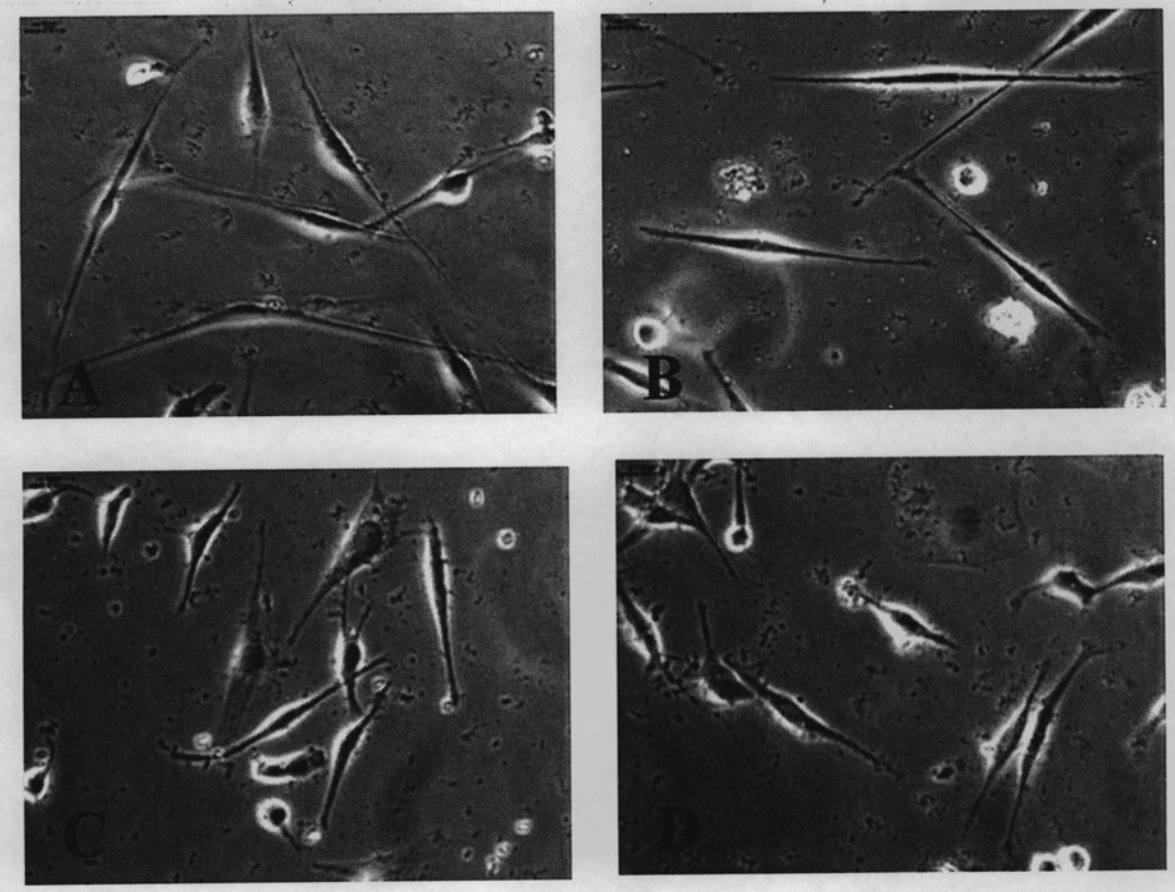

Figure 6. Representative phase contrast photomicrographs (magnification 400x) of olfactory epithelial neuronal cultures treated with (A) estradiol 100 nM, (B), PPT 0.5 nM, (C) DPN 0.3 nM, and (D) water alone.

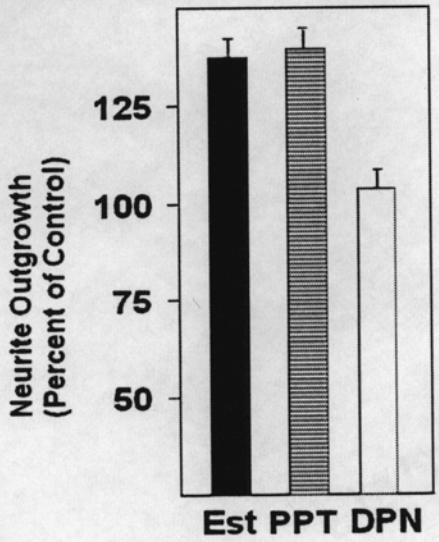

Figure 7. Quantification of the effect of estradiol (Est), PPT, and DPN on neurite outgrowth of ORN from wild type OEC expressed as percentage of control (mean ± SE).

Effect of estradiol on neurite outgrowth of ORN in estrogen receptor knockout olfactory epithelial cultures:

To determine by which estrogen receptor subtype estradiol promotes neurite outgrowth, olfactory epithelial cultures derived from either ERα KO mice or ERβ KO mice were treated with estradiol (100 nM) starting at 2 DIV. Estradiol was added again every 2 days when the growth media was changed. At 8 DIV, the cultures were fixed with 4% paraformaldehyde, and the neurite outgrowth of ORN was quantified. Estradiol treatment of OEC from ERβ KO mice had ORN with significantly ($p < 0.05$) longer neurite outgrowth compared to water-treated OEC from ERβ KO mice (Figure 8). Neurite outgrowth in ERα KO cultures treated with estradiol was similar to that in ERα KO cultures treated with water alone (Figure 8). Quantification of the results described above is shown in Figure 9.

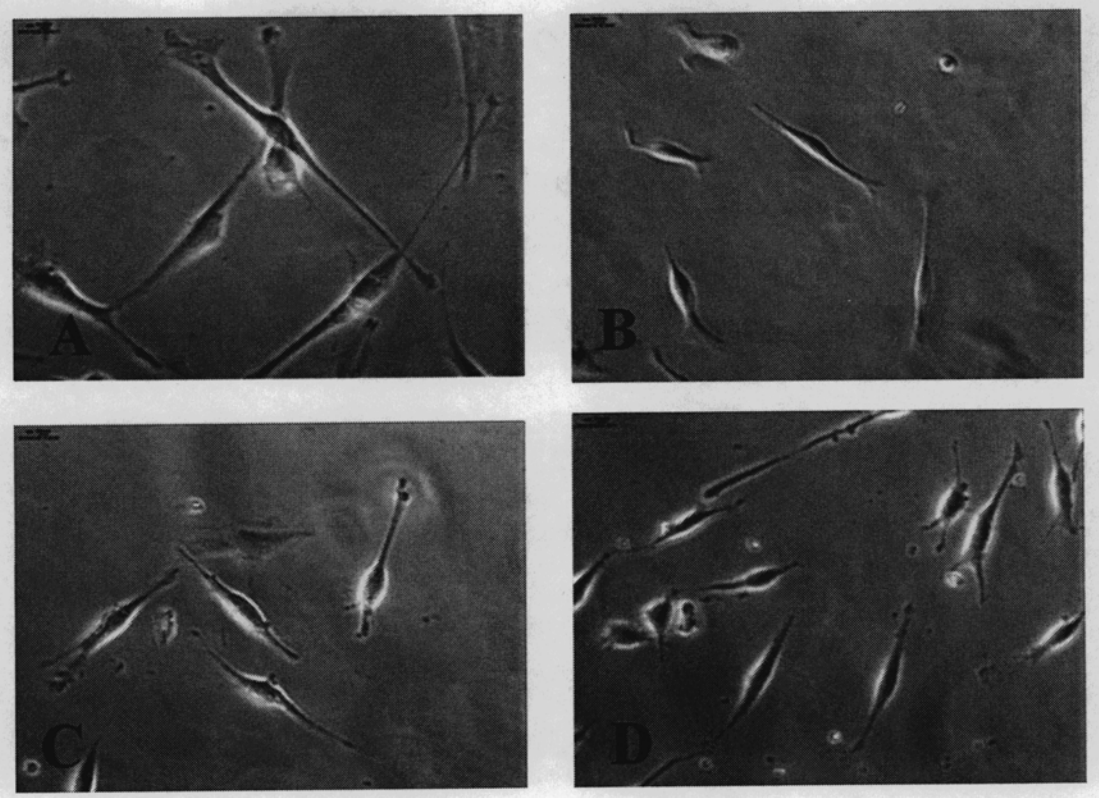

Figure 8. Representative phase contrast photomicrographs (magnification 400x) of olfactory epithelial neuronal cultures treated with (A) estradiol 100 nM from ERβ KO OEC, (B) water from ERβ KO OEC, (C) estradiol 100 nM from ERα KO OEC, and (D) water from ERα KO OEC.

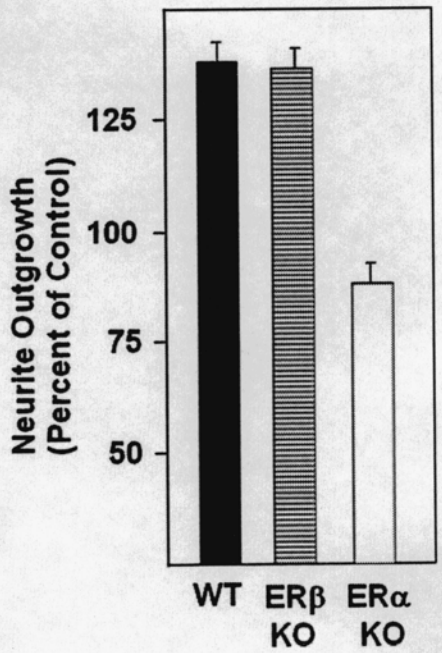

Figure 9. Quantification of the effect of estradiol on neurite outgrowth of ORN from ERα KO and ERβ KO OEC expressed as percentage of control (mean ± SE).

DISCUSSION

The present study focused on the neurotrophic and neuroprotective effects of estradiol in the olfactory epithelium through estrogen receptors. Olfactory receptor neurons in the olfactory epithelium regenerate continuously throughout the life of an organism, making the olfactory epithelium an important model to study neuronal growth and regeneration. The effects of several steroid hormones, mainly estradiol, on the neurite outgrowth of olfactory receptor neurons are discussed, along with the effects of estradiol on the cellular localization and expression of ERα and ERβ. Possible mechanisms of the observed effects of estradiol on neurite outgrowth are discussed with implications in the treatment of Alzheimer's disease.

The results of this study showed that *(1)* treatment of olfactory receptor neurons with estradiol promoted neurite outgrowth, *(2)* estradiol treatment increased the expression of ERα in the cell body and neurites and increased the expression of ERβ in the nucleus, and *(3)* ERα is the estrogen receptor that mediates the neurite outgrowth-promoting effects of estradiol.

Characterization of the olfactory epithelial culture (OEC):

The olfactory epithelium is a pseudostratified epithelium comprised of three types of cells: cuboidal basal cells, columnar supporting cells, and bipolar olfactory receptor neurons (22). Olfactory receptor neurons are the only type of neuron in the olfactory epithelium and comprise 80-85% of the cells in the epithelium (22). The supporting cells are analogous to neural glial cells that provide metabolic and structural support to the olfactory neurons. The basal cells are progenitor stem cells that divide and differentiate into either olfactory receptor neurons or supporting cells.

The olfactory epithelial explant culture in the present study was supplemented with basic fibroblast growth factor (FGF2), which has been shown to stimulate the growth of adult and embryonic neurons from an explant of the olfactory epithelium (14). The establishment of the olfactory epithelium begins at 1-2 DIV when cells start to migrate out of the explant and appear round. After 3-4 DIV, cells proliferate into a sheet surrounding the explant that consists of irregularly shaped flat supporting cells and basal cells. Neurogenesis can be documented at 4-5 DIV when bipolar neurons are observed surrounding the flat cell zone. Because neurons are not apparent until the establishment of the flat cell zone, neurogenesis can be explained by a process in which neurons originate from the basal cells surrounding the explant. Previous studies have shown that transplanted basal cells proliferate and differentiate into other basal cells, supporting cells, and mature olfactory receptor neurons when cells in the epithelium have been destroyed (23). Another explanation could be that the flat cell zone is formed to provide a favorable environment prior to the migration of olfactory receptor neurons from the explant.

Effect of steroid hormones on neurite outgrowth of olfactory receptor neurons (ORN) in OEC derived from wild type (WT) mice:

Estradiol treatment has been shown to promote neurite outgrowth, regeneration, neuroprotection, and cognitive function (2, 7, 11, 26). Since cholesterol, progesterone, and testosterone are precursor molecules in the synthesis of estradiol, we wanted to examine whether these compounds had an effect on neurite outgrowth.

Testosterone

It has been previously shown that high levels of testosterone (micromolar range) induce apoptosis in neuronal cells, but lower concentrations (nanomolar or lower) induce neuronal differentiation (27, 28). These results coincide with those of the present study in which neurite outgrowth decreased with increasing concentrations of testosterone. Neurons treated with 100 μM testosterone had significantly shorter neurites than those treated with lower concentrations of testosterone or vehicle. Our results suggest that high concentrations of testosterone reduce neurite outgrowth, but very low concentrations of testosterone may have a beneficial effect on neurite outgrowth. It has been previously shown that the effects of testosterone on neurite outgrowth are independent of the estrogen pathway and not caused by its metabolization into estradiol (28).

Progesterone

Progesterone has been shown to antagonize the neuroprotective effects of estradiol (31, 32), but progesterone treatment alone did not have a significant effect on the neurite outgrowth of neurons in the present study. Previous studies have shown that

in neuronal cultures with astrocytes and microglia present, progesterone did not affect neurite outgrowth, but when microglia were absent, progesterone supported neurite outgrowth (31). Microglia contain progesterone receptors that contribute to the antagonism of progesterone on estradiol-mediated neurite outgrowth (31). While the olfactory epithelial culture contains mostly olfactory receptor neurons, some glial cells are present, which could explain why progesterone did not affect the growth of neurons in this study. It should be noted that the olfactory epithelial culture in this study was supplemented with B27, which contains progesterone that could have masked the results of the progesterone in this experiment (15).

Cholesterol

Previous studies have shown that cholesterol depletion stimulated neurite outgrowth in hippocampal neurons, but decreased neurite outgrowth in cortical neurons, suggesting that the effects of cholesterol depend on the neuronal type (29). Cholesterol has been shown to promote synaptogenesis, but this effect is due to the conversion of cholesterol into estradiol (30). In the present study, cholesterol treatment did not have a significant effect on the neurite outgrowth of olfactory receptor neurons. Our results suggest that cholesterol was not converted into estradiol in the olfactory epithelial culture, as we showed that estradiol promotes neurite outgrowth.

Estradiol

Previous research has shown that estradiol promotes neurite outgrowth in various regions of the developing brain, a characteristic that is not normally exhibited in the adult

brain (2, 11). However, if normal estrogen levels are decreased, treatment with estradiol promotes the growth of neurites, even in the adult brain (2). Shorter neurite outgrowth may indicate poor neuronal plasticity and conduction, increasing the risk for a number of neurological problems. These findings suggest a neuroprotective effect of estradiol. In the present study, we investigated the effects of estradiol treatment on the growth of olfactory receptor neurons in order to better understand the role of estrogen in the brain. The results of our study showed that treatment of wild type olfactory epithelial cultures with estradiol (100 nM) produced olfactory receptor neurons with significantly longer neurites compared to water-treated cultures. These findings are consistent with our hypothesis that estradiol has an important role in the regeneration and growth of neurons; however, the exact mechanism facilitating the neurite outgrowth-promoting effect of estradiol is yet to be determined. After blocking estrogen receptors, a previous study found that estradiol did not promote neurite outgrowth, suggesting that the estradiol-induced neurite outgrowth is mediated by estrogen receptors (26).

Effect of estradiol on the cellular localization and expression of estrogen receptor α (ERα) and estrogen receptor β (ERβ) in OEC derived from WT mice:

As an initial step toward understanding how estradiol promotes neurite outgrowth, we needed to determine whether ERα and ERβ are expressed in olfactory receptor neurons. Consistent with our hypothesis, the results of our study showed that both ERα and ERβ are expressed in olfactory receptor neurons. We then determined whether treatment with estradiol affects the expression and cellular localization of ERα and ERβ. Our findings show that ERα is localized in the cell body of olfactory receptor neurons,

and treatment with estradiol increases ERα expression in the cell body as well as in the neurites. Treatment with estradiol in this study increased the expression of ERα in neurites three-fold higher than in water-treated neurons. ERβ was found only in the nucleus of olfactory receptor neurons, but treatment with estradiol did increase its expression, suggesting estradiol may act through ERβ to produce effects other than neurite outgrowth. A previous study found that estradiol treatment resulted in increased levels of ERβ mRNA but not ERα mRNA, and increased levels of both ERα and ERβ protein levels, suggesting that the increased expression of ERα may be due to increased mRNA translation (32). These results suggest that ERα may be important in the neurite outgrowth-promoting effect of estradiol since treatment with estradiol increased the expression of ERα in the neurites.

To better understand how estradiol affects neurite outgrowth through ERα, we performed a time course study in which olfactory epithelial cultures were treated with estradiol for 2, 24, 48, and 72 hours, and observed immunoreactivity for ERα. Our results indicate that ERα expression was increased in the cell body and neurites of olfactory receptor neurons after 2 hours of estradiol treatment, and ERα expression was observed in the nucleus of ORN after 24 hours of estradiol treatment. After 48 hours of estradiol treatment, ERα expression was not observed in the nucleus and after 72 hours of estradiol treatment ERα expression was decreased in the neurites as well. However, ERα expression in estradiol-treated ORN at any of the tested times was higher than that of water treated ORN. These results suggest that treatment with estradiol increases ERα expression in ORN within 2 hours of treatment compared to water-treated ORN. Within 24 hours of estradiol treatment, ERα expression increases in the neurites and localizes

into the nucleus of ORN, which may suggest the activation of genomic pathways that influence neurite outgrowth. After 48 hours, however, ERα moves out of the nucleus, and after 72 hours, its expression in the neurites is decreased. This may be caused by downregulation of ERα expression due to prolonged estradiol treatment. These results are consistent with a previous study that showed estradiol treatment for 24-48 hours reduced ERα levels, suggesting downregulation (36).

Identification of the ER receptor subtype that mediates the neurite outgrowth promoting effect of estradiol using selective estrogen receptor modulators (SERMs) and estrogen receptor knockout (ERKO) mice:

Because estradiol treatment clearly has a differential effect on ERα and ERβ, it was important to determine by which receptor subtype estradiol increases neurite outgrowth. Compounds that have been synthesized to selectively activate either ERα or ERβ were used to determine by which receptor subtype estradiol exerts the observed effects. The selective estrogen receptor modulators (SERMs) DPN and PPT were used. PPT exhibits a 400-fold higher affinity for ERα than ERβ, and DPN exhibits a 100-fold higher affinity for ERβ than ERα (25). DPN, an ERβ agonist, did not have an effect on the neurite outgrowth of olfactory receptor neurons, but treatment with PPT, an ERα agonist, produced neurons with significantly longer neurites compared to vehicle-treated cultures. These results suggest that neurite outgrowth is promoted by estradiol through ERα. PPT treatment has been shown to upregulate a recently identified gene, seladin-1, that exerts neuroprotective effects and is downregulated in AD-vulnerable brain regions (6). DPN did not produce this increase in seldain-1, suggesting that the expression of this

neuroprotective gene is mediated by ERα, and the binding of ERα could be important in preventing or treating AD.

Olfactory epithelial cultures derived from ERα and ERβ knockout mice were also used to confirm the receptor by which estradiol increases neurite outgrowth. Estradiol increased neurite outgrowth in ERβ knockout cultures but not in ERα knockout cultures, verifying that ERα is the receptor that facilitates the neurite outgrowth-promoting effects of estradiol. In a study exposing cells from human fetal olfactory epithelium to β-amyloid, decreased cell viability was observed, but treatment with estradiol increased their resistance to β-amyloid toxicity (6). The accumulation of β-amyloid plaques is one of the trademark indications of Alzheimer's disease, and these findings suggest a neuroprotective effect of estradiol with implications in AD. This neuroprotective effect of estradiol has been demonstrated in the brain of ovariectomized ERβ knockout mice, but not in ERα knockout mice, which is consistent with our findings that ERα has an important role in neuroprotection (6).

Possible mechanisms of the neurite outgrowth-promoting effect of estradiol:

Because the results of our ERKO and SERM experiments suggest that estradiol promotes neurite outgrowth through the ERα receptor, and estradiol treatment increased ERα expression in the neurites, we propose that ERα is the receptor by which estradiol increases neurite outgrowth. The mechanism by which this occurs is not clearly understood, but estradiol may activate transcription factors that increase ERα in neurites and promote neurite outgrowth. Proposed mechanisms by which estradiol and SERMs exert their effects on the brain include a genomic nuclear estrogen receptor-mediated

mechanism and a nongenomic mechanism involving membrane-associated estrogen receptors. The classic genomic estrogen activation pathway involves the ligand-activated dimerization of nuclear ERα and ERβ that bind to DNA to modulate gene transcription. The activation of these transcription factors could happen within 24 hours of estradiol treatment, as we observed ERα in the nucleus at that time. Studies have shown that 24 hours of estradiol treatment is required to observe neuroprotective effects against brain injury, suggesting a genomic mechanism (19). The mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3 kinase (PI3-K)/Akt pathways have been shown to be important signaling pathways associated with estradiol in the brain, and their rapid activation after estradiol treatment suggests a non-genomic mechanism (13, 17, 19, 20). Estradiol has also been shown to promote the release of intracellular Ca^{2+} within seconds to minutes after treatment, which cannot be attributed to genomic mechanisms (13). It has been suggested that estradiol may regulate gene expression using both genomic and non-genomic mechanisms, and the non-genomic pathways estradiol may activate could produce transcription factors that are involved in the genomic interactions of estrogen receptors in the nucleus (9). Therefore, it is difficult to conclude that the neurite outgrowth-promoting effects of estradiol can be attributed to any single mechanism, but most likely involve a complex relationship between genomic and non-genomic pathways.

A novel plasma membrane-associated estrogen receptor, ER-X, has recently been identified as unique and functionally distinct from ERα and ERβ (10). Using western blotting and immunoprecipitation, this study found an unidentified protein that was reactive with ERα antibodies but had a different molecular weight than ERα (10). This unidentified protein, termed ER-X, was also found in the membrane and cytoplasm of

neurons from ERα knockout mice, but not in the nucleus (10). The expression of ER-X was found to be at a maximum 7-10 days postnatal and was expressed very little in adults (10). However, following brain injury, ER-X and ERα were upregulated to levels comparable to those present during development (10). These findings complicate the traditional ERα and ERβ system, as membrane ERs previously identified as ERα may actually be ER-X. Future studies involving the effects of estradiol on neurite outgrowth should take into account the possibility of ER-X.

In the present study, estradiol did not increase neurite outgrowth in ERβ knockout cultures, and DPN did not increase neurite outgrowth in wild type cultures. However, since estradiol treatment did increase the expression of ERβ in the nucleus of olfactory receptor neurons but not in the neurites, ERβ may have a function other than the promotion of neurite outgrowth. A previous study has shown that estradiol treatment in ovariectomized wild type mice improved their performance in tasks mediated by the cortex and hippocampus, but not in ERβ knockout mice (24). The ERβ agonist DPN produced the same results, suggesting that ERβ may be important to enhance cognitive performance. Another study showed that ERβ was responsible for estradiol-mediated neuroprotection against NMDA toxicity, which has been proposed to lead to decreased synaptic plasticity in Alzheimer's disease (32). Future studies examining the role of estradiol in neuroprotection and treatment of neurodegenerative diseases should investigate the role of ERβ in areas such as cognition.

CONCLUSIONS

Estrogens are steroid hormones known to regulate reproduction and sexual differentiation, but there is evidence that the actions of estrogen are not restricted to areas of the brain related to reproduction, namely the hypothalamus (17, 18). The results of the present study, along with previous studies, suggest an important role of estrogen in neuronal repair and regeneration, as well as cognitive performance. This study demonstrated that estradiol promotes the neurite outgrowth of olfactory receptor neurons through ERα, and agonists of ERα produce a comparable effect. Increasing or maintaining neurite outgrowth has implications in the treatment or prevention neurodegenerative diseases such as AD. Neurons with shorter neurites may indicate poor neuronal plasticity and conduction, leading to a disruption of the normal communication of neurons. To maintain a healthy nervous system, a normal balance between neuronal injury and repair is necessary. With the progression of age, neurons are more susceptible to damage by neurotoxins, requiring efficient repair mechanisms. Although a definite causative relationship has not been established between decreased estrogen levels and the increased incidence of Alzheimer's disease, stroke, mood changes, and cognitive dysfunction in post-menopausal women, the correlation between estrogen and brain functioning is apparent (19).

Although hormone replacement therapy with estradiol has been shown to have positive effects on the symptoms of menopause and may also have neuroprotective effects in the brain, estradiol can stimulate breast and uterine tissue, and estrogen replacement therapy has been associated with an increased risk of developing breast and uterine cancer (19). Progestins are commonly included with estrogens in hormone

therapy because estrogen alone increases the risk of uterine cancer, but even progestin/estrogen therapy still comes with an increased risk of these cancers (31). Selective estrogen receptor modulators such as tamoxifen and raloxifene were developed to have antagonistic estrogenic effects in breast and uterine tissue and agonistic estrogenic effects in the brain, bone, and cardiovascular system (19). These SERMs have also been shown to have neuroprotective and neurotrophic effects in the brain (19). The development of SERMs that demonstrate the neuroprotective effects of estradiol without the increased risk of breast and uterine cancer have great potential in the treatment of AD. The present study showed that the activation of ERα is important in the growth of neurons, and a compound that activates ERα may be beneficial in treating or preventing neurodegeneration. Estrogens and estrogen receptors clearly have an important role in the maintenance of a healthy neural environment in the brain, and understanding the mechanisms by which estrogen exerts its effects on the brain is crucial to developing effective treatments for neurological diseases.

REFERENCES

(1) Mitra, S. W. (2003). Immunolocalization of Estrogen Receptor beta in the Mouse Brain: Comparison with Estrogen Receptor alpha. *Endocrinology*, *144*(5), 2055-2067. doi: 10.1210/en.2002-221069.

(2) Toran-Allerand, C. D., Singh, M., & Sétáló, G. (1999). Novel Mechanisms of Estrogen Action in the Brain: New Players in an Old Story. *Frontiers in Neuroendocrinology*, 20(2), 97-121. doi: 10.1006/frne.1999.0177.

(3) Hill, R. A., & Boon, W. C. (2010). Transgenic and Mutant Tools to Model Brain Disorders. (A. V. Kalueff & C. L. Bergner, Eds.)*Brain,* 44(6). doi: 10.1007/978-1-60761-474-6.

(4) Morrison, J. H., Brinton, R. D., Schmidt, P. J., & Gore, A. C. (2006). Estrogen, Menopause, And The Aging Brain: How Basic Neuroscience Can Inform Hormone Therapy In Women. *The Journal of Neuroscience*, 26(41), 10332-48. doi: 10.1523/JNEUROSCI.3369-06.2006.

(5) Garcia-Ovejero, D., Azcoitia, I., Doncarlos, L. L., Melcangi, R. C., & Garcia-Segura, L. M. (2005). Glia-Neuron Crosstalk In The Neuroprotective Mechanisms Of Sex Steroid Hormones. *Brain Research,* 48(2), 273-86. doi: 10.1016/j.brainresrev.2004.12.018.

(6) Benvenuti, S., Luciani, P., Vannelli, G. B., Gelmini, S., Franceschi, E., Serio, M., et al. (2005). Estrogen And Selective Estrogen Receptor Modulators Exert Neuroprotective Effects And Stimulate The Expression Of Selective Alzheimer's Disease Indicator-1, A Recently Discovered Antiapoptotic Gene, In Human Neuroblast Long-Term Cell Cultures. *The Journal of Clinical Endocrinology and Metabolism*, 90(3), 1775-82. doi: 10.1210/jc.2004-0066.

(7) Caruso, S., Grillo, C., Agnello, C., Di Mari, L., Farina, M., & Serra, A. (2004). Olfactometric And Rhinomanometric Outcomes In Post-Menopausal Women Treated With Hormone Therapy: A Prospective Study. *Human Reproduction*, 19(12), 2959-64. doi: 10.1093/humrep/deh465.

(8) Gillies, G. E., & Mcarthur, S. (2010). Estrogen Actions in the Brain and the Basis for Differential Action in Men and Women : A Case for Sex-Specific Medicines. *Pharmacological Reviews*, 62(2), 155-198. doi: 10.1124/pr.109.002071.155.

(9) Björnström, L., & Sjöberg, M. (2005). Mechanisms Of Estrogen Receptor Signaling: Convergence Of Genomic And Nongenomic Actions On Target Genes. *Molecular Endocrinology*, 19(4), 833-42. doi: 10.1210/me.2004-0486.

(10) Toran-Allerand, C. D., Guan, X., MacLusky, N. J., Horvath, T. L., Diano, S., Singh, M., et al. (2002). ER-X: A Novel, Plasma Membrane-Associated, Putative Estrogen Receptor That Is Regulated During Development And After Ischemic

Brain Injury. *The Journal Of Neuroscience*, 22(19), 8391-401. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12351713.

(11) Toran-Allerand, C. D. (2005). Estrogen and The Brain: Beyond ER-Alpha, ER-Beta, and 17beta-Estradiol. *Annals of the New York Academy of Sciences*, 1052, 136-44. doi: 10.1196/annals.1347.009.

(12) Woolley, C. S. (2007). Acute Effects Of Estrogen On Neuronal Physiology. *Annual review of pharmacology and toxicology*, 47, 657-80. doi: 10.1146/annurev.pharmtox.47.120505.105219.

(13) Nilsson, S., & Gustafsson, J.-Å. (2011). Estrogen Receptors: Therapies Targeted To Receptor Subtypes. *Clinical Pharmacology And Therapeutics*, 89(1), 44-55. doi: 10.1038/clpt.2010.226.

(14) MacDonald, K. P., Murrell, W. G., Bartlett, P. F., Bushell, G. R., & Mackay-Sim, a. (1996). FGF2 Promotes Neuronal Differentiation In Explant Cultures Of Adult And Embryonic Mouse Olfactory Epithelium. *Journal Of Neuroscience Research*, 44(1), 27-39. doi: 10.1002/(SICI)1097-4547(19960401)44:1<27::AID-JNR4>3.0.CO;2-K.

(15) Brewer, G J, Torricelli, J R, Evege, E K, et al. (1993). Optimized Survival Of Hippocampal Neurons In B27-Supplemented Neurobasal, A New Serum-Free Medium Combination. *Journal Of Neuroscience Research*, 35(5), 567-76.

(16) Dhong, H. J., Chung, S. K., & Doty, R. L. (1999). Estrogen Protects Against 3-Methylindole-Induced Olfactory Loss. *Brain Research*, 824(2), 312-5.

(17) Morissette, M., Le Saux, M., D□Astous, M., Jourdain, S., Al Sweidi, S., Morin, N., et al. (2008). Contribution Of Estrogen Receptors Alpha And Beta To The Effects Of Estradiol In The Brain. *The Journal Of Steroid Biochemistry And Molecular Biology*, 108(3-5), 327-38. doi: 10.1016/j.jsbmb.2007.09.011.

(18) McEwen, B. (2002). Estrogen Actions Throughout The Brain. *Recent Progress In Hormone Research*, 57, 357-84.

(19) Dhandapani, K. M. (2002). Protective Effects of Estrogen and Selective Estrogen Receptor Modulators in the Brain. *Biology of Reproduction*, 67(5), 1379-1385. doi: 10.1095/biolreprod.102.003848.

(20) Kuroki, Y., Fukushima, K., Kanda, Y., Mizuno, K., & Watanabe, Y. (2001). Neuroprotection By Estrogen Via Extracellular Signal-Regulated Kinase Against Quinolinic Acid-Induced Cell Death In The Rat Hippocampus. *The European*

Journal Of Neuroscience, 13(3), 472-6. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11168553.

(21) Mérot, Y., Ferrière, F., Gailhouste, L., Huet, G., Percevault, F., Saligaut, C., et al. (2009). Different Outcomes Of Unliganded And Liganded Estrogen Receptor-Alpha On Neurite Outgrowth In PC12 Cells. *Endocrinology*, 150(1), 200-11. doi: 10.1210/en.2008-0449.

(22) Gonzales, F., Farbman, a I., & Gesteland, R. C. (1985). Cell And Explant Culture Of Olfactory Chemoreceptor Cells. *Journal Of Neuroscience Methods*, 14(2), 77-90. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/3897728.

(23) Chen, X., Fang, H., & Schwob, J. E. (2004). Multipotency Of Purified, Transplanted Globose Basal Cells In Olfactory Epithelium. *The Journal Of Comparative Neurology*, 469(4), 457-74. doi: 10.1002/cne.11031.

(24) Walf, A. a, Koonce, C. J., & Frye, C. a. (2008). Estradiol Or Diarylpropionitrile Administration To Wild Type, But Not Estrogen Receptor Beta Knockout, Mice Enhances Performance In The Object Recognition And Object Placement Tasks. *Neurobiology Of Learning And Memory*, 89(4), 513-21. doi: 10.1016/j.nlm.2008.01.008.

(25) Morissette, M., Le Saux, M., D□Astous, M., Jourdain, S., Al Sweidi, S., Morin, N., et al. (2008). Contribution Of Estrogen Receptors Alpha And Beta To The Effects Of Estradiol In The Brain. *The Journal Of Steroid Biochemistry And Molecular Biology*, 108(3-5), 327-38. doi: 10.1016/j.jsbmb.2007.09.011.

(26) Schassen, C. von, Fester, L., Prange-Kiel, J., Lohse, C., Huber, C., Böttner, M., et al. (2006). Oestrogen Synthesis In The Hippocampus: Role In Axon Outgrowth. *Journal Of Neuroendocrinology*, 18(11), 847-56. doi: 10.1111/j.1365-2826.2006.01484.x.

(27) Estrada, M., Varshney, A., & Ehrlich, B. E. (2006). Elevated Testosterone Induces Apoptosis In Neuronal Cells. *The Journal Of Biological Chemistry*, 281(35), 25492-501. doi: 10.1074/jbc.M603193200.

(28) Estrada, M., Uhlen, P., & Ehrlich, B. E. (2006). Ca2+ Oscillations Induced By Testosterone Enhance Neurite Outgrowth. *Journal Of Cell Science*, 119(Pt 4), 733-43. doi: 10.1242/jcs.02775.

(29) Ko, M., Zou, K., Minagawa, H., Yu, W., Gong, J.-S., Yanagisawa, K., et al. (2005). Cholesterol-Mediated Neurite Outgrowth Is Differently Regulated Between Cortical And Hippocampal Neurons. *The Journal Of Biological Chemistry*, 280(52), 42759-65. doi: 10.1074/jbc.M509164200.

(30) Fester, L., Zhou, L., Bütow, A., Huber, C., Lossow, R. von, Prange-Kiel, J., et al. (2009). Cholesterol-Promoted Synaptogenesis Requires The Conversion Of Cholesterol To Estradiol In The Hippocampus. *Hippocampus*, 19(8), 692-705. doi: 10.1002/hipo.20548.

(31) Wong, A. M., Rozovsky, I., Arimoto, J. M., Du, Y., Wei, M., Morgan, T. E., et al. (2009). Progesterone Influence On Neurite Outgrowth Involves Microglia. *Endocrinology*, 150(1), 324-32. doi: 10.1210/en.2008-0988.

(32) Aguirre, C., Jayaraman, A., Pike, C., & Baudry, M. (2010). Progesterone Inhibits Estrogen-Mediated Neuroprotection Against Excitotoxicity By Down-Regulating Estrogen Receptor-B. *Journal Of Neurochemistry*, 115(5), 1277-87. doi: 10.1111/j.1471-4159.2010.07038.x.

(33) Paganini Hill, A, & Henderson, V W. (1994). Estrogen Deficiency And Risk Of Alzheimer's Disease In Women. *American Journal Of Epidemiology*, 140(3), 256-61.

(34) Stern, Y., Sano, M., & Mayeux, R. (1998). Postmenopausal Estrogen Use And Parkinson's Disease With And Without Dementia. *Neurology*, 1141-1143.

(35) Saunders Pullman, R, Gordon Elliott, J, Parides, M, et al. (1999). The Effect Of Estrogen Replacement On Early Parkinson's Disease. *Neurology*, 52(7), 1417-21.

(36) Bondar, G., Kuo, J., Hamid, N., & Micevych, P. (2009). Estradiol-induced estrogen receptor-alpha trafficking. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, *29*(48), 15323-30. doi: 10.1523/JNEUROSCI.2107-09.2009.