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Beneficial Effects Of Estradiol Are Mediated Through Apoe

Aseem Hussain
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BENEFICIAL EFFECTS OF ESTRADIOL ARE MEDIATED THROUGH apoE

HUSSAIN

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Beneficial effects of estradiol are mediated through apoE

(TITLE)

BY

Aseem Hussain

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DEDICATION

I would like to dedicate this dissertation to the Almighty for giving me all the opportunities, for his countless blessings, for giving me the strength and helping me through everything.

To my father, (late) Mohammed Hussain, for his unconditional love and my mother, Bashirunnisa Hussain for instilling the love for education in me and hope I have made both of you proud. Thank you both for making me the person I am today.

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ABSTRACT

Estradiol is the primary premenopausal hormone circulating in women. It is a powerful hormone in the female body since it sends signals to the brain, regulates ovulation and menstrual cycle and acts on the nervous system, heart, breast and many other tissues in the body. However all estradiol production is stopped after menopause. Post menopausal women can develop a host of neurodegenerative diseases such as Alzheimer's disease. If the onset of progressive dementia is after menopause during loss of estradiol, it seems logical to assume that the lack of estradiol should have a big role to play in the pathogenesis of Alzheimer's dementia.

Many epidemiological studies suggest that estrogen replacement therapy (ERT) decreased morbidity from different chronic neurological disorders. ApoE alleles have been proved to modify the risk for progression of the same diseases. Literature review suggests a close relationship between estradiol and apoE in the central nervous system. ApoE levels in mouse brain vary during the estrous cycle and increase in the presence of estradiol. Research has proved that ERT exerts neuroprotective effect by increasing resistance against β amyloid induced toxicity. We propose that these neuroprotective effects of estradiol are mediated by apoE.

I predicted that estradiol would increase neurite outgrowth in olfactory receptor neurons, and that this can be supported by increased levels of apoE in culture in the presence of

estradiol. Estradiol would also have differential effect on the neurite outgrowth of receptor neurons in the presence of different isoforms of apoE.

This hypothesis was tested on olfactory epithelial explant culture. 17β estradiol was added at 100 Pico Molar (pM) concentration every 2 days *in vitro* (*DIV*). Neurite outgrowth was measured using a stage micrometer and apoE levels were documented by immunocytochemistry. It was observed that the neurite outgrowth of olfactory receptor neurons derived from apoE producing transgenic Wild Type (WT) mice was significantly longer when compared to neurite outgrowth of ORN derived from apoE deficient Knockout (KO) mice. This may be due to the presence of apoE in the WT culture. To further test this hypothesis apoE3 was added to the apoE3 deficient KO cultures which restored the neurite outgrowth similar to the neurite outgrowth seen in WT ORN. Results also showed that estradiol increased apoE secretion in the olfactory explant epithelial culture derived from WT mice in a time dependant manner. Estradiol had neurite outgrowth promoting effect on WT ORN. Estradiol had no effect on neurite outgrowth of ORN derived from apoE deficient KO mice. The results indicate poor neurite outgrowth is due to the absence of apoE.

Estradiol had isoform specific effects on neurite outgrowth in the presence of purified recombinant human apoE in KO ORN. In the presence of apoE3 and apoE2, estradiol significantly increased neurite outgrowth, while it had no significant effect in the presence of apoE4. This supports the idea that beneficial effects of estradiol on neurite outgrowth are isoform specific. Estradiol also had no neuroprotective beneficial effects

on KO ORN cultures which inherently lack apoE. This supports the idea of interrelationship between estradiol and apoE, thus proving that all the neuroprotective effects of estradiol are mediated by apoE. The isoform specific effects of estradiol suggest that effects of ERT on chronic neurological diseases vary with apoE genotype. This can explain some of the contradictory findings in clinical studies in which no improvement was found in incidence of Alzheimer's dementia in post menopausal women treated with estrogen replacement therapy. Thus for ERT to be efficient in preventing dementia, identifying the apoE genotype and careful selection of the subjects might be needed.

1. INTRODUCTION

Apolipoprotein E (ApoE), a 34-kDa protein plays a fundamental role in the transport of lipids and in the maintenance and repair of neurons (3). It is the major apolipoprotein, in the brain and cerebrospinal fluid (CSF), where it is secreted by both astrocytes and microglia (4). ApoE is a polymorphic protein 299 amino acids long. It exists in three major isoforms apoE2, apoE3 and apoE4 that are produced by different alleles on chromosome 19. Three apoE alleles differ from each other only at two positions. ApoE3 which is the most common isoform (occurs in about 60% - 70% of the population) has cysteine and arginine at positions 112 and 158 respectively. ApoE4, the next most common isoform (occurs in 15% - 20% of the population) has arginine at both positions. ApoE2 the least common isoform (occurs in 5% - 10% of the population) has cysteines at both the positions (6). ApoE has two structural domains, the amino-terminal domain that facilitates binding with the receptor and the carboxyl-terminal domain used to bind with the lipids (8). ApoE acts through lipoprotein receptors on cell surface (7). Brain cells express six major receptors for apoE-containing lipoproteins: the low density lipoprotein (LDL) receptor, the LDL receptor-related protein (LRP), the very low density lipoprotein (VLDL) receptor, the glycoprotein (gp) 330, the LR 11 receptor, and the apoE receptor 2 (APOER2) (55,56). Binding of apoE to the receptor results in the uptake (apoE + Lipids)

and degradation of the lipoprotein by endocytosis, cholesterol bound to lipoprotein is metabolized, making lipid available for use in the regulation of intracellular cholesterol metabolism. (6). Mouse apoE, the species used in this study, is similar to human apoE3 in its structural and functional properties, including receptor binding and lipoprotein preferences (41).

ApoE is an indispensable player in cholesterol homeostasis and maintenance of normal central nervous system (CNS). Significance of ApoE: i) It is the major apolipoprotein in the brain and CSF where it is secreted by both astrocytes and microglia (4). ii) ApoE mRNA has been localized in astrocytes and few microglia (36). iii) Recent studies from our laboratory have proved that neuronal regeneration in mice that are deficient for the apoE gene (KO) is slow when compared to neuronal regeneration in wild-type mice (WT), proving that apoE facilitates neuronal regeneration and plasticity (35). iv) ApoE levels increase dramatically at the site of injury following nerve damage (34) v) ApoE exerts beneficial effects on a different variety of cultured neurons. ApoE3 treatment increases neurite outgrowth while treatment with apoE4 decreased neurite outgrowth in cultured adult mouse cortical neurons (30).

Alzheimer's disease (AD) is an irreversible, progressive brain disorder that occurs gradually and results in memory loss, unusual behavior, personality changes, and a decline in thinking ability. AD is the major form of dementia and is characterized by

senile plaques and neurofibrillary tangles in the brain and loss of cholinergic neurons in the basal forebrain.

Numerous studies have shown that apoE4 is the major risk factor for Alzheimer's disease (AD) (54). ApoE4 allele is a dose-dependent risk factor for AD. Moreover, AD patients with apoE4 usually showed an earlier age of onset and a more rapid progression of the disease. A similar increase in risk and earlier age of onset of dementia is observed in apoE4 carriers in Parkinson's disease (PD). In addition, risk of developing dementia increases in apoE4 individuals following a head trauma. Despite intense research in the last few years to unravel the association of apoE genotype with these diseases, the mechanism whereby apoE4 contributes to the poorer prognosis of these diseases is not known.

Previous studies have also shown that AD patients with apoE4 allele have elevated neuronal degeneration and impaired neuronal remodeling than those lacking the apoE4 allele. Studies have demonstrated that in individuals with one copy of the apoE4 allele a four fold increase in the incidence of AD is seen, when compared to individuals with two copies of apoE4 showing 15 fold increase in the incidence of AD. 40% to as many as 80% of AD patients have at least one apoE4 allele, thus it is strongly linked to AD pathology. They also show a significantly early onset of AD when compared to the individuals with apoE2 or apoE3 alleles (18). Results from transgenic mice expressing

human apoE isoforms are consistent with human studies. One possible mechanism whereby apoE4 may lead to the pathogenesis of these diseases is by compromising neuronal plasticity. Inheritance of apoE2 allele proves to be protective against dementia (13) and inheritance of apoE2 and apoE3 are associated with decreased risk of AD, (3) supporting the idea of apoE3 being beneficial and apoE4, detrimental. (11). However the specific mechanism by which apoE4 leads to the pathogenesis of AD is not yet known.

Along with the apoE genotype, estradiol plays an important protective role in the central nervous system. Estradiol is the primary premenopausal sex hormone secreted by ovaries. It plays a major role in regulating ovulation and menstrual cycle. Significance of estradiol: i) estradiol controls the brain, heart, breast and many other tissues in the body. ii) Studies have proved that it regulates expression of genes related to cell proliferation, growth and death. (19) iii) estradiol exerts neuroprotective and trophic effects in the adult brain (15, 22, and 23). iv) It has been shown to regulate and interact with neurotrophins and their receptors (16, 23). v) estradiol also seems to protect the brain against neurodegeneration caused by β-amyloid toxicity through both receptor and nonreceptor mediated mechanisms (15, 22). vi) estradiol is known to affect learning and memory, behavior and cognition (21).

Alzheimer's disease is most common in postmenopausal women, and decreased estradiol levels after menopause are a risk factor for the disease (24). Over the past few decades,

life span of women has increased, but the age of the menopause has remained fixed. Therefore women are living substantial amount of their lives in hypoestrogenic and postmenopausal state leading to increased risk of host of neurodegenerative diseases (23).

The most common form of estradiol found in human body is 17β–estradiol. When used in experimental studies 17β–estradiol has been proven to increase apoE levels in both *vivo* and *in vitro*. Estradiol increases apoE levels in a time and dose-dependant fashion *in vitro* (29). In mixed glial cultures estradiol significantly increases apoE levels, and this increase was associated with a concomitant increase in markers for astrocytes (51). Estradiol treatment had no effect on apoE mRNA levels in monotypic cultures of either astrocytes or microglia. However, in mixed glial cultures estradiol treatment increased both apoE mRNA and protein, suggesting that interaction of various cell types in a culture are important to elicit estradiol's upregulation of apoE. Many animal studies have shown that estradiol regulates apoE expression. ApoE levels in the CNS tend to vary during the estrous cycle in mice (31). These studies have been supported by other studies suggesting that estradiol exerts its beneficial effects via apoE- dependant mechanism (32). Our laboratory was the first one to show that apoE level was at its peak, in several brain regions, when estradiol levels were highest during the estrous cycle (29). Other studies have also shown that exogenous estradiol also increases regional and whole-brain levels of mRNA message for apoE (43, 44). In contrast, a previous study reported that estradiol increased apoE protein, although the total apoE mRNA message did not change

(52). A discrepancy between message and protein has been previously noted (53).

Recent studies have found that estradiol treatment may decrease the risk or delay the onset of AD in postmenopausal women. Epidemiological studies suggest that a history of ERT reduces the likelihood of developing late-life dementia (24, 25). ERT also has been proved to delay either the onset or progression of Parkinson disease (26, 27). However, prospective studies with estradiol neither slowed the progress of dementia nor protected against cognitive decline in the Women's Health Initiative Memory study (33). Resolving these conflicting studies of ERT clearly requires clarification of how estradiol affects the nervous system. These findings suggest isoform specific effects of apoE on neurogenesis and neuronal differentiation. This can be attributed to the fact that beneficial effects of estradiol are mediated via apolipoprotein and different isoforms of apoE differ in their efficacy in promoting neurotropic effects. A previous study showed that ERT reduced cognitive decline in women other than carriers of apoE4 allele (37). These isoform specific effects of estradiol suggest that for optimal efficacy of ERT on chronic neurological diseases selection of the subjects might be needed. How ever the exact mechanism still remains vague. Pathway describing relationship between estradiol and apoE hold a lot of value in treatment of AD.

Interestingly, apoE allele status is associated with olfactory dysfunction in Alzheimer's disease. Pioneering studies performed by Dr. Claire Murphy and collaborators have clearly

shown that apoE4 individuals have a significant decline in odor threshold and odor identification, and have delays in processing of olfactory information (54). The etiology of this delay is open to question; however, it is likely that the lack of apoE in the OE greatly diminished neuronal differentiation resulting in a delay in nerve regeneration. In this project we explored this possibility by examining the effects of apoE isoforms on neuronal differentiation in OEE culture.

Some of the *in vitro* studies have shown that apoE3 increased and apoE4 decreased neurite outgrowth in adult mouse cortical culture (14) The mechanism underlying these isoform specific effects of apoE on olfactory function is not clear. However, it is tempting to suggest that normal repair and axon regeneration may underlie these deficits. Together, these data suggest a tremendous role for estradiol in neurological health, which is modulated by apoE genotype. The Olfactory epithelium is one of those few rare tissues in an adult animal, where neurogenesis and neuronal differentiation is known to persist, and therefore serves as a useful model to investigate molecules that regulate these processes. Olfactory receptor neurons (ORN) in an adult OE continuously regenerate from basal cells throughout the life of the organism to support the ongoing turnover of receptor cells in this system.

Present study examined neuronal growth, proliferation and regeneration of olfactory receptor neurons in the olfactory epithelial culture derived from WT and KO mice.

Effects of estradiol and different isoforms of apoE on the olfactory receptor neurons were studied to better understand the mechanism involved in beneficial effects of estradiol and possible implications to AD.

The olfactory receptor neurons in the epithelium regenerate from the stem cells continuously through out the life of an organism. This incredible ability of the ORN to regenerate can be used as a model for studying the mechanism of neuronal growth, plasticity and interactions between proteins involved in this process. Olfactory epithelium derived from the KO mice can be used to test efficacy of different isoforms of apoE in modulating neurite growth thus making the olfactory epithelium a perfect model for the present study.

1.1 GOAL AND HYPOTHESIS

1. **Characterization of the olfactory epithelial culture:** Because my project dealt with olfactory epithelial explant culture, I characterized the epithelial culture with respect to cell population and cell number.

2. **Effect of estradiol on apoE levels in wildtype olfactory epithelial culture:** Since estradiol increases apoE levels in vivo and in vitro, I hypothesized that it might increase apoE levels in wildtype olfactory epithelial explant culture.

3. **Effect of estradiol on neurite outgrowth from olfactory receptor neurons (ORN) in olfactory culture from wildtype mice:** I hypothesized that estradiol would increase neurite outgrowth in wildtype ORN. Since increase in neurite outgrowth calls for enhanced supply of lipids, endogenous apolipoprotein E present in the wildtype culture would accommodate for the effect.

4. **Effect of estradiol on number of neurons in wildtype olfactory epithelial culture:** I hypothesized that estradiol would have synergistic effect on wildtype olfactory epithelial explant cultures and increase the neuronal numbers. Moreover neurogenesis can be easily accommodated due to the presence of apolipoprotein E to supply the much needed lipids.

5. **Effect of estradiol on neurite outgrowth from olfactory receptor neurons (ORN) in Olfactory Epithelial Culture (OEC) from KO mice:** I hypothesized that estradiol would not have any effect on neurite outgrowth. Due to the absence of apolipoprotein E in knockout culture, lipid supply to the neurons is restricted. In the presence of estradiol increase in neurite outgrowth would need increased lipid supply which cannot be met. Thus estradiol would not increase neurite outgrowth in knockout epithelial explant culture.

6. **Effect of estradiol on neuronal numbers in knockout olfactory epithelial culture:** I hypothesized that estradiol will not have any effect on neuronal numbers in knockout culture. Since neurogenesis needs increased lipid supply, neuronal numbers in knockout culture would not increase due to the absence of lipoprotein to meet with the need for increased lipids.

7. **Effect of different isoforms of apoE on neurite outgrowth from knockout olfactory epithelial culture:** I hypothesized that different isoforms of apoE would yield differential results since apoE2 and E3 are beneficial in promoting neurite outgrowth in a variety of cultured neurons where as apoE4 is not (3, 13, 14).

8. **Effect of different isoforms of apoE on neuronal numbers in knockout olfactory epithelial culture:** I hypothesized that treatment of KO ORN with different isoforms of apoE would have isoform-specific effects on neuronal number. Because apoE2 and apoE3 are more efficient in lipid transport when compared to apoE4, apoE2 and apoE3 would increase neuronal numbers where as apoE4 would reduce or not have significant effect at all.

9. **Effect of estradiol on neurite outgrowth of ORN from knockout olfactory epithelial culture in the presence of different isoforms of apoE:** I hypothesized that estradiol would increase neurite outgrowth from knockout ORN in the presence of apoE2 and apoE3 but not in the presence of apoE4. Since apoE isoforms differ in their efficacy for transporting lipids, apoE2 and apoE3 have been proved to promote neurite outgrowth in cortical culture. ApoE4 however has not been so beneficial (14).

10. **Effect of estradiol on neuronal numbers of knockout olfactory epithelial culture in the presence of different isoforms of apoE:** I hypothesized that estradiol would increase neuronal number in knockout olfactory epithelial culture in the presence of apoE2 and apoE3 but not in the presence of apoE4. Since knock out mice lack apoE, addition of exogenous apoE will help in the neurogenesis induced by estradiol. How ever apoE4 will not have any effect since it does not promote lipid transport as good as apoE2 and apoE3 (14).

2. MATERIALS AND METHODS

2.1 Olfactory Epithelial Explant culture:

Homozygous apoE KO mice (C57BL/6-Apoe$^{<tmiUnc>}$) bred 10 generations onto C57BL/6 background and control mice (C57BL/6) were obtained from Jackson laboratory (Bar Harbor, MA). Seven to eight post natal pups (two days old) were used for each experiment. Two hours prior to the experiment, coating solution was prepared by dissolving fibronectin (Invitrogen Grand Island, NY) in Neurobasal–A (NBA) media (Invitrogen Grand Island, NY) to make 50μg/ml fibronectin solution and 24 glass slips were coated with the fibronectin solution just enough to cover the surface of the glass slips. These glass slips were placed in the incubator for two hours so that the slips would be evenly coated with fibronectin. After the incubation the slips are rinsed with NBA and returned back to the incubator with NBA media containing B27 supplement (Invitrogen Grand Island, NY) so that the fibronectin coating on the slips does not crack. The pups are then decapitated using a sterile surgical scissors and their nasal cavity was cut open sagitally using a sterile razor blade exposing the nasal olfactory epithelium. Then the OE was dissected and placed in ice-cold 5 ml of Hanks' Balanced Salt Solution (Invitrogen Grand Island, NY), containing gentamycin (100 μg/ml) and glucose (6 mg/ml). The OE was then sliced into explants of about 200 μm thickness in NBA media containing B27 supplement (concentration: 20μl/ml) and glutamine (0.5 mM). The explants were transferred to a 24-well plate containing slips coated with fibronectin (Invitrogen Grand Island, NY). The explants were placed on the slips without media for 30 minutes in a

humidified incubator at 37 ºC and 5% CO2. Following incubation, 500 µl of growth media (Neurobasal-A medium with 5 ng/ml FGF2, and B27) was added to the each well. Medium was changed every two days. On 8 DIV, neurite outgrowth assays were performed as described below.

2.2 Measurement of Neurite Outgrowth and Neuronal number :

The test reagents and their concentration used in this study are: (1) 100 pM estradiol (dissolved in 95% ethanol; Sigma, St.Louis, and MO), (2) ethanol (control, vehicle for estradiol 1µl/100µl of media (3) human apoE isoforms (5 µg/ml; Panvera, Madison, WI), (4) RAP (5 µg/ml; a generous gift from Dr. Dudley Strickland at American Red Cross, Rockville, MD), and (5) LAC (10 ug/ml; Sigma, St.Louis, MO). Two DIV OEE cultures were treated with the test reagents either individually or in combinations. The media was replaced every two days with readdition of the test reagents. Neurite outgrowth and neuronal number measurements were made on the 8 DIV.

(1) Method of collecting data for neurite outgrowth: Olfactory epithelium was collected from postnatal 2 day old pups and olfactory epithelial explant culture was prepared as described above. Freshly prepared media was added every 48 hr. Starting from 2 DIV, reagents (estradiol, ethanol, apoE3, apoE2, apoE4, apoE2+ estradiol, apoE3+ estradiol, apoE4 + estradiol) were added with every media change. At 8 DIV, the cultures are fixed with 4% paraformaldehyde. Neurons were identified by their bipolar morphology and/or by staining with tubulin III antibody (neuronal marker) and the neurite outgrowth (length

of each neurite and combined length of all neurites) in a neuron is measured with a stage micrometer.

(2) Method of counting neuronal number: Olfactory epithelium was collected from postnatal 2 day old pups and olfactory epithelial explant culture was prepared as described above. Freshly prepared media was added every 48 hr. Starting from 2 DIV, reagents (estradiol, ethanol, apoE3, apoE2, apoE4, apoE2+ estradiol, apoE3+ estradiol, apoE4 + estradiol) were added with every media change. At 8 DIV, the cultures are fixed with 4% paraformaldehyde. Neurons were identified by their bipolar morphology and/or by staining with tubulin III antibody (neuronal marker) and the total numbers of neurons were counted per field of view.

2.3 Immunocytochemistry:

Cells from 8-day old cultures were rinsed with warm PBS (37°C) and fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. After rinsing with PBS, cells were permeabilized with 0.5% Triton for 30 minutes, and then incubated for 1 hour in room temperature in blocking solution (PBS containing 1% BSA and 10% normal serum of the species in which secondary antibody was produced). Cells were then incubated in PBS at 4°C overnight with goat anti-human apoE (1:200; Calbiochem, San Diego, CA), anti-β- tubulin III (1:200; Sigma, St.Louis, MO), goat anti-OMP (1:500; Wako, Richmond, VA.), or goat anti-GAP43 (Millipore, Danvers, MA.). Following overnight incubation, slips were rinsed with PBS and incubated in blocking solution for 30 minutes at room temperature with respective secondary antibody- FITC conjugated

donkey anti-goat (1:200; for apoE Jackson immunoresearch, west groove, PA), TRITC conjugated donkey anti-mouse in (1:200; for tubulin III, Jackson immunoresearch, west groove, PA), Cy3-conjugated donkey anti-goat (1:500; for OMP Jackson immunoresearch, west groove, PA) or Alexa-488 Donkey anti mouse (1:200, for GAP43, Invitrogen, Eugene, OR). Appropriate controls were performed in which the primary antibodies were replaced with non-immune serum. Slips were then rinsed with PBS, and mounted with anti-photo-bleach medium (Vectashield; Vector labs, Burlingame, CA) and stored in the dark. Digital images were captured using a fluorescence microscope equipped with a digital camera (Penguin Pixera, San Jose, CA), maintaining identical exposure settings for all images.

2.4 Statistical Analysis:

All experiments were repeated at least three times using different preparations of OEE cultures and reagents. The data in individual experiments were presented as the mean $\pm$ standard error, and statistical analysis (One way ANOVA, Post-hoc Bonferroni Corrected t-tests) was performed using Sysstat.

3. RESULTS

3.1. Characterization of Olfactory epithelial culture (OEC):

Olfactory epithelial culture was done by modifying the techniques previously described (2). When observed under the microscope at 8 DIV, the culture had cells with different morphologies since it is a mixed culture originating from epithelial explant. The explant culture is divided into three zones (as shown in figure 1). Epithelial zone – adjacent to the explant this zone is composed of flat cells, with epithelial morphology, almost forming a dense sheet of cells surrounding the explant. Intermediate zone – This zone has a roughly equal mixture of flat cells and bipolar cells and Neuronal zone – most of the cells in this zone have a bipolar morphology, reminiscent of the olfactory sensory neurons in vivo. Four cell types in OEC were identified by immunocytochemistry. Table 1 shows percentage of different kinds of cells found in the culture at 8DIV. Fig. 3 and Fig. 4 show images representating different kinds of immunofluorescent labeling of cell types in respective zones of the culture. In the inner halo or the flat cell zone, most of the flat irregular shaped cells were Globose Basal Cell-1 positive. Some of them were Growth Associated Protein-43 (GAP-43) positive. In the outer halo or neuronal zone, majority of the cells stained for neurofilament anti tubulin III. Among the cells stained for neurofilament in the exterior zone most of the cells also stained for anti Olfactory Marker Protein (OMP, marker for mature neurons) and some of them stained for GAP-43 (marker for immature neurons). These four immunocytochemistry markers identified an average of about 95% of the cells present in an eight-day old culture. Neurofilament positive cells and OMP positive cells displayed bipolar structure with long and short neurites. Globose Basal Cell -1 positive cells were irregular shaped. Some of the GAP-43

cells had irregular morphology and others had a bipolar structure. Fluorescent pictures of the representative morphologies of the different cell types in the OEC can be seen in Fig.3 and Fig .4. Percentages of different cell types found in the different zones in the culture are described in Fig.2 and Fig.5.

3.2 ApoE receptors in the Olfactory Epithelial Culture:

Olfactory epithelial explant culture was prepared by modifying the techniques previously described (2). The culture was fixed with 4% paraformaldehyde at 8 *DIV,* immunocytochemistry to identify apoE receptors was performed. After completing immunocytochemistry, when observed under the microscope. Both LDL (Low Density Lipoprotein receptor) and LRP (Lipoprotein Related Protein) receptors were found in the culture. Fig. 6 and Fig. 7 represent the different kinds of receptors found in the culture.

3.3 Effect of estradiol on apoE secretion from ORN derived from wild type (WT) mice:

Olfactory epithelial cultures from WT mice were incubated with 100pM estradiol as described in the methods section. Immunocytochemistry for apoE was performed and digital images were captured using a fluorescence microscope equipped with a digital camera. OEC cultures treated with 100pM estradiol secreted more apoE than the cultures grown in medium alone. Difference in the intensity of immunofluorescence in different zones of the culture is shown in Fig.8.

3.4 Effect of estradiol on apoE secretion of OEC derived from WT mice at different time points:

Effect of estradiol on apoE levels in WT olfactory epithelial culture at different time points was tested by incubating the culture with 100pM estradiol in medium for 0, 0.5, 1.0, 4.0, 16.0, 24.0, 36.0 hours. Following incubation, immunocytochemistry was performed to detect the elevated levels of apoE in the OEC. It was documented that elevation of apoE levels started after 4 hrs and reached a stable plateau after 16 hr incubation and stayed elevated until 36hrs. Fig. 9 shows images of increasing apoE levels with respect to treatment with estradiol for different time periods. These data suggest a time dependant increase in apoE secretion as a function of estradiol incubation.

3.5.1 Comparison of neurite outgrowth between Olfactory Receptor Neuron (ORN) derived from wild type olfactory epithelial culture and ORN derived from knock out olfactory epithelial culture:

Olfactory epithelial cultures from WT mice had ORN with significantly longer neurite outgrowth when compared to ORN derived from KO olfactory epithelial culture. Both the cultures were grown in medium alone for 8 *DIV* and neurite outgrowth was measured as described in methods. Respective images showing the differences in the neurite outgrowth are shown in Fig.10. Graph representing these differences can be seen in Fig.11.

3.5.2 Comparison of neuronal numbers between wild type olfactory epithelial culture and knock out olfactory epithelial culture:

Olfactory epithelial cultures from WT mice had significantly higher neuronal number when compared to cultures derived from KO olfactory epithelium. The cultures were grown in medium alone for 8 *DIV* and neuronal numbers were analyzed as described in methods section. Fig. 12 shows graph representing these differences in the neuronal numbers between WT and KO cultures.

3.6.1 Effect of estradiol on neurite outgrowth of olfactory receptor neurons (ORN) in olfactory epithelial culture (OEC) derived from WT mice:

Olfactory epithelial cultures from WT mice were treated with 100pM of estradiol starting from 2 *DIV* till 8 *DIV*. Combined neurite extension of the neurites in the culture were quantified. Estradiol concentrations were added at every 48 hrs. Olfactory epithelial culture treated with 100pM estradiol had ORN with significantly (P value>0.01) longer neurite outgrowth as compared to the control. The differences in the neurite outgrowth can be seen in Fig. 13. Quantification of neurite outgrowth is represented in graph shown in Fig.14.

3.6.2 Effect of estradiol on neuronal numbers of olfactory epithelial culture derived from WT mice:

Olfactory epithelial cultures from wild type mice were treated with 100pM estradiol starting from 2 *DIV* till 8 *DIV* and neuronal numbers in the cultures were quantified. Estradiol concentrations were added at every 48 hrs. Results demonstrated that estradiol did not have any significant effect ($P < 0.01$) on neuronal numbers of olfactory epithelial culture derived from wild type mice. Quantification of results are represented in the form of graph in Fig. 15

3.7.1. Effects of estradiol on neurite outgrowth of ORN in olfactory epithelial culture derived from apoE deficient knock out (apoE KO) mice:

To test if the effect of estradiol is apoE dependant, olfactory epithelial cultures from apoE deficient KO mice were incubated with 100pM estradiol. After 6 days of incubation the neurite outgrowth was monitored. Results demonstrated that estradiol had no significant effect on neurite outgrowth of ORN derived from apoE KO mice. Respective images can be seen in Fig. 16. Quantification of these results can be seen in the graph, Fig. 17.

3.7.2. Effect of estradiol on neuronal numbers of olfactory epithelial culture derived from apoE deficient (KO) mice:

Olfactory epithelial cultures from apoE deficient KO mice were treated with 100pM estradiol starting from 2 *DIV* till 8 *DIV* and neuronal numbers in the cultures were

quantified. Estradiol concentrations were added at every 48 hrs. Estradiol had no significant effect on neuronal numbers of olfactory epithelial cultures derived from KO mice. ($P<0.01$). These results are quantified in graph shown in Fig. 18.

3.8.1. Effects of recombinant human apoE isoforms on neurite outgrowth of ORN in olfactory epithelial culture derived from apoE deficient KO mice:

In this study we tested the effect of apoE isoforms on neurite outgrowth in apoE deficient KO ORN. Incubation of the apoE deficient KO olfactory epithelial culture with apoE3 or apoE2 significantly (Pvalue> 0.01) increased combined neurite outgrowth. In contrast addition of apoE4 did not have significant effect on neurite outgrowth (Pvalue<0.01). Fig. 19 contains phase contrast pictures representing the findings. Quantification of the results is represented as graph in Fig. 20.

3.8.2. Effects of recombinant human apoE isoforms on neuronal number in olfactory epithelial culture derived from apoE deficient KO mice:

In this study we tested the effect of apoE isoforms on neuronal number in apoE deficient KO olfactory epithelial culture. Incubation of the olfactory epithelial culture with apoE3 or apoE4 significantly (P value> 0.01) increased neuronal numbers. In contrast addition of apoE2 did not have significant effect on neuronal number (P value< 0.01). Quantification of these results is represented as a graph in Fig. 21.

3.9.1 Effect of estradiol on neurite outgrowth of ORN from OEC derived from KO mice in the presence of recombinant human apoE isoforms:

In the presence of apoE3 or apoE2, estradiol significantly increased combined neurite outgrowth (Pvalue>0.01) of ORN in the KO OEC. Estradiol had no significant effect on the neurite outgrowth of ORN in the presence of apoE4. (Pvalue <0.01). Phase contrast pictures supporting the data are shown in Fig. 22. Quantification of these results can be seen in Fig.23.

3.9.2. Effect of estradiol on neuronal number of apoE deficient KO OEC in the presence of recombinant human apoE isoforms:

In the presence of apoE3 or apoE2, estradiol significantly increased neuronal numbers in the apoE deficient KO olfactory epithelial culture. Estradiol had no significant effect on the neuronal numbers of KO OEC in the presence of apoE4.(Pvalue< 0.01).Quantification of these results can be seen in Fig. 24.

4. FIGURES

FIGURE 1

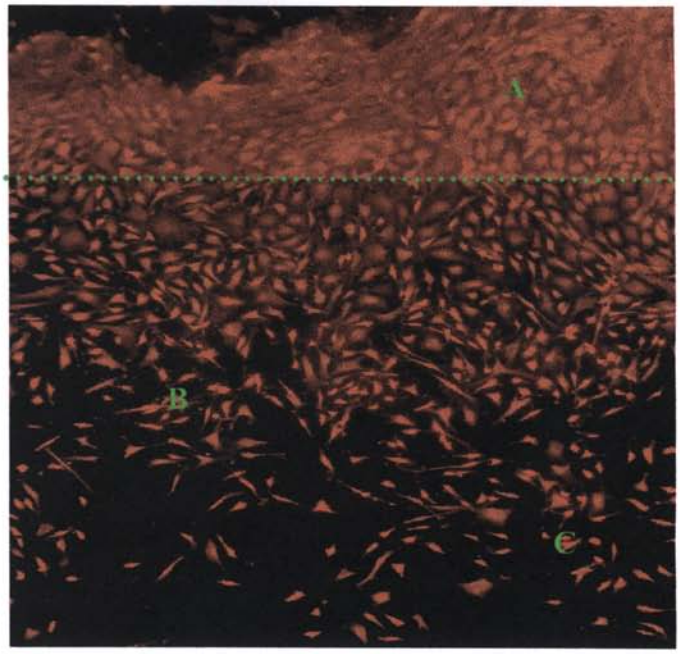


Fig. 1. Low power micrographs of the OE explant culture (OEC) on day 8 *in vitro*. This picture is a montage of two micrographs (dashed line indicates the joint) to show the characteristics of the three different zones of cells. A. Epithelial zone - This zone is composed of flat cells, with epithelial morphology, almost forming a dense sheet of cells surrounding the explant. B. Intermediate zone – This zone has a roughly equal mixture of flat cells and bipolar cells. C. Neuronal zone – Most of the cells in this zone have a bipolar morphology, reminiscent of the olfactory sensory neurons *in vivo*.

TABLE 1

Different zones	GBC-1	GAP-43	OMP	Tubulin
Epithelial zone	**80% +ve**	**16% +ve**	**3% +ve**	**20% + ve**
Neuronal zone	**10% +ve**	**13.02% + ve**	**71.08% + ve**	**85% + ve**

Table. 1. Immunofluorescent staining of cell types in the OEE cultures. A. OEE cultures were grown for 8 days, fixed and stained for GBC-1, GAP43, and OMP and anti Tubulin-III. For each antigen, three cover slips were immunostained, and immunopositve cells in 12 fields / slip were counted and expressed as a percentage of total viable cells.

FIGURE 2

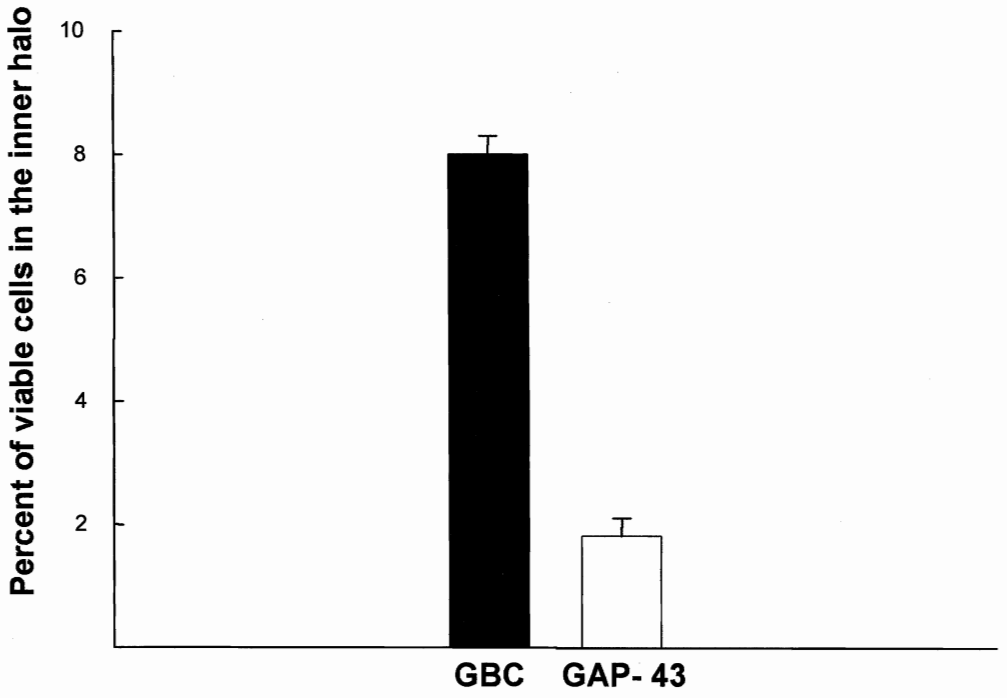
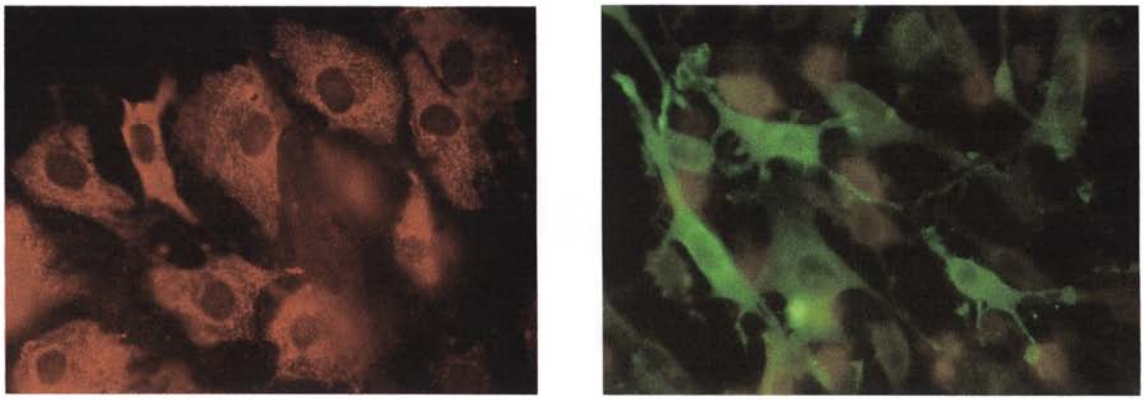


Fig. 2. Percentage of viable cells after immunofluorescent labeling of cell types in the inner halo of olfactory epithelial culture.

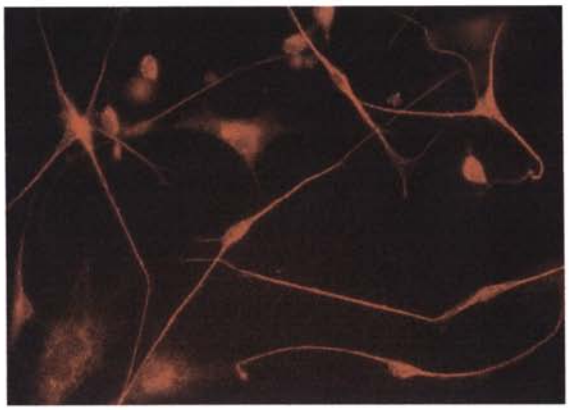
FIGURE 3



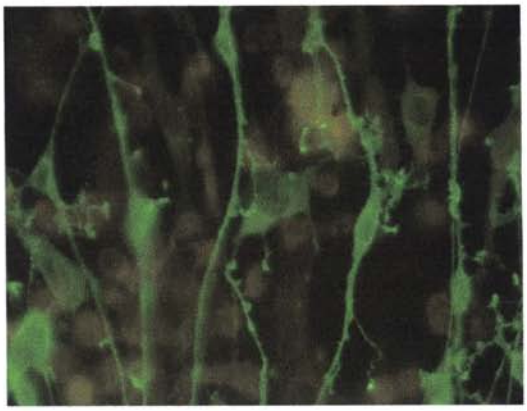
GBC-1 **GAP-43**

Fig. 3. Representative morphologies of immunostained cells for GBC-1 and GAP-43 in the inner halo of olfactory epithelial culture.

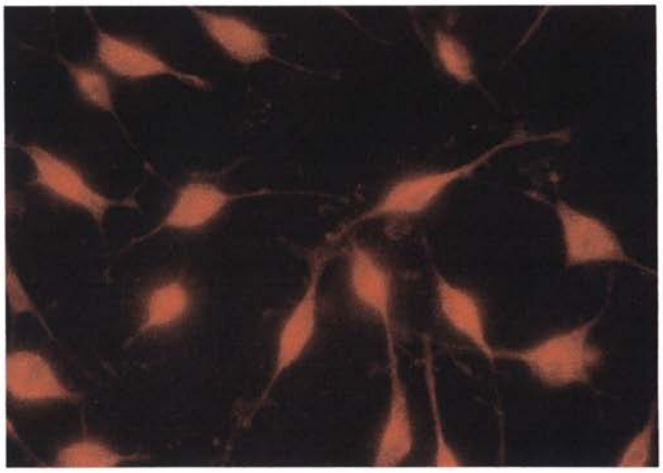
FIGURE 4



Anti-beta III Tubulin



GAP- 43



OMP

Fig. 4. Representative morphologies of immunostained cells for Anti tubulin III, GAP-43 and OMP in the outer halo of olfactory epithelial culture.

FIGURE 5

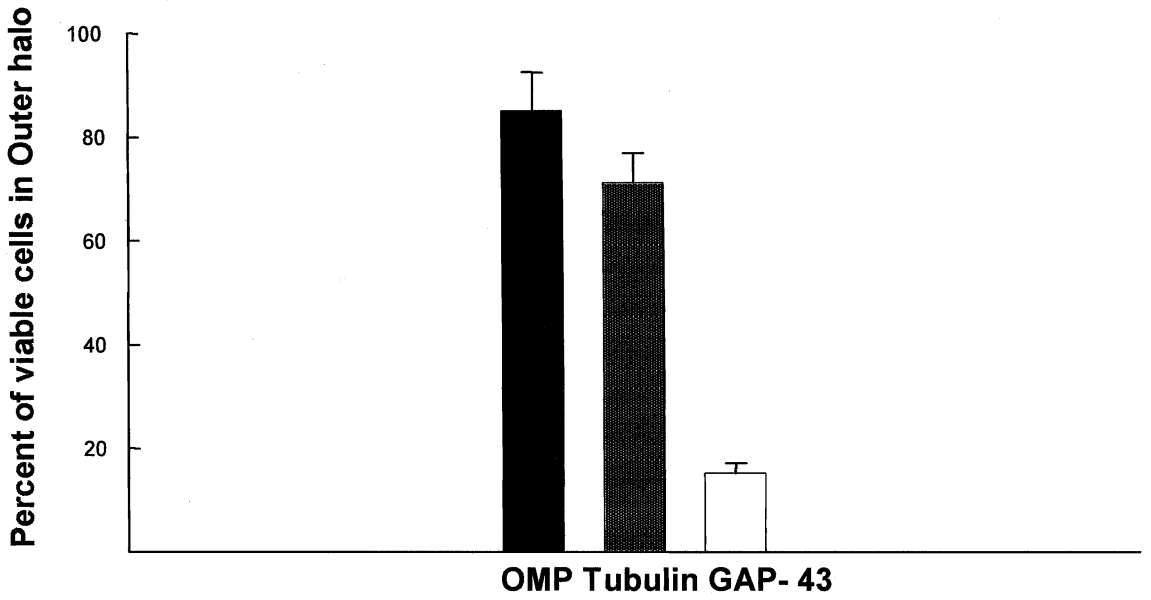


Fig. 5. Percentage of viable cells after immunofluorescent labeling of cell types in the outer halo of olfactory epithelial culture.

FIGURE 6

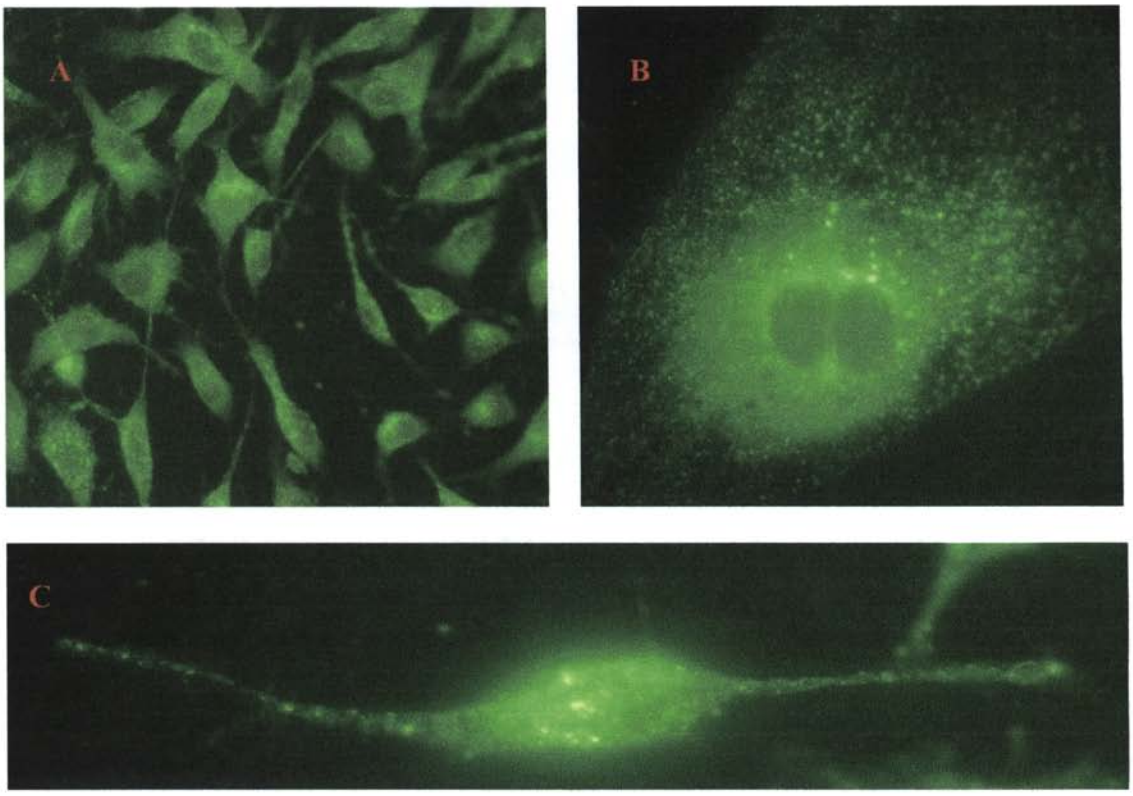


Fig. 6. A. Low power shot of culture with different kinds of cells expressing LRP receptors. B. Epithelial cell with LRP receptors. C. Bipolar neuron with LRP receptors.

FIGURE 7

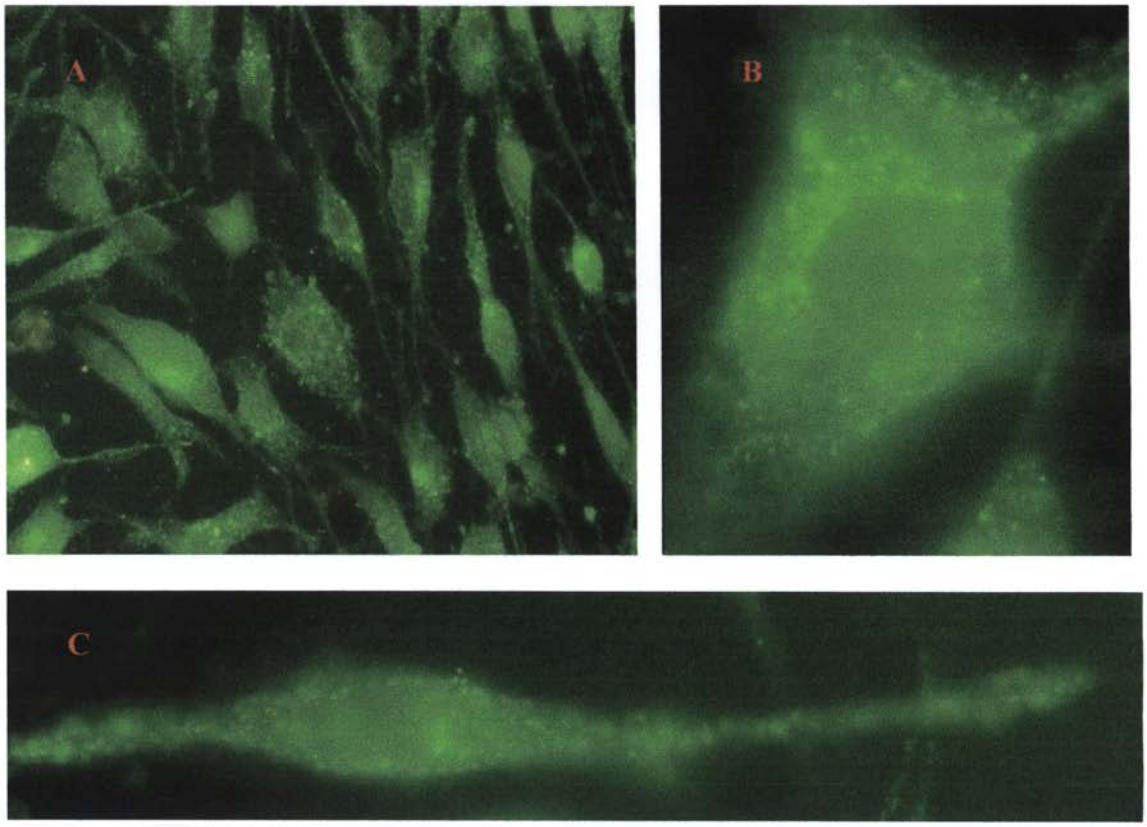


Fig. 7. A. Low power shot of culture with different kinds of cells expressing LDL receptors. B. Epithelial cell with LDL receptors. C. Bipolar neuron with LDL receptors.

FIGURE 8

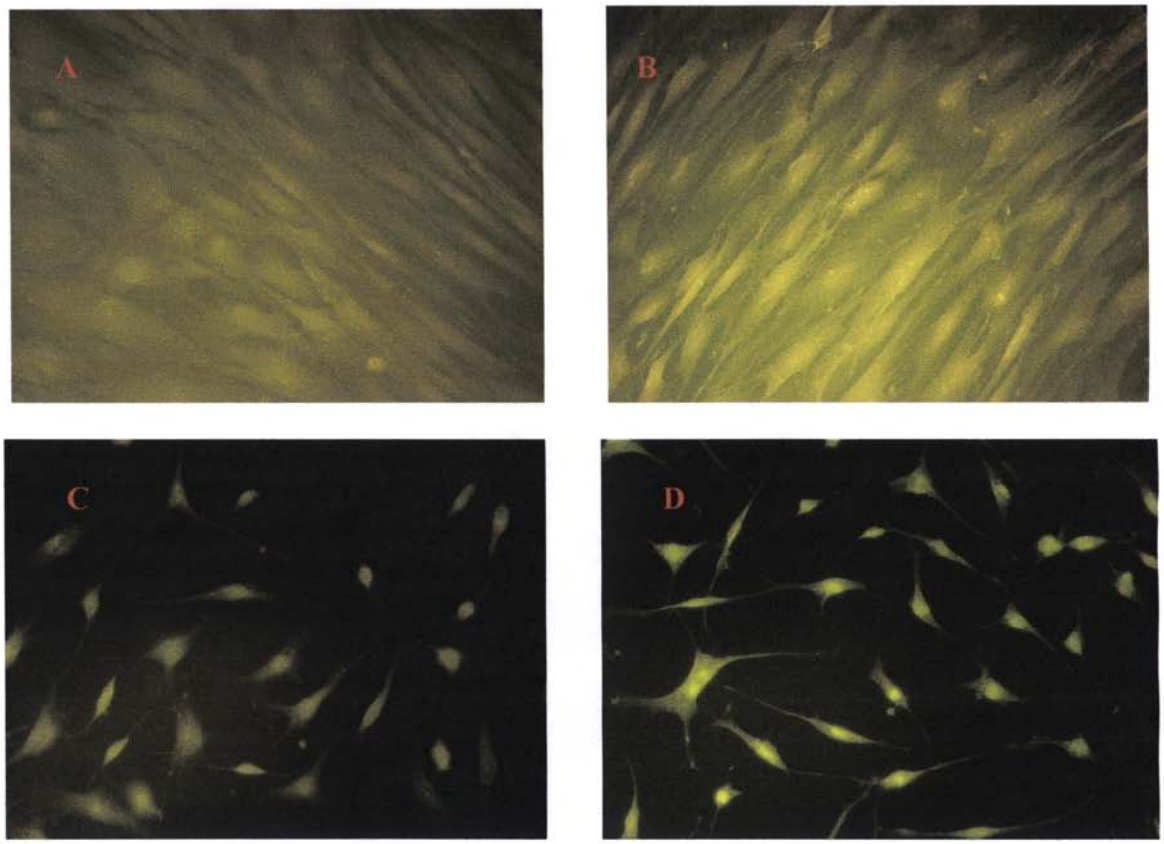
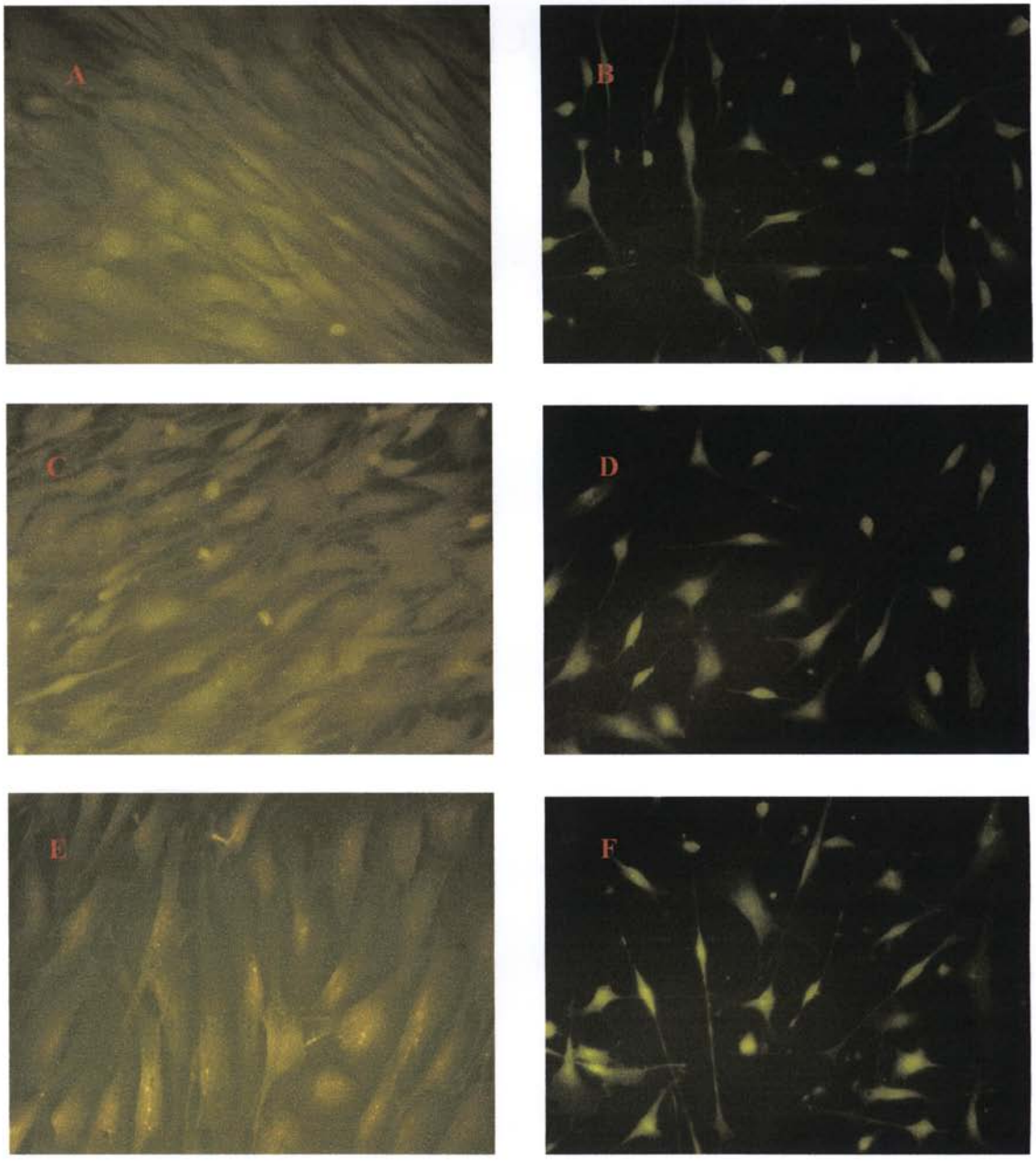
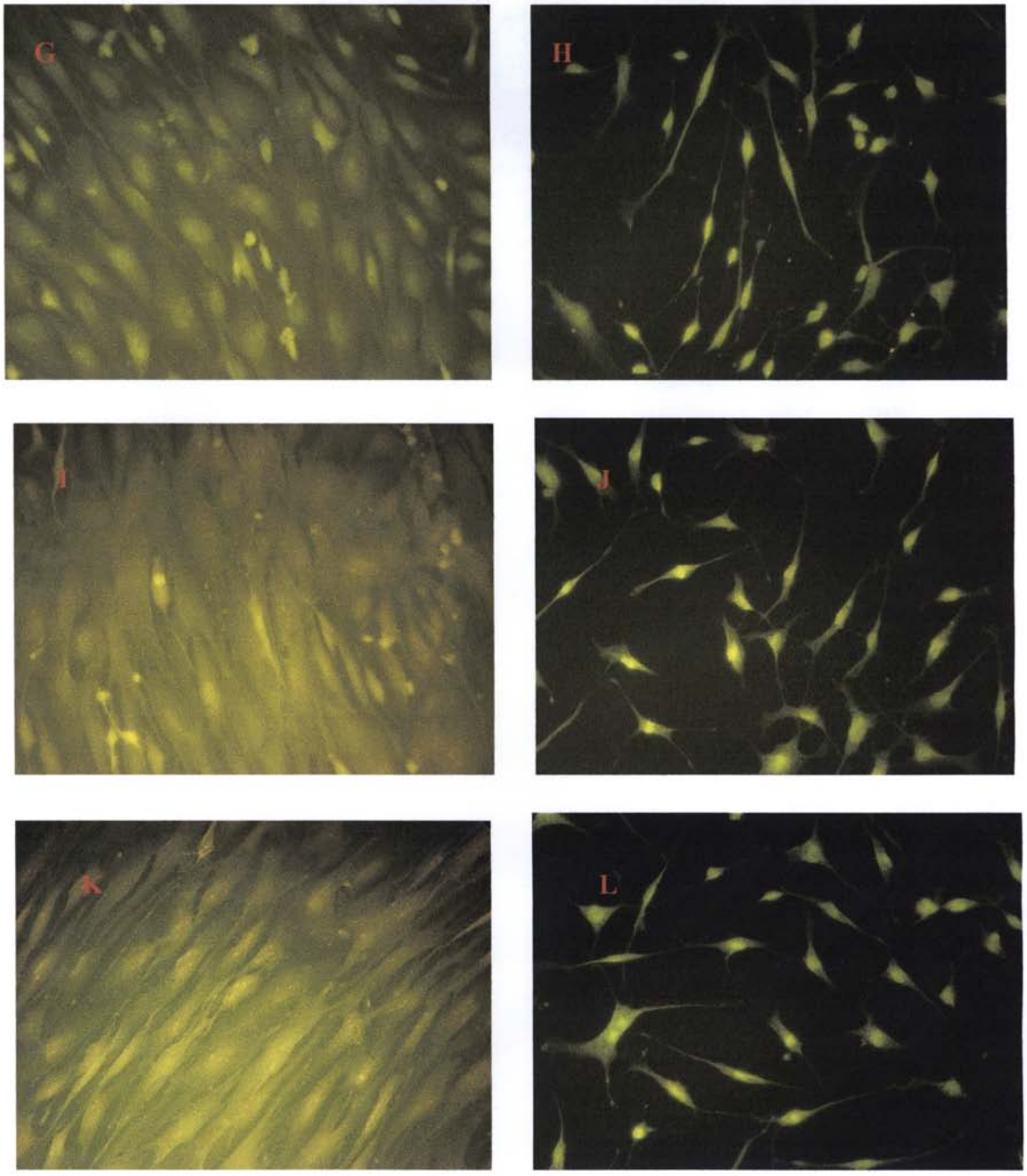


Fig. 8. Effects of estradiol on apoE secretion from olfactory epithelial culture. OEC from wild type mice was incubated for 24hrs in medium alone or in medium containing 100pM of estradiol. Cells were fixed and immunocytochemistry was performed as described in methods section. A. Photograph of inner halo in OEC derived from wild type mice incubated with medium alone at 24hrs. B. Photograph of inner halo in OEC derived from wild type mice incubated with 100pM estradiol in medium for 24hrs. C. Photograph of outer halo in OEC derived from wildtype mice incubated with medium alone at 24hrs. D. Photograph of outer halo in OEC derived from wild type mice incubated with 100pM estradiol in medium for 24hrs.

FIGURE 9




G
H
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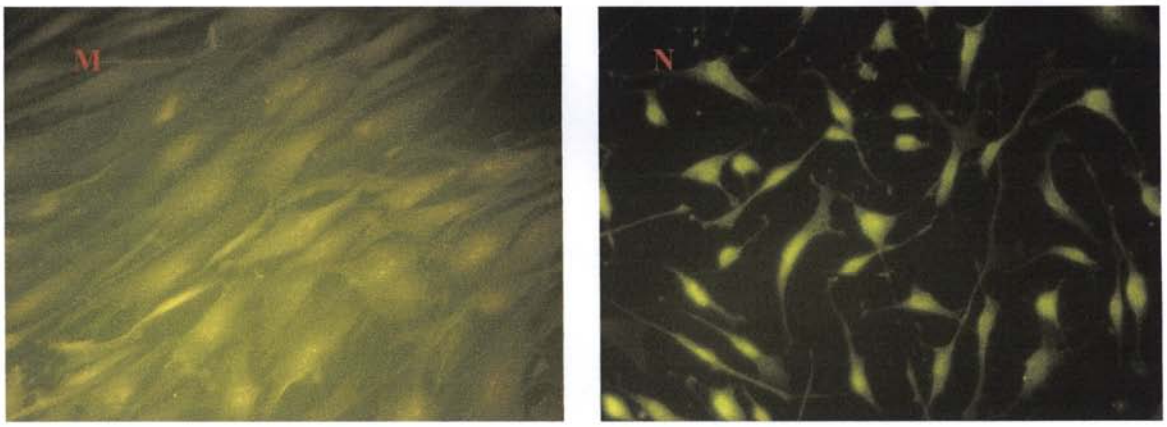
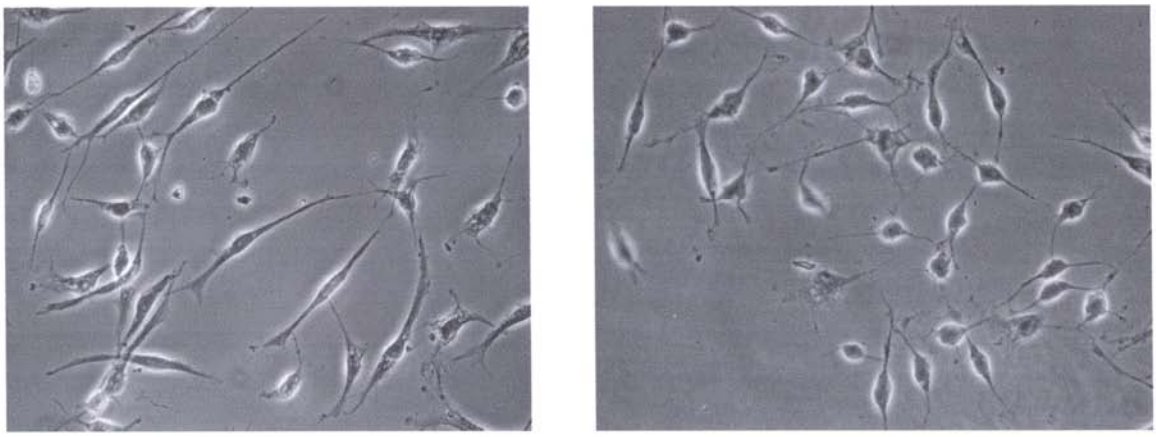


Fig. 9. Effects of estradiol on apoE secretion from olfactory epithelial culture. OEC from wild type mice was incubated in medium alone or in medium containing 100pM estradiol for different time periods. Cells were fixed and immunocytochemistry was performed to detect elevated levels of apoE as described in methods section. A,B. Photographs of inner and outer halo respectively in OEC derived from wild type mice incubated with medium alone. C, D. Photographs of inner and outer halo respectively in OEC derived from wild type mice incubated with 100pM estradiol in medium for 30mins. E, F. Photographs of inner and outer halo in OEC derived from wildtype mice incubated with 100pM estradiol for 1 hour. G, H. Photographs of inner and outer halo respectively in OEC derived from wild type mice incubated with 100pM estradiol in 4 hrs. I, J. Photographs of inner and outer halo respectively in OEC derived from wild type mice incubated with 100pM estradiol for 16 hrs. K, L. Photographs of inner and outer halo respectively in OEC derived from wild type mice incubated with 100pM estradiol for 24 hrs. M, N. Photographs of inner and outer halo respectively in OEC derived from wild type mice incubated with 100pM estradiol for 36 hrs.

FIGURE 10



Wild type **Knock out**

Fig. 10. Comparison of neurite outgrowth between Olfactory Receptor Neuron (ORN) derived from wild type olfactory epithelial culture and ORN derived from knock out olfactory epithelial culture

FIGURE 11

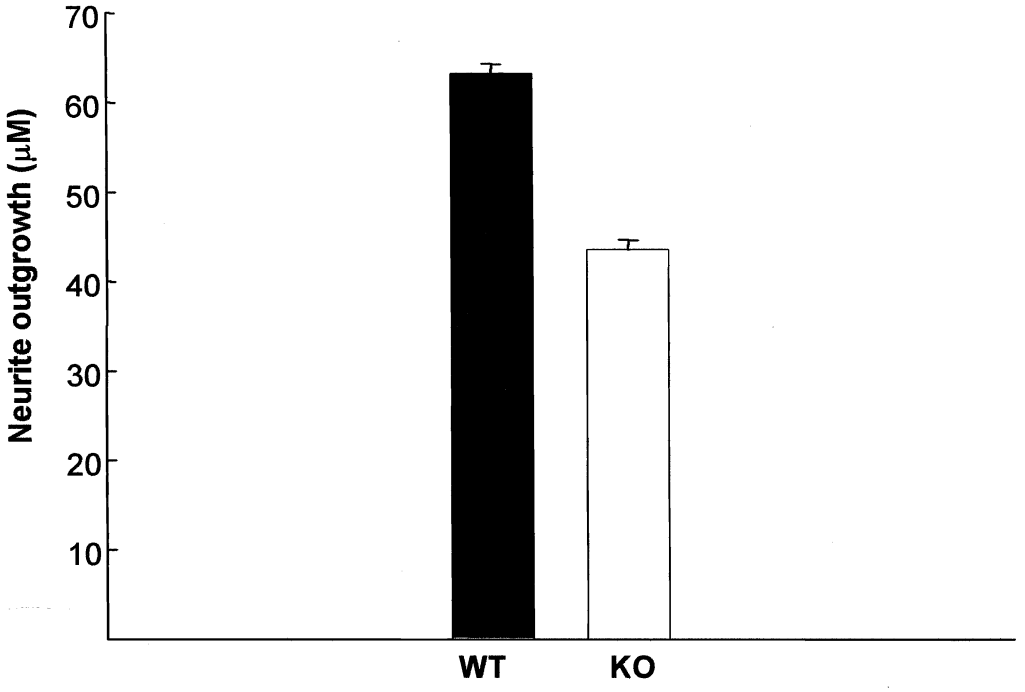


Fig. 11. Comparison of neurite outgrowth between ORN in olfactory epithelial culture derived from WT mice to cultures derived from KO mice. Neurite outgrowth for 60 neurons from each group as described under methods section.

FIGURE 12

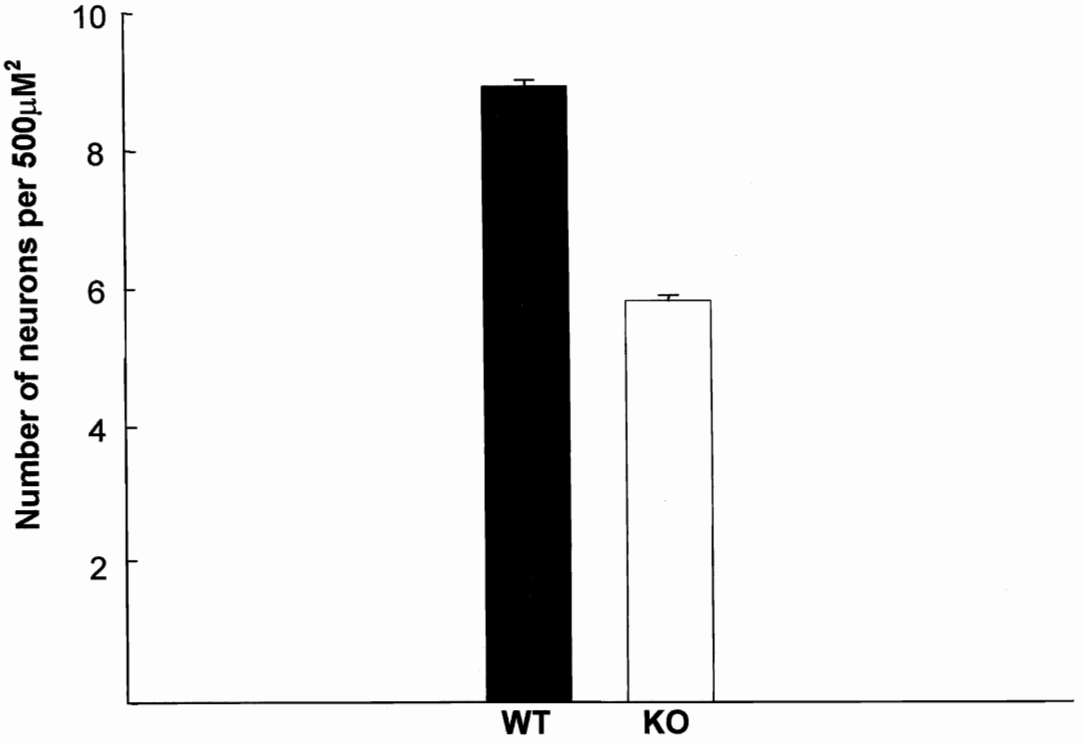
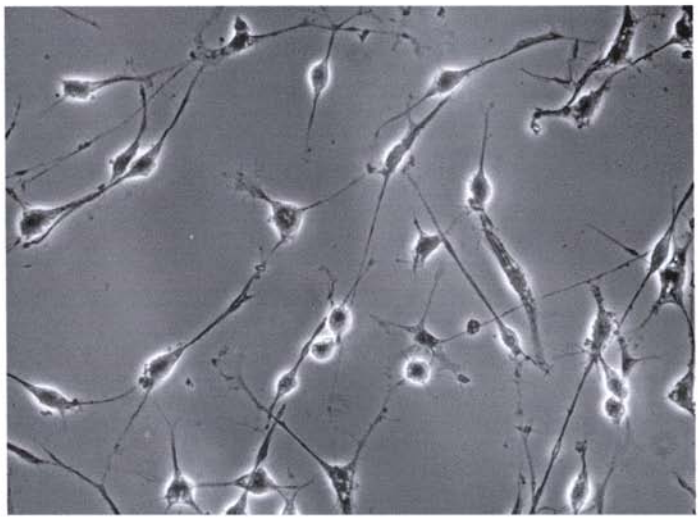
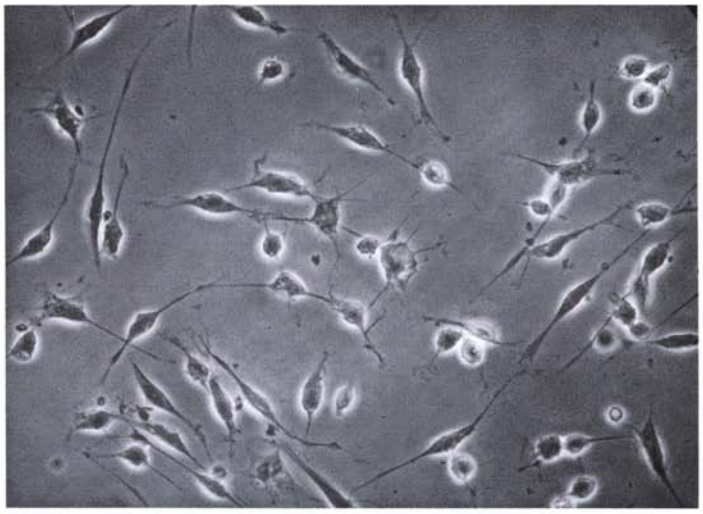


Fig. 12. Comparison of neuronal numbers between ORN in olfactory epithelial culture derived from WT mice to cultures derived from KO mice.

FIGURE 13



Estradiol



Ethanol

Fig. 13. Effect of estradiol on neurite outgrowth of ORN in olfactory epithelial culture derived from WT mice. Phase contrast photograph representing ORN from wild type mice grown for 6 days in medium containing 100pM estradiol dissolved in ethanol or 100pM Ethanol alone.

FIGURE: 14

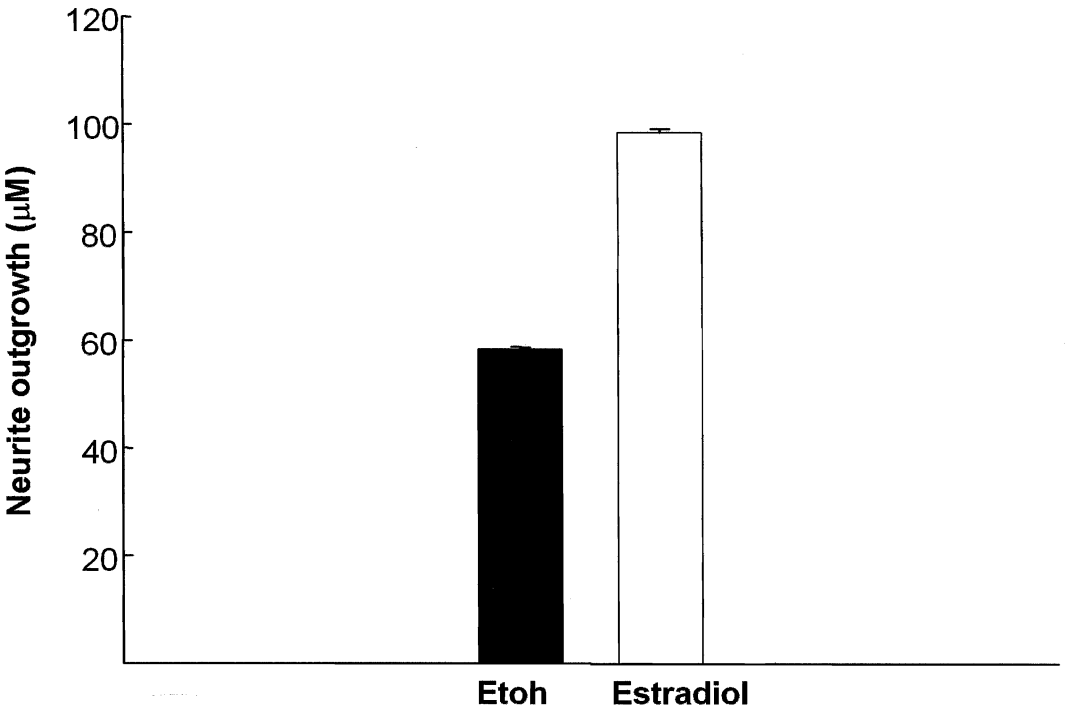


Fig. 14. Quantification of the effects of estradiol on neurite outgrowth in ORN from wild type OEC. Neurite outgrowth for 60 neurons from each group as described under methods section.

FIGURE 15

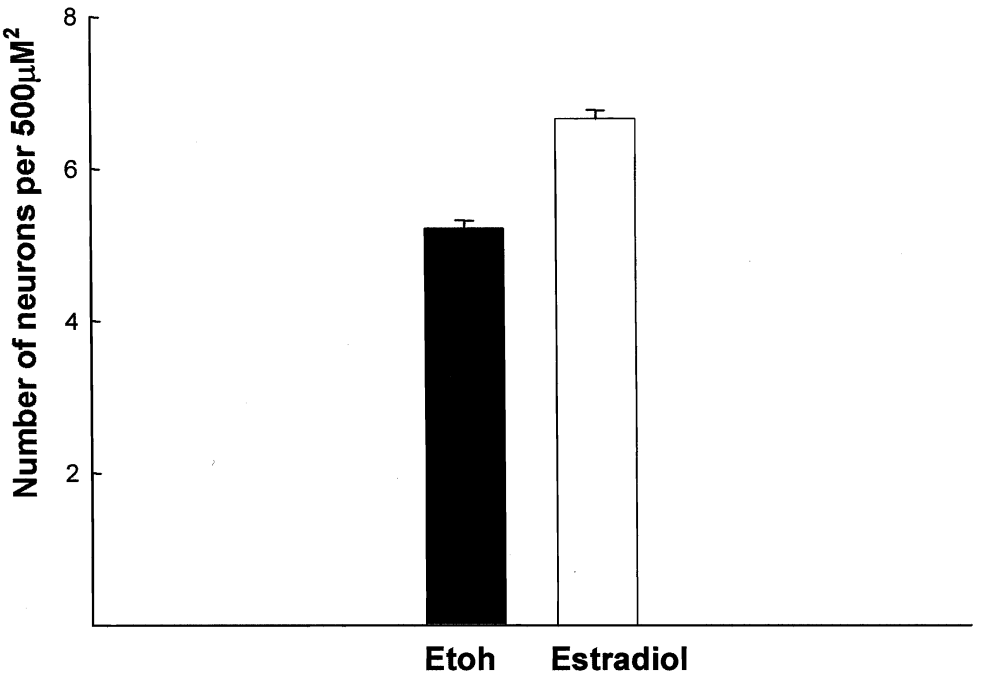
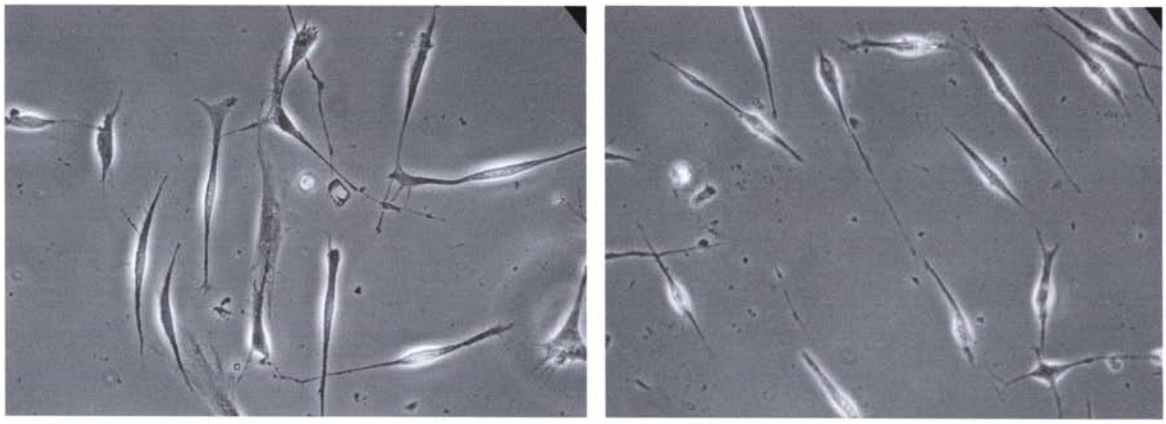


Fig. 15. Quantification of the effects of estradiol on neuronal number in olfactory epithelial culture derived from wild type. Neuronal numbers from 30 groups were collected as described under methods section.

FIGURE 16



Estradiol **Ethanol**

Fig. 16. Effect of estradiol on neurite outgrowth of ORN in OEC derived from apoE deficient knock out mice. Phase contrast photograph representing ORN from apoE deficient knockout mice grown for 6 days in medium containing 100pM estradiol alone.

FIGURE 17

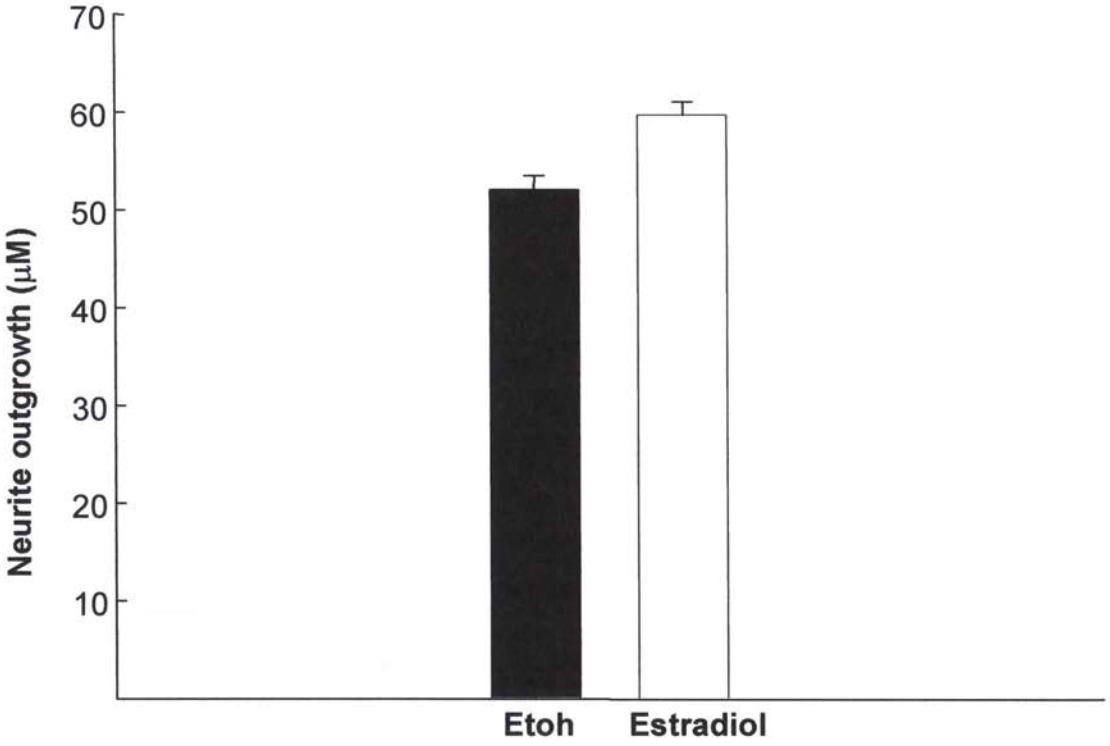


Fig. 17. Quantification of the effects of estradiol on neurite outgrowth in ORN from apoE deficient KO OEC

FIGURE 18

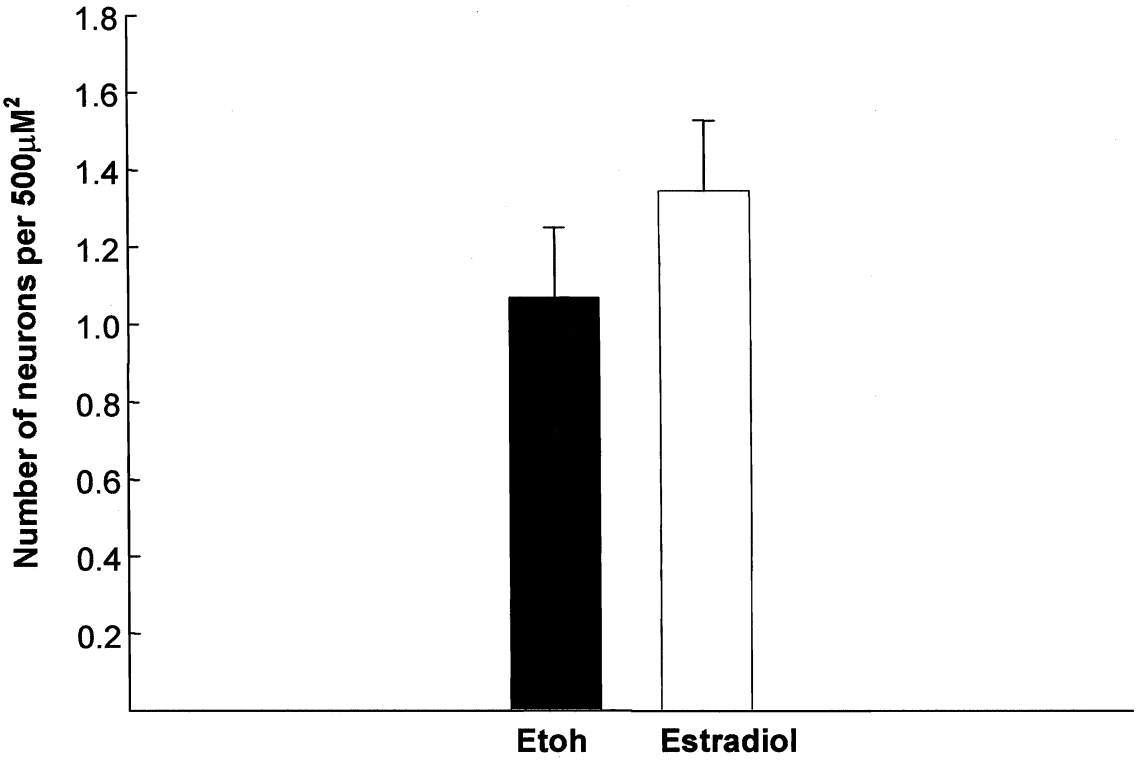
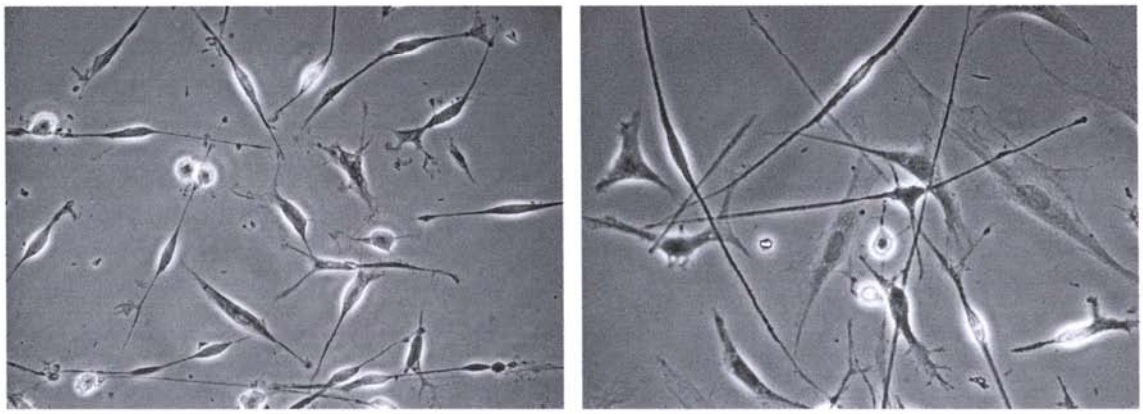
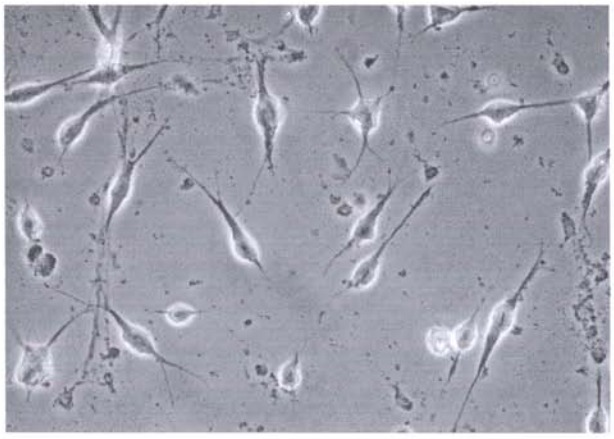


Fig. 18. Quantification of the effects of estradiol on neuronal number in OEC from apoE deficient KO OEC

FIGURE 19



ApoE2 **ApoE3**



ApoE4

Fig. 19. Effect of recombinant human apoE isoforms on neurite outgrowth of ORN in apoE deficient KO OEC. Phase contrast photographs representing ORN from apoE deficient knockout OEC grown for 6 days in medium containing 3µg/ml apoE2, 3µg/ml apoE3, 3µg/ml apoE4.

FIGURE 20

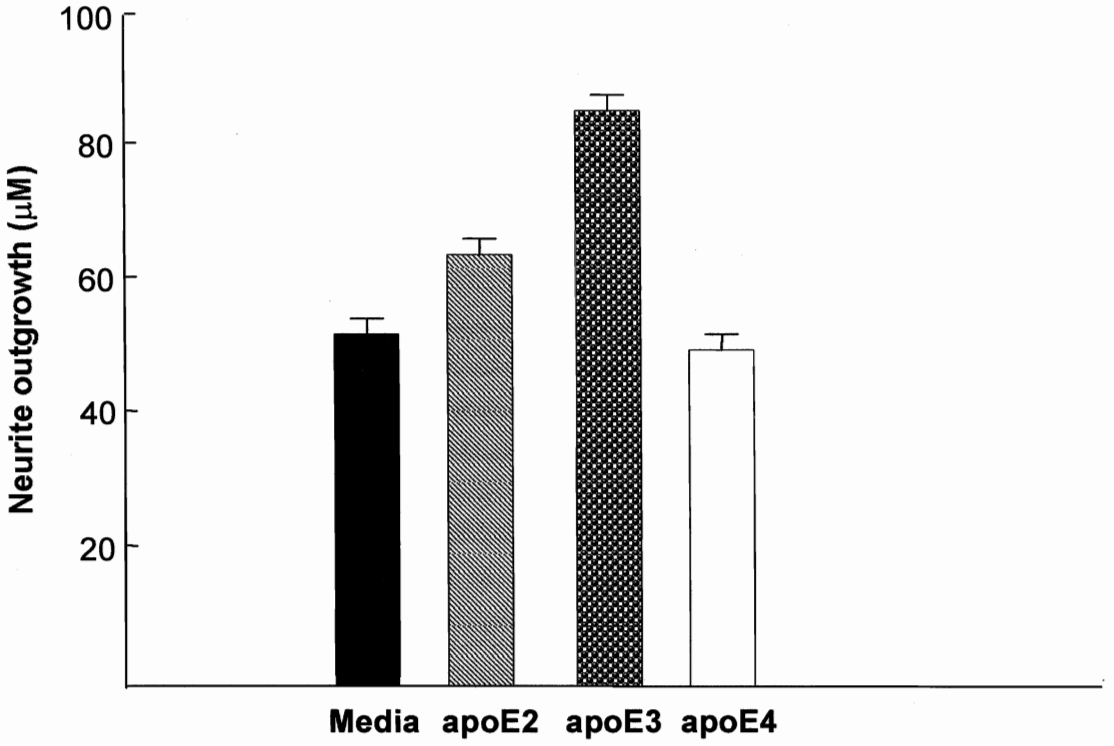


Fig. 20. Quantification of effects of recombinant human apoE isoforms on neurite outgrowth of ORN from apoE deficient KO OEC.

FIGURE 21

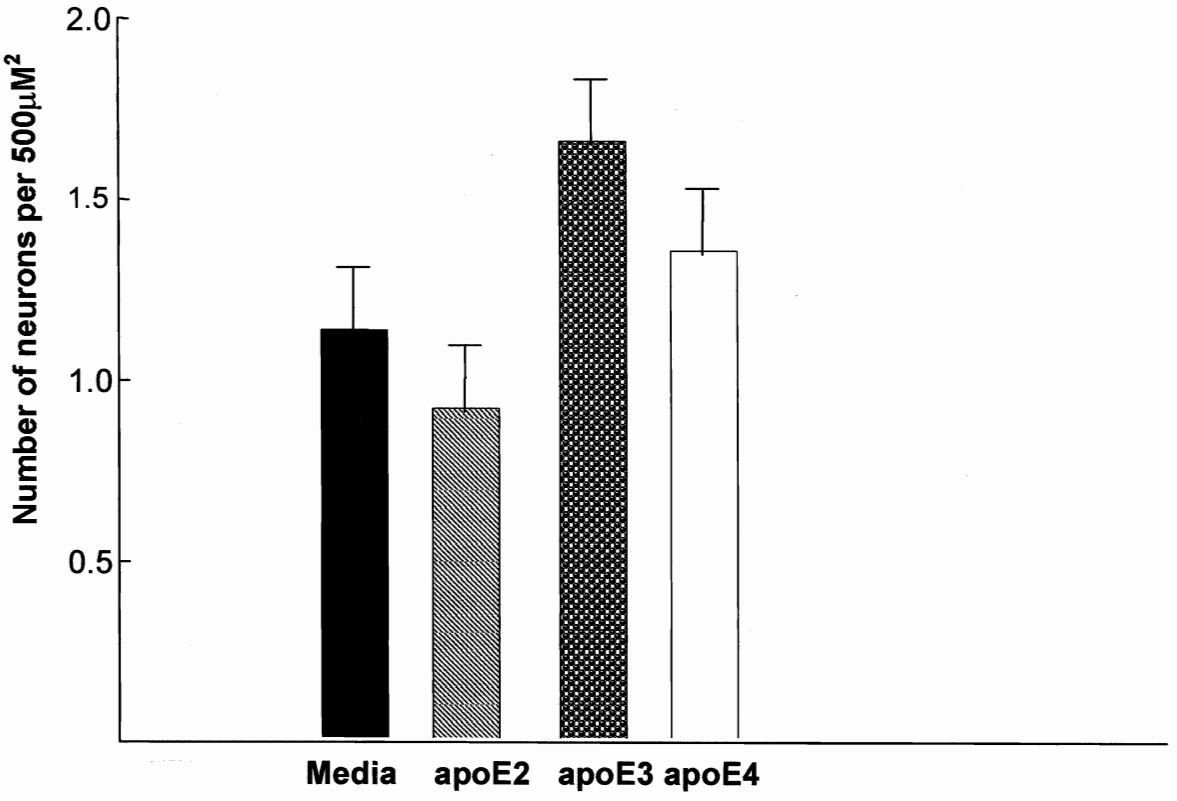
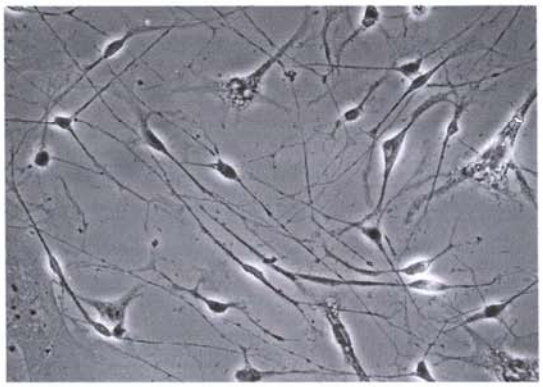
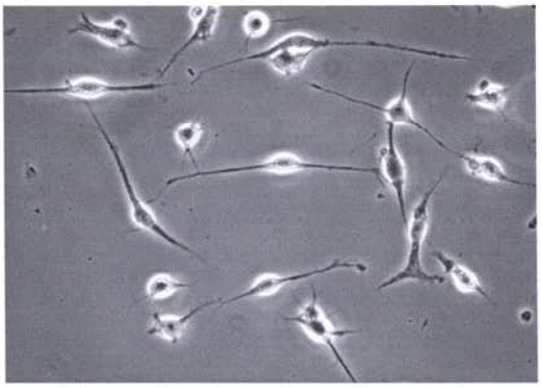


Fig. 21. Quantification of effects of recombinant human apoE isoforms on neuronal numbers of OEC from apoE deficient KO OEC.

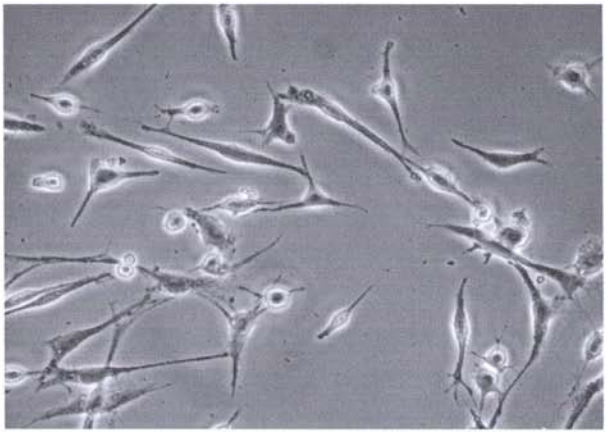
FIGURE 22



ApoE3+ Est



ApoE2+ Est



ApoE4 + Est.

Fig. 22. Combined effect of estradiol and recombinant human apoE isoforms on neurite outgrowth of ORN in apoE deficient KO OEC. Phase contrast photographs representing ORN from apoE deficient knockout OEC grown for 6 days in medium containing 100pM estradiol, 3μg/ml apoE2, 3μg/ml apoE3 and 3μg/ml apoE4.

FIGURE 23

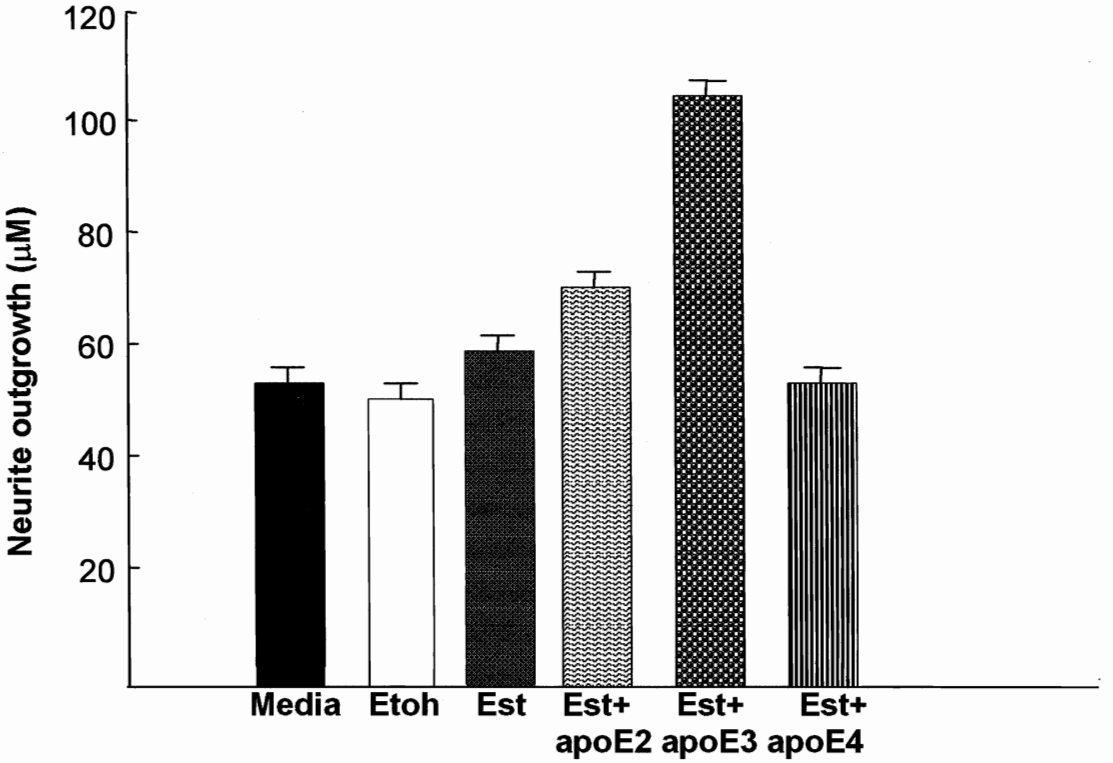


Fig. 23. Quantification of combined effects of estradiol and recombinant human apoE isoforms on neurite outgrowth of ORN form apoE deficient KO OEC.

FIGURE 24

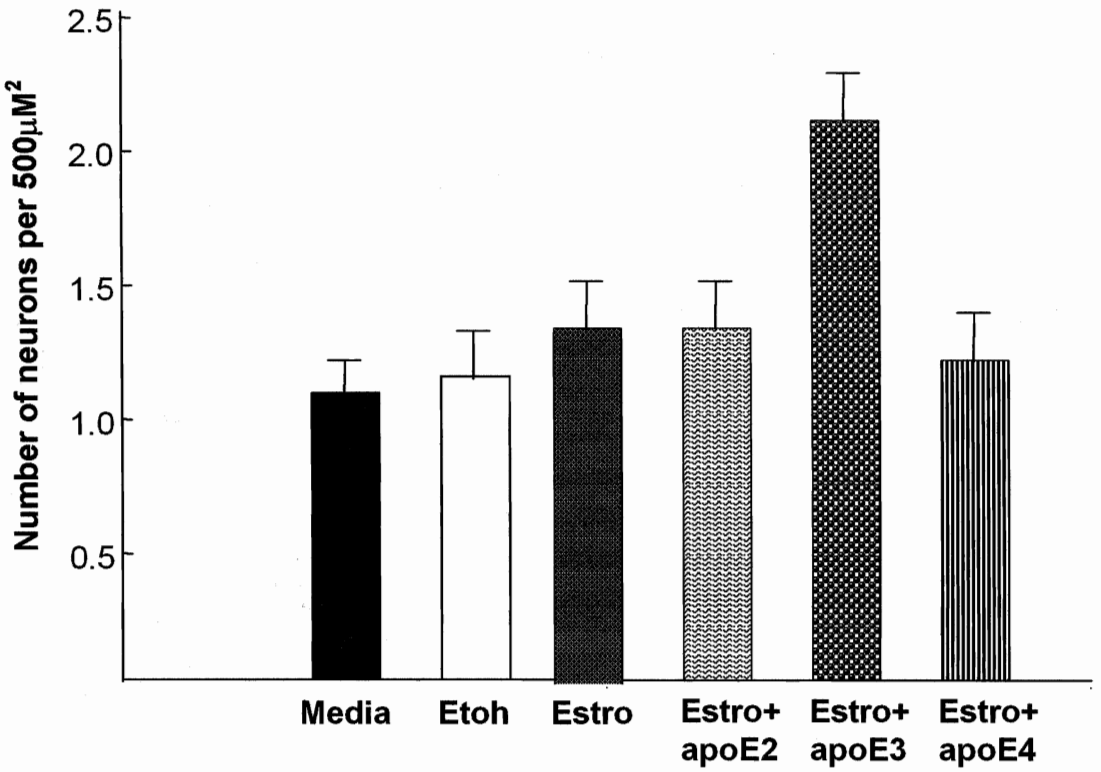


Fig. 24. Quantification of combined effects of estradiol and recombinant human apoE isoforms on neuronal number of apoE deficient KO olfactory epithelial culture.

5. DISCUSSION

My research focuses on the neurotrophic and neuroprotective actions of estradiol in the olfactory epithelium. Olfactory receptor neurons in the epithelium regenerate continuously throughout the life of the epithelium making it a perfect model to study mechanisms involved in neurogenesis and neurite outgrowth (57). Role of apoE as the mediator for synergistic effects of estradiol has also been studied. Finally, a multicellular model of actions of estradiol mediated by apoE in the regulation of neurogenesis and neurotrophism is discussed, as a potential future direction for the field of treatment for AD.

This is the first study to show that the lipid transporting protein apoE modulates neuronal differentiation in culture. The results revealed that (1) at 8 *DIV* the olfactory epithelial culture contained globose basal cells, mature olfactory receptor neurons, immature olfactory receptor neurons. (2) ApoE and its major receptors, the LRP and LDL receptor are expressed in the OEE cultures, (3) apoE levels of the OE culture derived from WT mice were elevated in the presence of estradiol and in time dependant manner. (4) deficiency of apoE in OEE cultures derived from apoE KO mice lead to significantly shorter neurite outgrowth, increased precursor cell numbers, and decreased bipolar neuronal numbers, (5) incubation of KO OEE cultures with recombinant human apoE3, but not human apoE4, significantly increased neurite outgrowth and neuronal number, and (6) Incubation of WT OE cultures with estradiol increased neurite outgrowth and neuronal numbers. (7) Estradiol had isoform specific effects on the neurite outgrowth and neuronal numbers of KO ORN in the presence of different

isoforms of apoE. Together, these results lend strong support for a novel role for apoE in neuronal differentiation.

1. Characterization of Olfactory epithelial culture (OEC):

Structurally, the olfactory neuroepithelium resembles the germinative neuroepithelia of the embryo that gives rise to the central nervous system (CNS) but is much simpler in that it produces only one type of neuron, the olfactory receptor neuron. This characteristic makes it possible to study the molecular regulation of neurogenesis in OE (57). Results from my characterization studies of the olfactory epithelial culture revealed that it comprised globose basal cells, immature neurons and mature neurons (ORN). Globose basal cells, horizontal basal cells and ensheathing glial cells form bundles around olfactory receptor neurons. The present study had considerable population of olfactory receptor neurons and a percentage of globose basal cells (GBC). This could be because the culture was grown in Fibroblast Growth Factor (FGF) from 0 DIV, fibroblast growth factor aids in neuronal recovery after the culture preparation (61). Previous studies have shown that globose basal cells are more responsive to FGF when compared to horizontal basal cells (60, 61) thus explaining the predominant population of globose basal cells and receptor neurons in the olfactory epithelial culture. In the early stage (1-3 DIV) of these cultures, cells with epithelial morphology move out of the explant and proliferate to form a sheet surrounding the explant. In the later stage (3-8 DIV), cells that stain positive for the immature neuronal marker, GAP-43, appear within the epithelial sheet. These immature neurons travel away from the epithelial sheet and attain bipolar morphology,

reminiscent of its *in vivo* counterpart in the OE. These bipolar cells stain positive to mature ORN marker, OMP. Reduced number of neurons in the KO mice culture could represent an isolated deficit in any one of these processes or a combination thereof. Slower proliferation and establishment of the epithelial sheet could have contributed to fewer neurons in OE culture from KO mice. Since most of the cells in the epithelial sheet are positive for GBC-1 (marker for olfactory sensory neuron precursor), a reduced/delayed proliferation of these precursor cells in KO culture would contribute to the decreased neuronal numbers. Results from this study are in striking contrast to this possibility. Infact, the GBC-1+ cell numbers were higher in OE cultures from KO mice as compared WT cultures. Since apoE could play a variety of roles to facilitate neuronal differentiation in the OE cultures. These data suggest that apoE deficiency reduced differentiation of precursor cells to neurons, which results in more precursor cells and fewer neurons in KO cultures versus WT cultures. It should also be considered that the present culture might also contain a small population of ensheathing glial cells and supporting SUS cells. Immunofluorescence labeling needs to be done to denote their percentage in the culture for further studies.

Interesting finding in this experiment was evidence of round, neurofilament positive cells with no neurites, scattered randomly in the inner halo. It looked like they formed a thin layer on top of the flat cell zone or the inner halo. Neuronal zone or the outer halo has most of the staining for Tubulin III but scattered labeling was also found in the inner halo as well. Moreover interestingly these round Tubulin III positive cells also stained

positive for anti OMP, a marker for mature receptor neurons. Due to all of the observations listed above it is tempting to propose a series of events leading to the establishment of olfactory epithelial culture (OEC) with two distinct halo zones. Starting at 0 *DIV*; Culture preparation, 1-2 *DIV*; Cells start to migrate out of the explant and at this stage all the cells are round and shiny. 3-4 *DIV*; Flat cell zone formation is in process which includes mostly irregular shaped flat glial cells and globose basal cells (preparing to aid the neuronal survival in the culture). 4-5 *DIV*; Neurogenesis is documented by the appearance of growing exterior neuronal zone. It is noticeable that considerable amount of neurons appear only on 4-5 *DIV* after the establishment of the inner halo or flat cell zone. This can be explained by a process where neurogenesis might have originated from the flat cell zone. Studies have proved that putative stem cell for the olfactory receptor neurons lies among the globose basal cells (62). This idea is supported by recent experiments in which purified GBCs, transplanted into the OE of methyl bromidelesioned host mice, appear to give rise to both ORNs and SUS cells (62). Another explanation may be that the flat cell zone is formed prior to the migration of the receptor neurons from the epithelial explant to provide favorable microenvironment for the receptor neurons to migrate out of the epithelium and recover in the culture. This explains the scattered Tubulin III staining found on top of the flat cell zone, as the receptor neurons migrate out of the explant.

2. ApoE receptors in the Olfactory Epithelial Culture:

Brain cells express six major receptors for apoE-containing lipoproteins: the low density

lipoprotein (LDL) receptor, the LDL receptor-related protein (LRP), the very low density lipoprotein (VLDL) receptor, the glycoprotein (gp) 330, the LR 11 receptor, and the apoE receptor 2 (APOER2). How ever results from this study have shown that two kinds of receptors were found in the OE culture. Low density lipoprotein receptor (LDL) and LDL receptor related protein (LRP) were found in both the epithelial cells and olfactory receptor neurons. Results from the receptor inhibition studies suggest that both apoE3 and apoE4 are using the LRP to modulate neurite outgrowth. However, binding of apoE4 to LRP leads to shorter neurite outgrowth and fewer bipolar neurons. The mechanism underlying the isoform specific effects of apoE neurite outgrowth and increased neuronal differentiation is not clear from this study.

3. Effect of estradiol on apoE secretion from OEC derived from wild type (WT) mice:

Previous studies have demonstrated that apoE is expressed throughout the OE and its underlying lamina propria in mouse and human tissue. In particular, intense apoE immunoreactivity was observed in the end feet of Sus cells, found in close proximity to the basal cells (65). I investigated the effect of estradiol on levels of apoE in WT OEC by comparing the levels of apoE in the presence of estradiol in WT OEC and at different time periods. Results of my studies show that estradiol treatment increases apoE levels in WT OEC and also that estradiol treatment increases apoE levels at different time intervals in ascending manner starting at 4hrs and reaching a plateau at 16hrs and remaining the same until 36hrs. The result of this study is consistent with my hypothesis and results of the

previous study from our laboratory (29). It can be justified with the findings of the recent studies that have proved that apoE mRNA levels in the brain are induced by estradiol (43, 44) thus leading to increased apoE levels in turn.

4. Comparison of neurite outgrowth and neuronal numbers between cultures derived from wild type mice and cultures derived from knock out mice:

Results from this study, taken together with data from previous studies, suggest a direct relationship between apoE and neuronal process growth (63). This study has shown that KO cultures deficient in apoE resulted in having shorter neurite outgrowth and fewer numbers of neurons when compared to the WT culture. In the presence of human apoE3, however, the KO cultures had comparable neurite outgrowth and neuronal numbers to WT cultures, suggesting that the these effects observed in the KO culture are not due to the intrinsic properties of the neurons. When the neuronal numbers were evaluated in the OEC in the absence of estradiol, more neuronal number was documented in WT OEC when compared to KO OEC. It may be justified by the universal fact that apoE is present in the WT culture leading to better prognosis for neuronal recovery after culture preparation and aiding neurogenesis due to efficient lipid supply. Consistent with the present study it was also observed that there was a large population of GBC-1 positive cells in KO OE culture when compared to the WT culture. Since it has been proved that apoE influences neurite outgrowth and differentiation of neurons (53); one potential role

of apoE in Sus endfeets is to facilitate basal cell differentiation to neurons in the adult OE. Such a hypothesis is compatible with the published studies that have examined the impact of apoE deficiency in apoE KO mice on neuronal repair (63). In general, these studies showed that KO mice have poor or delayed regeneration capacity than their WT littermates. Furthermore, olfactory nerve regeneration and recovery in the OB post OE lesion were significantly delayed in KO mice. While the precise mechanism for his delay is unclear, it is tantalizing to propose that the absence of apoE in the end feet of Sus cells, surrounding the basal cells, would have resulted in delayed or reduced proliferation and differentiation of basal cells to neurons. Therefore, delayed regeneration of receptor neurons in KO mice could represent slower differentiation of basal cells in the OE post injury leading to increased population of undifferentiated basal cells in the KO OE cultures.

5. Effect of estradiol on neurite outgrowth and neuronal numbers of olfactory epithelial culture (OEC) derived from WT and KO mice

When the effect of estradiol on neuronal numbers in WT OEC was evaluated it had no significant effect. It was contradictory to some of the studies where estradiol increased neuronal numbers, indicating increased neuronal numbers in cultures with estradiol levels close to proestrous period or highest physiological levels of estradiol (64).

Another part of my study examined the effect of estradiol on neurite outgrowth of ORN derived from WT and KO OEC. The results of this study proved that estradiol

significantly increases neurite outgrowth in ORN derived from WT OEC and also that estradiol does not have any effect on neurite outgrowth of ORN derived from KO OEC. In this study I propose that protective effects of estradiol are mediated by apolipoprotein E, since apoE is a very important lipoprotein in the nervous system. These results can be explained by the fact that the presence of apoE in OEC derived from WT mice helps estradiol to promote neurite outgrowth. Another result which showed that estradiol did not have any effect on neurite outgrowth in ORN derived from KO ORN supports the idea of apoE being indispensable in supporting the process of neurite outgrowth. My findings were consistent with the previous studies performed in our laboratory (63) and other studies which proved a significant relationship between apoE and estradiol in mediating nerve regeneration (32). Inspite of all the studies about estradiol being neuroprotective, the exact mechanism leading to this protective effect is yet to be determined. I hypothesized that estradiol would aid in increased neurite outgrowth by boosting apoE secretion. This has been proved by my other experiment where effects of estradiol on apoE levels of WT OEC were evaluated and the levels increased in the presence of estradiol in time dependant manner. Thus, it can be explained that apoE plays a pivotal role in mediating beneficial effects of estradiol, rendering estradiol useless on KO ORN.

6. Effects of recombinant human apoE isoforms on neurite outgrowth and neuronal numbers of KO olfactory epithelial culture (OEC):

In this present study I have also evaluated the effect of different recombinant human

apoE isoforms on neurite outgrowth and neuronal numbers of KO OEC. Results of my study revealed that different isoforms of apoE had differential effects on neurite outgrowth of ORN derived from KO OEC. ApoE2 and apoE3 significantly promoted neuronal numbers and neurite outgrowth in KO OE cultures when compared to apoE4 which did not have any significant effect on promoting neurite outgrowth or neuronal numbers. This was consistent with the study from our laboratory (14, 47). There has been no evidence that neurons synthesize apoE. Since neurons do not produce apoE but can take up and internalize exogenous apoE. (59). Thus, the difference in the neurite outgrowth is due to the different isoforms of apoE picked up by the neurons. This disparity in effects can be explained by the fact that apoE4 is much less effective than apoE3 in intracellular transport of lipids (29). Studies have also shown that apoE3 accumulation in the neurons is more rapid when compared to apoE4 (48) leading to a better approach in increasing neurite outgrowth. Growth and regeneration of neurites depends on the supply of fatty acids and lipids to the neurons and apoE plays a vital role as a transport lipoprotein that ensures supply (49, 50). Previous studies from our laboratory have also shown that apoE4 accumulates at a slower level when compared to apoE3 (29, 30), indicating apoE4 not being able to transport lipids with efficacy as much as apoE3, forming the basis of inhibition of neurite outgrowth in apoE4 treated neurons Thus the shorter neurite outgrowth may indicate poor neuronal plasticity and repair and inheritance of apoE4 alleles increasing the risk of occurrence of AD. It has also been shown that in the presence of a source of cholesterol, apoE3 promoted neurite outgrowth in cultured dorsal root ganglion cells (47). ApoE3 is also involved in response to

neuronal injury (45, 46). It has been proved to increase neurite extension in a variety of cultured neurons and is involved in response to neuronal injury (30, 34). ApoE4 treatment, on the other hand, had no significant effect on neurite outgrowth. It is possible that apoE3 and apoE4 differ in their ability to perform their fundamental function that is, transporting lipids from the outside cellular compartment into cells. An alternative possibility is that apoE4 could be toxic to neurons. ApoE4 has been reported to show toxic effects in culture, and disrupts microtubule polymerization at 10-fold higher concentration of that used in this study. Studies have also shown severe olfactory impairments in a variety of neurological disorders where apoE4 allele is a major risk factor [3, 10, 53, 55, 57]. For example, severe olfactory deficit is one of the first signs of AD, and has been proposed as a predictor of the disease [17, 56]. Interestingly, apoE 4 allele status is associated with olfactory dysfunction in AD [26, 55]. ApoE4 individuals have a significant decline in odor threshold and odor identification, and have delays in processing of olfactory information [3, 56, 97, and 99]. The mechanism underlying these isoform specific effects of apoE on olfactory function is not clear. We propose that the presence of apoE3 will facilitate the continuous turn over of receptor cells in the OE by promoting basal cell differentiation to mature neurons, whereas apoE4 may not be very effective in carrying out this critical role, and thus leads to olfactory deficits in apoE4 carriers.

7. Effect of estradiol on neurite outgrowth of ORN and neuronal numbers of KO OEC in the presence of recombinant human apoE isoforms:

Another study evaluated the effect of estradiol on the neurite outgrowth and neuronal numbers of KO OEC in the presence of different isoforms of apoE. Estradiol had significant effect on neurite outgrowth and neuronal numbers of KO OEC in the presence of apoE3 but not in the presence of apoE4 and apoE2. This can be explained by the process that estradiol increases neurite outgrowth via apoE dependant mechanism (28, 29) and the difference in efficacy of apoE isoforms in promoting neurite outgrowth is due to the difference in internalization, uptake and regulation. How ever it was interesting to note that estradiol did increase neurite outgrowth in the ORN in the presence of apoE2 but not as much as it did in the presence of apoE3. This proves that beneficial effects of estradiol are mediated very well by apoE3 when compared to apoE2 and apoE4. I also studied role of apoE and estradiol in neuronal proliferation (or) neurogenesis. When the effect of different isoforms of apoE were evaluated on neuronal numbers of KO OEC, it was found that apoE3 treated KO OEC had most neuronal numbers when compared to apoE2 and apoE4. It was surprising to find that apoE2 had fewer neurons than apoE4 treatment of KO OEC. In the presence of estradiol, cell proliferation is encouraged. Enhanced cell proliferation signals for increased lipid supply which can only be met in the presence of efficient lipid carrier. ApoE3 has been proved to be better lipid carrier than apoE4 or apoE2 leading to the differential results. How ever estradiol combined with

apoE2 was not as effective in promoting neuronal numbers as estradiol and apoE3 together were. This can be explained by the fact that rate of uptake of apoE3 is faster than that of apoE2 and hence more lipids are delivered to the target site (29).It was surprising to see that apoE4 was more effective increasing neuronal number when compared to apoE2, it has to be noted that apoE2 had neurons with longer neurite outgrowth in the culture when compared to apoE4 thus limiting the number of neurons one can evaluate through the microscope. In the presence of apoE4 cells have shorter neurite outgrowth thus making it easier for them to spread out in a more compact fashion when compared to apoE2 treated culture, leading to differential count results.

Based on my results I have developed a model that might explain the potential role of apoE and estradiol in pathogenesis of AD. The model proposes that a normal balance is needed between injury and repair of neurons to maintain healthy nervous system and prevent onset of AD. With progression of age neurons are more susceptible to the damages by neurotoxins, needing effective repair. Since my study has proved that estradiol induces apoE secretion, in a premenopausal woman, presence of estradiol induces maintenance of apoE in the central nervous system. Protecting premenopausal women from neurodegeneration. Since the genotype of apoE present in brain differs from individual to individual, presence of apoE2 and apoE3 support the repair processes efficiently when compared to apoE4 which does not support repair, leading to the early onset of AD in women with apoE4 genotype. Similarly for ERT to be effective, genotype

of the subject has to be known since beneficial effects of estradiol are mediated differentially through different isoforms of apoE. My research makes a strong argument that apoE has a critical role in mediating the effects of estradiol. My results have been reproducible and have shown that estradiol has no beneficial effects in KO mice lacking apoE, it induces production of apoE in wild type mice with apoE gene, and there is a distinct and unequivocal difference in the way estradiol effects neurite outgrowth in the presence of different subsets of apoE. In fact, there has been a hasty effort to relegate estradiol to treatment of just hot flashes and other symptoms instead of the great promise that it once held in preventing the increasing incidence of dementia, coronary artery disease, and stroke in post menopausal women. It has been relegated to the side without taking into consideration the complex interaction of genetic factors and susceptibility involved in the mediation of effects of estradiol. Our research elucidates many of these finer complexities involved in the effects of estradiol. From the results, one can presume that what is needed is elucidating the apoE allele determination in participating subjects, avoiding subset of women with apoE 4 where estradiol will worsen or precipitate dementia and the realization that the combination of these different alleles confers a varying risk of dementia. From my research, it is obvious that a woman with homozygous apoE 3 will show the greatest benefit with estradiol supplementation. Heterozygozity with apoE 4 will obviously lower the benefit and homozygous apoE 4 women will fare worse when supplemented with estradiol. The contradictory effects of estradiol on heart disease and stroke might have similar underpinnings.

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