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Long-term effects of estradiol replacement in the olfactory system

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Abstract

Olfactory dysfunction often precedes other clinical symptoms in chronic neurodegenerative diseases like Alzheimer's and Parkinson's disease. Estrogen deficiency and apoE genotype are known risk factors in these diseases and these factors also affect olfaction. Therefore we examined the effects of estradiol replacement following ovariectomy on expression of apoE and markers of cell proliferation, neuronal maturation, synaptogenesis and reactive gliosis in the primary olfactory pathway of wild-type (WT) and apoE knockout (KO) mice. Estradiol replacement increased apoE staining in the olfactory nerve and glomerular layers. Estradiol increased astrocyte density and olfactory epithelium (OE) thickness regardless of the genotype. In addition estradiol treatment increased the number of mature neurons in the OE and glomerular synaptophysin in both genotypes, but the magnitude of increase was greater in the WT than in the KO mice. These data suggest that estrogen and apoE act synergistically to minimize the loss of mature sensory neurons and synapses following ovariectomy.

Keywords

estrogen; apoE; olfactory; glia; glial proteins; knockout mice

Introduction

Estrogen deficiency is a known risk factor for neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD) (Henderson, 2006, Paganini-Hill and Henderson, 1994, Tang, et al., 1996). Olfactory dysfunction often precedes clinical symptoms in these diseases (Bacon, et al., 1998, Calhoun-Haney and Murphy, 2005, Gilbert and Murphy, 2004, Murphy, et al., 1998, O'Hara, et al., 1998). Replacing estrogen lost during menopause reduces disease risk and also improves olfactory function (Caruso, et al., 2008, Deems, et al., 1991). These observations lead to the possible relation of estrogen loss to the progression of these diseases. Olfactory sensitivity to common odors in post-menopausal women was significantly better following estradiol treatment (Caruso, et al.,

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2008) and a retrospective study (Deems, et al., 1991) found that only four of the 99 post-menopausal women taking estrogen showed deficits in olfactory function, which is relatively low when compared to the prevalence of olfactory dysfunction in women in the general population. In essence these studies suggest that estrogen may influence olfactory function in humans. Part of these deficits may be mechanical since the inhalation and exhalation rhinomanometric values were higher in post-menopausal women following estradiol treatment (Caruso, et al., 2008). However, mechanical deficits appear inadequate to explain all deficits.

Recent studies suggest that apolipoprotein E (apoE) genotype modifies the beneficial effects of hormone therapy on olfactory and cognitive function (Bacon, et al., 1998, Calhoun-Haney and Murphy, 2005, Gilbert and Murphy, 2004, Murphy, et al., 1998, O'Hara, et al., 1998). Moreover, apoE genotype is a major risk factor for several neurological diseases including AD (Corder, et al., 1993). ApoE is a protein component of lipoproteins and humans have three major isoforms of apoE (apoE2, apoE3, and apoE4) that are produced by three alleles, ε2, ε3, and ε4, at a single gene locus on chromosome 19 (Davignon, et al., 1988, Hallman, et al., 1991, Menzel, et al., 1984, Weisgraber, 1994). The ε4 allele is linked to a higher risk and also to an earlier age of onset of AD (Corder, et al., 1993, Saunders, et al., 1993). The ε4 allele is also associated with impairment in olfactory function in the early stages of AD. Non-demented ε4-carriers showed dramatic decline in olfactory functioning as compared to individuals without ε4 allele (Sundermann, et al., 2007, Sundermann, et al., 2008). Decline appears to be specific to inheritance of ε4 allele, as AD patients with ε4 allele showed greater deficits in olfactory tests than siblings without ε4 allele (Handley, et al., 2006). Results from longitudinal studies also suggest that inheritance of ε4 allele leads to poor scores in olfactory tests (Calhoun-Haney and Murphy, 2005). In essence, these findings show that apoE plays a key role in olfactory function.

Estrogen administration appears to ameliorate olfactory dysfunction in apoE4 individuals. Sunderman *et al* showed that replacement of estrogen alone or combined with progesterone in women who had undergone hysterectomy improved olfactory functioning only in apoE4 carriers, but not in apoE4-negative individuals (Sundermann, et al., 2008). Together these studies suggest a role for estrogen in olfactory function, which is complexly modulated by apoE genotype. The mechanism underlying the interaction of estradiol on olfactory function is not clear.

We showed that treatment of estradiol in ovariectomized mice significantly increased apoE production in the olfactory bulb (McAsey, et al., 2006). We also found that apoE levels varied throughout the estrus cycle in the olfactory bulb of mice (Struble, et al., 2003). Increase in apoE production during estrus followed the surge of estradiol during proestrus, suggesting that estradiol increase may prime apoE production.

In the present study, we asked a simple but important question: What effect does estradiol replacement have following ovariectomy on expression of apoE and markers for cell proliferation, reactive gliosis, neuronal maturation, and synaptogenesis in the primary olfactory pathway? We used wild-type (WT) and apoE knockout (KO) mice to understand the impact of apoE deficiency on estradiol's effect.

We found that estradiol treatment increased apoE staining in the olfactory nerve and glomerular layers. Replacement was associated with a significant increase in process density of astrocyte, the primary producers of apoE in the CNS. However, apoE was not necessary for estradiol induced OE thickness. Estradiol increased the number of mature neurons in the OE and the glomerular synaptophysin in both genotypes, and the presence of apoE amplified the estrogen effect. These data suggest that estrogen and apoE act synergistically to

minimize the loss of mature olfactory sensory neurons and their synapses following ovariectomy.

Materials and Methods

Animals

Breeding pairs of WT C57BL/6 strain and homozygous apoE KO mice were purchased from the Jackson Laboratories, Bar Harbor, ME. ApoE genotype of the litters were verified by PCR and confirmed by immunoblotting using anti-apoE as described below. Four months old mice were used in this study.

Ovariectomy

WT and KO littermate mice, four months of age at the start of this study, were used. Mice were ovariectomized (OVX) by a dorsal, bilateral approach under ketamine (100 mg/kg) and xylazine (50 mg/kg) (Sigma, St. Louis, MO) anesthesia and sterile operating conditions. The animals recuperated for five days prior to estradiol/vehicle pellet placement. In previous studies we have shown that at five days post-OVX, estradiol levels were undetectable in mice (Cheng, et al., 2007, McAsey, et al., 2006). The animals were randomly assigned to either replacement with a pellet containing estradiol (0.36 mg, 60 day release, Innovative Research of America, Sarasota, FL) or a pellet containing only the binder (vehicle). Estradiol- or vehicle-pellets were placed subcutaneously using a trochar at the mid-scapular level. Preliminary results from our laboratory, and published results from several laboratories, have demonstrated that the estradiol pellets maintain 17 β-estradiol at a constant proestrus level for at least 60 days (Cheng, et al., 2007, Katovich and O'Meara, 1987, McAsey, et al., 2006, Rosenblum, et al., 1985).

Tissue preparation

Mice were sacrificed three days following implantation of estradiol- or vehicle-pellets. For fluorescence immunohistochemistry, mice were anesthetized as described above and transcardially perfused with cold saline (0.9% NaCl), followed by 4% paraformaldehyde in 0.1M PBS. Olfactory turbinates were removed and cryoprotected overnight in 30% sucrose in 0.1 M PBS. After cryoprotection, the turbinates were frozen with dry ice and sections were cut on a cryostat at 18 μm, and air dried for 2 h at room temperature.

CV Staining

Sections were rinsed in distilled water for 10 minutes and placed in the oven for 2 hours at 37°C. The sections were then defatted with xylene for 30 minutes. The sections were hydrated in a series of ethanol (100%, 95%, and 70%) for 10 minutes each. Sections were rinsed in water and stained in cresyl violet acetate solution (Sigma, St. Louis, MO) for 4 minutes. Sections were rinsed in water and in a series of ethanol (70, 95, and 100%). Following incubation in xylene for 30 minutes, sections were coverslipped using permount (Fisher Scientific, Fair Lawn, NJ).

Immunohistochemistry

Sections on slides were rinsed in 0.1 M PBS, and permeabilized with 0.2 % Triton X-100 (Sigma, St. Louis, MO) in PBS for 30 minutes at room temperature. The slides were rinsed once with PBS and treated with 70, 90, 100, 90, and 70% ethanol for two minutes each (Jang, et al., 2003). Non-specific immunoreactivity was attenuated by incubation in 2.25% gelatin in 0.1 M PBS for 1 h, followed by overnight incubation with primary antisera solution at 4°C (see Table 1 for source and dilution used). The sections were washed three times in PBS, and incubated for 1 hour at room temperature with secondary antibody

solution as listed in Table 1. The sections were washed three times in PBS, mounted in Vectashield (Vector labs, Burlingame, CA).

We chose primary antibodies that are thoroughly characterized and are highly specific to their respective antigen (Baker, et al., 1989, Buttini, et al., 2002, Castejon, et al., 2002, Keller and Margolis, 1975, Morrison and Prayson, 2000, Wang, et al., 2006, Wiedenmann and Franke, 1985). Moreover, for apoE staining, KO mice were also processed in parallel with WT mice. As shown in Figs. 1 and 2, apoE immunostaining was absent in the olfactory epithelium and olfactory bulb of apoE KO mice. In addition, specificity of all antibodies was evaluated by incubation with normal serum in place of the primary antisera which resulted in no staining.

Stained sections were examined using an Olympus BX-50 microscope. Images were captured using a Pixera Digital Camera (Pixera, Los Gatos, CA) and saved as high resolution TIF files. Figures from images were assembled using Photoshop (Adobe, San Jose, CA). Image analysis was performed using Scion Image software (Scion Image, Frederick, MD).

Quantification

Morphological thickness of the OE was determined from image calibration of a stage micrometer in Scion Image. Thickness was repeatedly measured from the horizontal basal cell layer to the head of the sustentacular cells. The number of OMP^+ cells in ten 100 μm segments of OE and $BrdU^+$ cells in 1 mm of OE was counted utilizing Scion Image.

GFAP process density was calculated by a point count method of grid intersections (Struble, et al., 2006). A 5 X 5 cell grid, 25 μm/cell side, was overlaid on the live image and each immunostained process (but not cell body) intersecting the grid was marked (Fig. 3). At least three nonoverlapping samples were obtained and averaged to give a single intersection score for each area.

Statistical Analysis

All quantification procedures were performed using three mice per genotype (WT, KO) and three mice per treatment (estrogen, vehicle). A total of 10 measurements were taken from each animal. The data in individual experiments were presented as mean ± standard error and statistical analysis (ANOVA, Repeated Measures ANOVA) was performed using SYSTAT. Post-hoc testing at each day was performed with t-tests using $p<0.001$ as the significant difference to protect against type I error. A blinded procedure was employed in all experiments so the experimenter was unaware of the genotype (WT versus KO) and treatment (estradiol versus vehicle) received by the animals.

Results

ApoE

ApoE immunostaining was comparable in estradiol- and vehicle-treated WT mice over the time course of this study (Fig. 1). In both groups, apoE immunoreactive processes were present between the olfactory sensory neuron (OSN) bundles and at the olfactory epithelial surface where the cell bodies of the sustentacular cells reside. As noted in a previous study, the immunoreactive processes in the estradiol-treated mice terminated on faintly stained globular structures above the unstained basal lamina. We previously showed that these globular structures expressed GBC-1, a marker for globose basal cells (Nathan, et al., 2007). In the vehicle group, apoE immunostaining was concentrated on oblong cells that were arranged on a plane parallel to the unstained basal lamina. Whether or not these oblong cells

are horizontal basal cells was not determined (Holbrook, et al., 1995). ApoE staining in the lamina propria was intense in the endothelial cells of blood vessels in both estradiol- and vehicle-treated groups. Diffuse apoE staining was observed throughout the core of the nerve bundles in estradiol-treated mice; whereas, only very faint apoE staining was observed in the vehicle-treated mice.

In contrast to the OE, estradiol treatment increased apoE staining in the olfactory nerve and glomerular layers in the OB throughout the duration of the study (Fig. 2). ApoE immunostaining was weak throughout the OB in the vehicle-treated mice. ApoE staining in estradiol-treated mice was observed in the olfactory nerve. Large olfactory nerve fascicles consistently stained, and were demarcated from each other by densely stained cellular processes. Glomeruli were clearly outlined by immunostained cells in the septae surrounding the glomeruli as previously described (Nathan, et al., 2001, Struble, et al., 1999). These studies suggest that estradiol treatment has major effect on apoE expression in the OB, but not in the OE of ovariectomized mice.

Glial fibrillary acidic protein

To identify the source of apoE increase in the OB of estradiol-treated mice, we examined astrocytes that are the primary producers of apoE in the CNS. Quantification of the glial fibrillary acidic protein (GFAP, reactive astrocyte marker) process density revealed that estradiol treatment significantly ($F_{1,40}$ =122.92; $p<0.001$) increased process density in both the WT and KO mice (Fig. 3). GFAP process density in WT mice appeared to be greater than that in the KO mice, although this did not reach standard levels of significance ($F_{1,40}$=3.19; $p<0.08$).

OE thickness

The OE thickness was measured in CV stained sections. The OE was thicker at all time points in the WT mice than the KO mice (F1,40=6.21; $p<0.001$) (Fig. 4). Estradiol treatment increased OE thickness by about 28% ($_{F1,40}$ =58.62; $p<0.001$) over the course of the study. ApoE presence or absence did not modulate this estradiol effect.

Olfactory marker protein

Olfactory marker protein (OMP) labels mature OSN in the OE (Margolis, 1972). We found a significant three-way interaction between genotype, day and estradiol treatment in the OMP data (F5,40=6.90; $p<0.001$) raising a complex interpretation; therefore Bonferroni-corrected post-hoc testing among groups was performed at each day (Fig. 5). Estradiol replacement was associated with an increased density of OMP cells on day 3 in both genotypes. Initial increase was followed by decline reaching a nadir on day seven. By day 21, the estradiol-replaced WT was significantly greater than the other three groups that were statistically equivalent to each other. At 42 days the estradiol-treated WT was greater than the estradiol-treated KO which was greater than both the vehicle-treated genotypes. Finally at day 56, the estradiol-treated KO had caught up with the estradiol-treated WT, followed by the vehicle-treated WT then the vehicle-treated KO. In essence, estradiol replacement was associated with increased OMP density and this effect was facilitated by the presence of apoE.

Synaptophysin

In general Syn staining in the glomeruli of the OB followed the density of mature olfactory neurons in the OE as measured by OMP. At 21 days the WT estradiol treated mice were greater than any of the groups (Fig. 6). The estradiol-treated KO mice recovered approaching the WT estradiol levels by 42 days. In essence, the presence of estradiol and

apoE acted synergistically to minimize the loss of synaptophysin seen following ovariectomy

Discussion

It is important to consider the cellular dynamics of OE changes following OVX. Our study started five days following OVX at which time we found no difference between genotypes for CV, OMP or GFAP. Syn was less in the apoE KO as we and others have previously reported (Masliah, et al., 1995, Nwosu, et al., 2008). We then implanted estradiol or vehicle and started sampling treated groups at 3 days (eight days post OVX). For analysis, we expressed our data as a percent of five days post-OVX. Our data suggest that the OE shows significant changes 8 days following OVX. This change is not indicated by OE shrinkage, which samples both mature and immature OSN. Estrogen replacement transiently increased the density of OMP^+ cells, which was followed by a decline. We hypothesize that this transient increase represented immature OSN cells *in situ* promoted by estradiol to display a mature phenotype (OMP^+). However, this maturation is transient resulting in a nadir at seven days. A similar pattern was seen for OB synaptophysin, with the nadir reached at 21 days and then followed by a progressive recovery. Hence we speculate that OVX results in loss of mature OSN that begins to become apparent at 8 days post-OVX.

Several studies have shown that estradiol increases apoE expression in both neuronal and non-neuronal tissues (Nathan, et al., 2004, Srivastava, et al., 2001, Srivastava, et al., 1996, Srivastava, et al., 1997, Stone, et al., 1997, Wang, et al., 2006). In contrast to these studies apoE expression levels in the OE were comparable in estradiol- and vehicle-treated mice at all time points post-treatment. In the vehicle-treated mice, apoE staining was at low levels and was diffusely located in the olfactory nerve and glomerular area. Estradiol treatment increased apoE expression in the olfactory nerve and the glomerular layer of the OB. The intensely stained cellular structure in the olfactory nerve layer resembles ensheathing glia and their processes. The immunostained cells around the glomeruli appear to be astrocytes, as previous studies have shown that astrocyte and its network of processes surrounds the neuropil of the glomeruli. The estradiol induced increase of apoE in the OB and olfactory nerve remained elevated throughout the entire course of the study, and no major intraday variation in intensity and localization pattern was evident.

Astrocytes are the primary producers of apoE in the CNS (Boyles, et al., 1985). We found that estradiol treatment significantly increased GFAP process density in the glomerular layer in WT mice throughout the entire time course of this study regardless of genotype. It is possible, therefore, that activation of astrocytes lead to increased production of periglomerular apoE.

Estradiol treatment significantly increased OE thickness in both WT and KO mice. We and others have shown that estradiol can increase basal cell proliferation (Barha, et al., 2009, Beites, et al., 2005, Nathan, et al., 2010, Pawluski, et al., 2009). Basal cell proliferation and subsequent differentiation to sensory neurons could potentially lead to an increase in OE thickness. Alternatively, estradiol treatment may have also increased the number of mature OSN in the OE by facilitating synapse formation. Our OMP data discussed below correspond with this assumption.

The OMP is a marker for mature OSN that have established functional synapses with the OB neurons (Graziadei, et al., 1980, Sydor, et al., 1986). Estradiol-treated mice of both genotypes had significantly more OMP^+ cells on the day 3 than vehicle-treated mice. Estradiol could have protected mature neuronal death induced by loss of ovarian hormones. Alternatively, estradiol treatment could also have pushed residual OSN into expressing an

adult phenotype by promoting axonal growth and synapse formation of the immature OSN. The increase of OMP cells at 21 days may represent this increased growth. The robust neuroprotection by estradiol did not persist on treatment day seven as the number of OMP^+ cells in the both groups reached the lowest level. Following this sharp decline estradiol treatment promoted maturation of OSN in both genotypes, however, the recovery of OMP^+ cells was faster and greater in the WT mice as compared to KO mice. This data is consistent with previous studies showing a synergistic effect of apoE and estradiol in axonal growth and maturation of neurons (Nathan, et al., 2004, Teter, et al., 1999).

Estradiol replacement was associated with increase in density of presynaptic marker, synaptophysin, which is clearly evident in the WT mice. There are several possible explanations for this increase. First, estradiol replacement could preserve Syn terminal from degeneration as a consequence of ovariectomy which has been previously noted in other parts of the brain (Masliah, et al., 1995, Nwosu, et al., 2008). Our studies extend these findings to the olfactory bulb. Importantly, the Syn we measured sampled terminals both intrinsic to the OB and extrinsic (OSN). The absence of apoE in KO mice appears to diminish the protective function of estradiol on Syn. Second, estradiol replacement could increase synaptogenesis. Previous studies have shown estradiol induced increase in synaptic proteins, including Syn (Frick, et al., 2002, McAsey, et al., 2006, Rune, et al., 2002, Sharma, et al., 2007). Third, estradiol treatment could potentially increase either the number of synaptic vesicles per terminal or the number of Syn molecules per vesicle without altering the number of synapses. In addition to these possibilities, our results could be explained by proposing varying role for estradiol in synaptic structures during the course of the study. Estradiol treatment could have protected synaptic structures in the glomeruli during the early days after ovariectomy, but could have facilitated synaptogenesis in later days.

To our knowledge very few studies have looked at the effects of estradiol fluctuations during estrus cycle on olfactory structure and function in animal models. Olfactory function varied during estrus cycle in mice, with heightened olfactory sensitivity to detect a buried food during the estrus stage of the cycle (Kumar and Archunan, 1999). One study has looked at the effects of estradiol on the olfactory system repair, and the results showed that estradiol replacement significantly improved recovery of olfactory discrimination performance post OE injury in rats (Dhong, et al., 1999). The physiological basis underlying this latter finding is not known.

The results from this present study suggest that estradiol deprivation by ovariectomy may result in degeneration of the OE that could, in part and transiently, be inhibited by replacing estradiol. Recovery to presumed adult levels (56 days) was facilitated by both estradiol and apoE. Future studies need to be performed to dissect the molecular pathways behind the interaction of estradiol with apoE in the olfactory system.

Given that estradiol replacement can result in both apoE dependent and independent processes in the OB and OE, it is not surprising that human studies are complex. Moreover, estrogen replacement in very elderly patients, where OE is substantially atrophic, may complicate interpretations (Trojanowski, et al., 1991). ApoE3, which is similar to mouse apoE, may promote basal cell proliferation, differentiation, and axonal growth and thereby protects from olfactory decline in women. In contrast, if apoE4 is produced in the olfactory system, estrogen use may not help repair the atrophic OE, which may explain the dramatic decline in olfactory function in individuals with apoE4-allele (Calhoun-Haney and Murphy, 2005, Sundermann, et al., 2007, Sundermann, et al., 2008).

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Abbreviations

ApoE	apolipoprotein E
OB	olfactory bulb
OE	olfactory epithelium
OSN	olfactory sensory neuron
GBC	globose basal cells
Sus	Sustentacular cells
OMP	olfactory marker protein
GFAP	glial fibrillary acidic protein
Syn	synaptophysin

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Highlights

- Estradiol treatment increases apoE expression in the olfactory system.
- Estradiol interacts with apoE to reduce olfactory neuronal loss post-ovariectomy.
- Estradiol increases neuronal and synaptic density in the olfactory system.

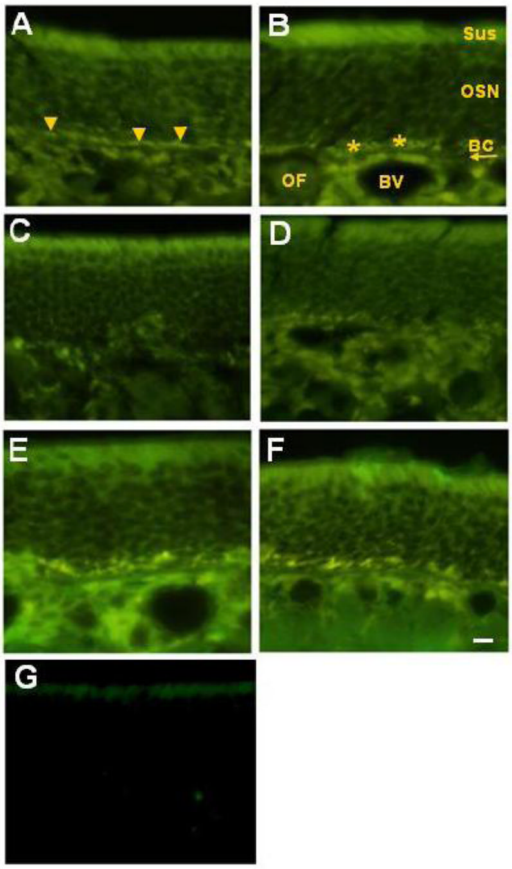

Fig. 1.

Effects of estradiol on apoE expression in the adult mouse olfactory epithelium. ApoE immunoreactivity in ovariectomized wild-type mice implanted for 3 (A, B), 21 (C, D) and 56 (E, F) days with either a vehicle (A, C, E) or estradiol pellet (B, D, F). Sus, sustentacular cells, OSN, olfactory sensory neuron, BC, basal cell zone, BV, blood vessel, OF olfactory fascicle. Arrow indicates basal lamina. ApoE immunoreactivity in vehicle- and estradiol-treated mice was intense in the perikarya of the sustentacular cells and was faint around the OSN. Intense apoE staining above the basal lamina was present in oblong cells (arrow heads) in the vehicle group, and in punctas surrounding globose cells in the estradiol treated group (asterisks). Endothelial cells of the blood vessels in both treatment groups were strongly stained. ApoE immunostaining was present throughout the core of the olfactory fascicle in estradiol treated mice, and was very faint in the vehicle treated mice. Absence of apoE staining in the olfactory epithelium of apoE knockout mice (G). Scale bars = 10 μm.

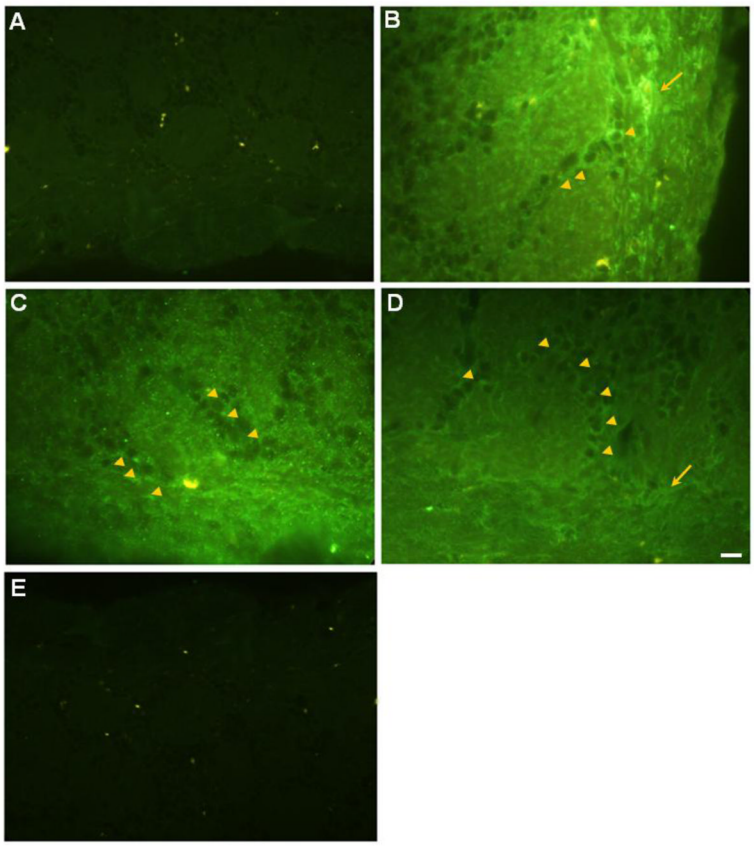

Fig. 2.

ApoE immunostaining in the olfactory nerve and glomerular layer of the OB of ovariectomized WT mice implanted with either vehicle (A) or estradiol (B, C, D) pellet. ApoE immunostaining was weak throughout the OB in mice implanted for 3 days with a vehicle pellet (A). In mice implanted with estradiol pellet for 3 (B), 21 (C), and 56 (D) days, dense apoE immunoreactivity was observed in cellular processes (arrows) in the olfactory nerve fascicles and in cells surrounding the glomeruli (arrow heads). Absence of apoE staining in the olfactory bulb of apoE knockout mice (E). Scale bars = 10 μm.

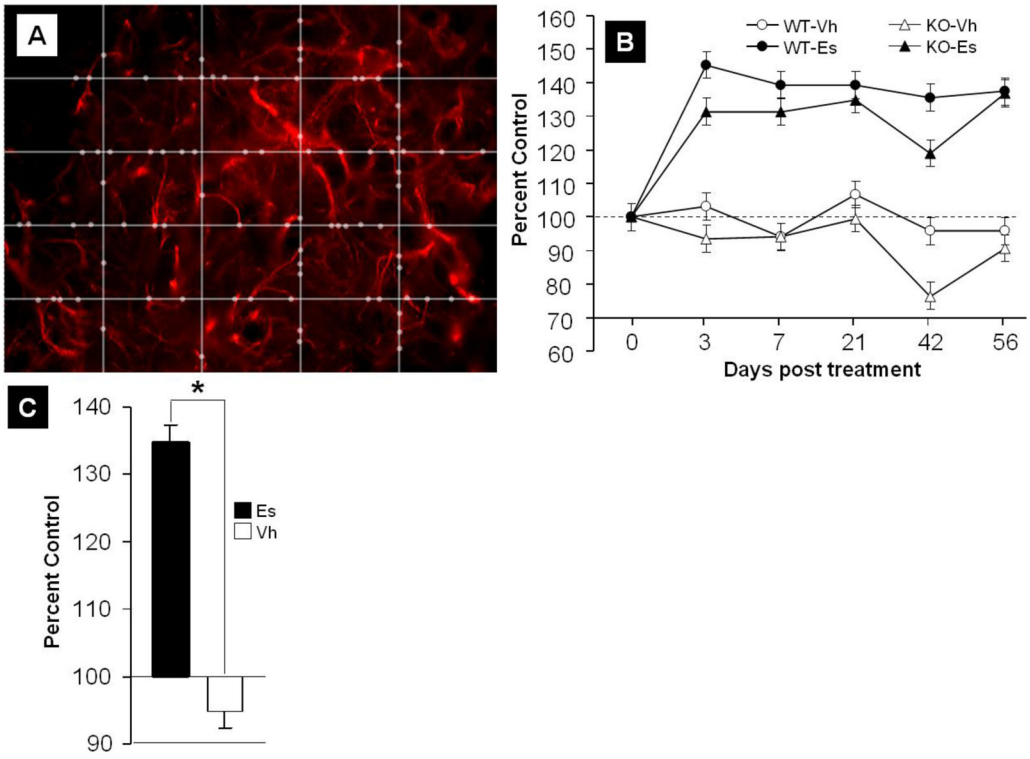

Fig. 3.

GFAP immunoreactivity in the glomerular layer of the olfactory bulb. (A) GFAP immunoreactive processes density was calculated by superposition of a computer generated grid over the microscopic section. Each intersection of a GFAP immunoreactive process is marked with a circle. (B) Quantification of GFAP immunoreactive process density in vehicle (Vh) and estradiol (Es) treated WT and KO mice (Mean and SE). (C) Collapsing the genotype effects across the time points shows that estradiol treatment significantly (* $p<0.001$) increased GFAP immunoreactive processes as compared to vehicle treatment.

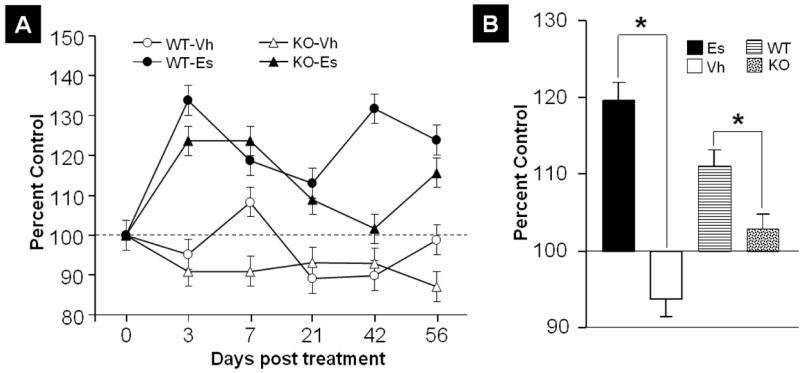

Fig. 4.

(A) Quantification of olfactory epithelium thickness in WT and KO mice treated with estradiol (Es) or vehicle (Vh) (Mean and SE). (B) Pooling the genotype effects across the time points shows that estradiol treatment significantly (*p<0.001) increased OE thickness as compared to vehicle treatment. In addition, WT mice had thicker OE (*p<0.001) as compared to apoE KO mice, irrespective of the treatment.

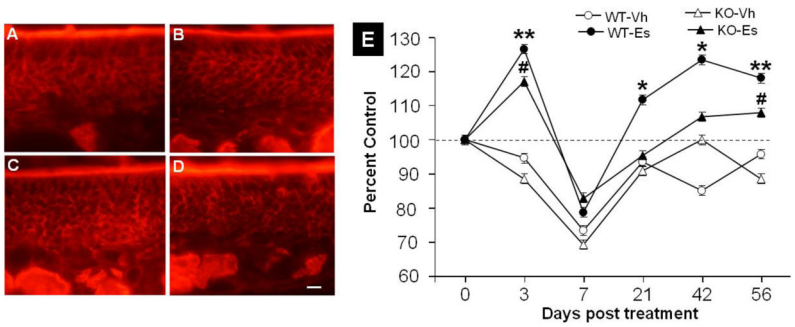

Fig. 5.

OMP stained sections of olfactory epithelium from WT (A, C) and apoE KO (B, D) mice implanted with an estradiol (C, D) or a vehicle (A, B) pellet for 21 days. Irrespective of the genotype, estradiol treatment increased OMP^+ cells; however, estradiol-treated WT mice had significantly more OMP labeled cells than the estradiol-treated KO mice. Scale bars = 10 μm. (E) Quantification of OMP^+ cells in WT and KO mice treated with estradiol (Es) or vehicle (Vh) (Mean and SE). WT-Es versus all other groups (* $p<0.001$), WT-Es versus vehicle groups (** $p<0.001$), KO-Es versus vehicle groups (# $p<0.001$).

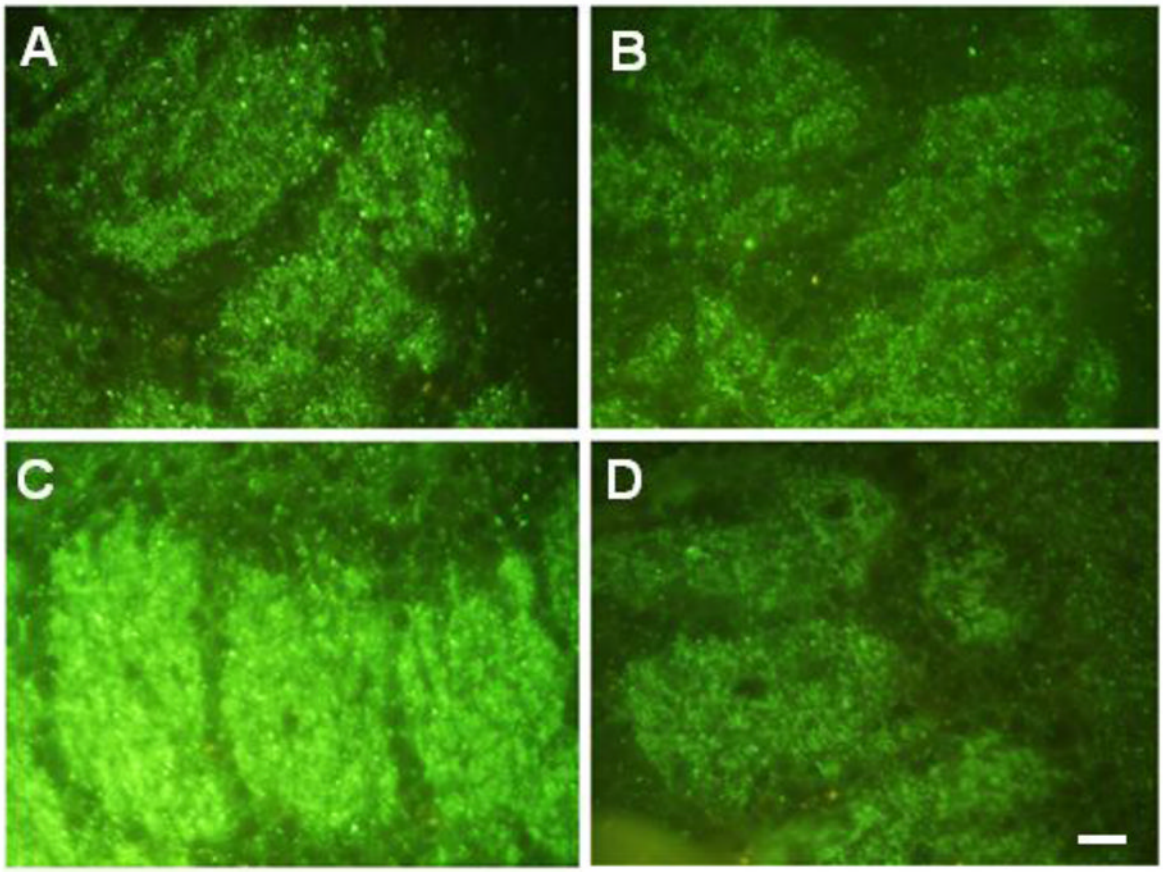

Fig. 6.
Syn stained sections of olfactory bulb from WT (A, C) and apoE KO (B, D) mice implanted with an estradiol (C, D) or a vehicle (A, B) pellet for 21 days. The WT estradiol treated mice were greater than any of the other groups. Scale bars = 10 μm.

Table 1

List of primary and secondary antibodies used in this study

Antibodies	Host	Source	Dilution
ApoE	Goat	Calbiochem, San Diego, CA	
OMP	Goat	Wako, Richmond, VA	1:500
Syn	Rabbit	Cell Marque, Rocklin, CA	1:500
GFAP	Mouse	Accurate, Westbury, NY	1:500
FITC-anti goat	Donkey	Jackson, West Grove, PA	1:500
Cy3-anti goat	Donkey	Jackson, West Grove, PA	1:500
Alexa-anti rabbit	Donkey	Invitrogen, Eugene, OR	1:200