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Interaction Of Apoe With Estrogen In The Olfactory System During Nerve Maintenance And Recovery

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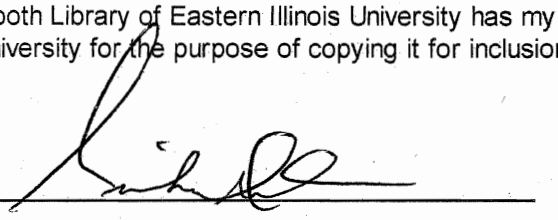

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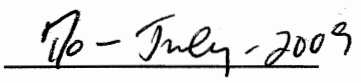

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Interaction of ApoE with Estrogen in the Olfactory System During Nerve Maintenance and Recovery

BY

Michael M. Tonsor II

THESIS

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MASTER IN BIOLOGICAL SCIENCES

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CHARLESTON, ILLINOIS

2009
YEAR

I HEREBY RECOMMEND THAT THIS THESIS BE ACCEPTED AS FULFILLING THIS PART OF THE GRADUATE DEGREE CITED ABOVE

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Abstract

Estrogen replacement profoundly aids in nerve regeneration after injury in adult mammalian models. Estrogen replacement also protects against olfactory dysfunction in mammals. The mechanism underlying estrogen's neuroprotective effects remains ambiguous. My goal is to investigate and better clarify if apolipoprotein E (apoE), a lipid transporting protein, is a critical intermediary to estrogen's effects on nerve maintenance and regeneration.

My hypothesis is that apoE is a mediator of estrogen's beneficial effects. This hypothesis is based on the following observations from previous studies. First, olfactory nerve regeneration was delayed in apoE-gene knockout (KO) mice as compared to wild type (WT) mice. Second, 17β-estradiol replacement in ovariectomized (OVX) mice resulted in a significant increase in levels of apoE and its receptor, LRP, in the olfactory bulb. Third, estradiol treatment increased both apoE and neurite outgrowth in cortical and olfactory neuronal cultures. Fourth, estradiol treatment had no effect on neurite outgrowth in cultures derived from apoE KO mice. This thesis study focuses on *in vivo* studies in the olfactory system.

I evaluated estrogen and apoE in the olfactory nervous system during normal nerve maintenance and during nerve repair post injury over a period of 56 days. WT and KO mice were treated with either a placebo or 17β-estradiol pellet. The olfactory epithelium (OE) and the olfactory bulb (OB) were examined by means of cresyl violet staining and immunohistochemistry. Staining performed in the OE investigated thickness, apoE, cell division, immature olfactory receptor neurons (ORNs), and mature ORNs. Staining in the OB investigated OMP and SYN immunoreactivity and astrocytes.

My results indicated that estrogen and apoE are critical in supporting olfactory nerve maintenance and regeneration in mice. Estrogen increased apoE which in turn facilitated maturation of olfactory neurons in the olfactory system over time. Also, nerve repair was faster and more efficient in the presence of estrogen. Estrogen increased cell division early in the repair process via an apoE requiring mechanism. Additionally, estrogen repaired the olfactory nerve beyond normal maturation in WT mice. In contrast to WT mice, KO mice nerve repair was slower and incomplete. Furthermore, estrogen had no effect on the rate of olfactory nerve regeneration in KO mice.

Dedication

I would like to thank my advisor, teacher, and friend Dr. Britto Nathan for giving me the opportunity to conduct research under his guidance. Dr. Nathan always saw the maximum potential in me, and I am grateful he was there to counsel me through my academic pursuits at Eastern Illinois University.

I would like to thank Dr. Gary Bulla and Dr. Ann Fritz, my committee members, and Dr. Costa for support and guidance throughout my graduate studies.

I would like to thank Sarita Upadhyay for her instruction and aid in the laboratory. Sarita was there for me not only as a guru of histology but as a friend.

I would like to thank my fellow students: Sreenivas Nannapaneni and Ikemefuna Nwosu who spent countless hours with me passing on their expertise and knowledge. Minh Luong, Aseem Hussain, Salina Gairhe, Mallory Stenslik, Stephen Clark and Preeti Dhakal for sharing countless hours with me in the research lab offering cooperation, friendship, and encouragement.

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I would like to thank my parents for their ongoing support. They have been there for me throughout pursuit of my dreams and backing me with every decision I make. I am forever grateful that God has blessed me with two wonderful parents.

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Dr. James Schwob, Tufts University, Boston, for generously providing antibodies for immunohistochemistry.

Table of Contents

Page

List of Figures

Figures by Chapter Page

Glossary of Terms

AD-Alzheimer's Disease

ApoE- Apolipoprotein E

BrdU- Bromodeoxyuridine

CNS-Central Nervous System

CSF-Cerebrospinal Fluid

ET-Estrogen Therapy

Gap43- Growth Associated Protein-43

GFAP- Glial Fibrillary Acidic Protein

KO- Knock Out

OB- Olfactory Bulb

OE- Olfactory Epithelium

OMP- Olfactory Marker Protein

ORN- Olfactory Receptor Neuron

OVX- Ovariectomy

PD-Parkinson's Disease

SYN- Synaptophysin

WT- Wild Type

Introduction

ApoE

The primary function of apoE is to redistribute cholesterol and other lipids through interaction with lipoprotein receptors on its target cells (1-5). Binding to target cells triggers uptake and degradation of lipoproteins, which ensures lipid availability to be used in intracellular metabolism. The protein structures of apoE from 10 different species have been identified, and the human species has three common isoforms: apoE2, apoE3, and apoE4. Human isoforms differ by amino acids in positions 112 and 158 in protein structure (6-9). ApoE2 has cysteine in both positions, and apoE4 has arginine in both positions. ApoE3 has cysteine in position 112 and arginine in position 158. ApoE3 is the most common isoform in humans, and mice have a single form of apoE similar to that of apoE3. ApoE in mice is similar in structural, functional, and binding properties (6, 10).

ApoE in the Nervous System

ApoE is the major apolipoprotein in the brain and the CSF and is produced by the astrocytes and microglia cells (11-13). 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) (14, 15). ApoE has been demonstrated as the only apolipoprotein in the CSF that can interact with lipoprotein receptors (12-13). Previous studies have determined that apoE containing lipoproteins in the CSF bind to LDL receptors. The LRP and LDL receptors mediate the binding and internalization of apoE-containing lipoproteins in cultured neurons (16).

These findings are significant because they show that apoE is important in normal functioning neurons through lipoprotein transport, binding, and internalization.

ApoE and Neuronal Repair

ApoE has been suggested as a possible mechanism for nerve maintenance and repair (1, 17-20). ApoE has been shown to increase nerve substantially in a variety of injury studies. Results from previous studies demonstrated that OB levels of apoE increased two-fold at 3 days post OE injury in mice (21). ApoE levels remained elevated by approximately 1.5 normal levels at 7 and 21 days after injury before returning to normal levels at 56 days. Both immunoreactive astroglia and microglia had large amounts of apoE in the OB and OE post injury (21).

An increase in the amount of ApoE in the nervous system seems to aid in neuronal repair. In a previous study, it was found that mice with apoE deficiency, KO mice, experienced nerve regeneration at a slower rate compared to WT mice (22). ApoE has also been considered as supporting neuronal repair following nerve lesion in other injury models. Currently, these studies suggest that apoE is produced at the injury site and searches for lipid released from degenerating cells. ApoE provides lipids to growth cones of sprouting axons for formation of new membranes in degenerating cells, thus supporting the nerve regeneration process (22-25).

Estrogen and Neuronal Repair

Current widespread findings testify to estrogen's beneficial effects on neuroprotection and neuronal repair. A number of studies have shown various beneficial effects of estrogen on the brain. A series of studies by McEwan and Woolley have determined that an influx of estrogen during the estrus cycle corresponded with an

increase in hippocampal spine density. Furthermore, researchers found that ovariectomy decreased spine density when normal blood level estrogen was depleted (26, 27). Estradiol treatment facilitated presynaptic density (SYN immunoreactivity) following entorhinal lesion (25, 28). Estradiol also aided in regeneration of hamster facial nerve after injury (29). A comparable enhanced effect of estradiol on sciatic nerve regeneration and muscle innervations after injury was observed in mice and rabbits (30, 31). In the basal forebrain, estradiol treatment encouraged axonal repair of cholinergic neurons post IgG-saporin induced injury (32). Several experiments have demonstrated that estradiol treatment prevented hippocampal neuronal loss induced by excitotoxin kainite (33, 34). Lastly, a group of studies have shown protection of several areas of the brain from ischemic injury due to estradiol treatment (35-38).

Few studies have examined whether or not estradiol treatment results in improvement of neuronal function. In one such study, spatial memory was improved by estrogen treatment. It was determined that estrogen increased hippocampal dendritic spine density (39). Moreover, estradiol which was intrahippocampally injected enhanced spatial memory in rats (40).

Only one study has examined the effects of estradiol on olfactory system repair. In this study, estradiol treatment showed significantly better and faster repair of olfactory discrimination performance post OE injury (41). The physiological basis for this finding has not yet been determined.

Estrogen Receptors in Olfactory System

Published studies have shown expression of estrogen receptors (ER) in the olfactory system (42-44). The alpha subtype of ER (ERα) is primarily expressed in cells

of the granule cell layer (42, 43). Whether these ERα expressing cells are granule cells or glia has to be examined. The second major type of estrogen receptor, ERβ, is highly expressed in glomerular, external plexiform, and granule cell layers of the OB (42, 44). Mitral cells of the rat express RNA message for ERβ (45). Nerve fibers in the olfactory nerve and external plexiform layers intensely stain for ERβ (43). The role of ER in the olfactory system is not known.

Estrogen and Neurological Diseases – The ApoE Link

Currently, conflicting results exist from clinical trials which have examined the benefits of estrogen in decreasing the risk of neurological diseases. Epidemiological studies imply that estrogen therapy (ET) protects against Alzheimer's and Parkinson's diseases (46-48). In a Women's Health Initiative Memory study, estrogen neither slowed the progress of dementia nor protected against decline in cognition (49, 50). This thesis seeks to clarify how estrogen affects the nervous system using olfactory nerve models, and hopefully solve conflicting studies of ET.

Recent studies have suggested the benefits of ET are modulated by apoE isoforms in the nervous system. Assessment of apoE genotypes and ET on learning and memory in postmenopausal women determined that higher learning and memory performance was observed in women taking ET who were not carriers of the apoE4 allele (51). Women with apoE4 taking ET performed the same on memory testing as those women not receiving ET. Moreover, ET has been shown to only reduce cognitive decline in women who were not carriers of the apoE4 allele (52).

ApoE alleles are associated with olfactory malfunction in Alzheimer's disease. Many studies have shown that apoE4 individuals have a significant decline in odor

threshold and identification. These subjects also experience delays in the processing of olfactory information (53-57).

Furthermore, ApoE4 has been shown to increase neurofibrillary tangles and amyloid plaques in the brain (58, 59). These are both associated with AD. Studies have shown increased degeneration of neurons and slowed dendritic growth in the brain of apoE4 individuals (60).

The resulting explanation for apoE specific isoform effects on olfactory dysfunction and cognition is not known. Estrogen and apoE genotype together play an important role in neurological health. The purpose of this thesis is to uncover the underlying mechanism between apoE and estrogen.

The Olfactory System as a Model for Neuronal Regeneration

The olfactory system in mice is a unique model used to study the effects of estrogen in normal nerve function and repair. The olfactory system regenerates throughout the life of the organism. Regeneration occurs in the olfactory epithelium (OE) were olfactory receptor neurons (ORNs) are constantly dying off and being replaced (61, 62). The OE mainly consists or immature and mature neurons. Mature ORNs synapse with the mitral and tufted cells in the olfactory bulb (OB) (Figure A). Therefore, a complete analysis of olfactory nerve can be generated. Following nerve lesion, ORNs begin to repopulate themselves from dividing stem cells in the basal lamina of the OE giving rise to developing immature and mature ORNs (61). ORN repair can be observed through immunohistochemical analysis over an existing time course of 56 days.

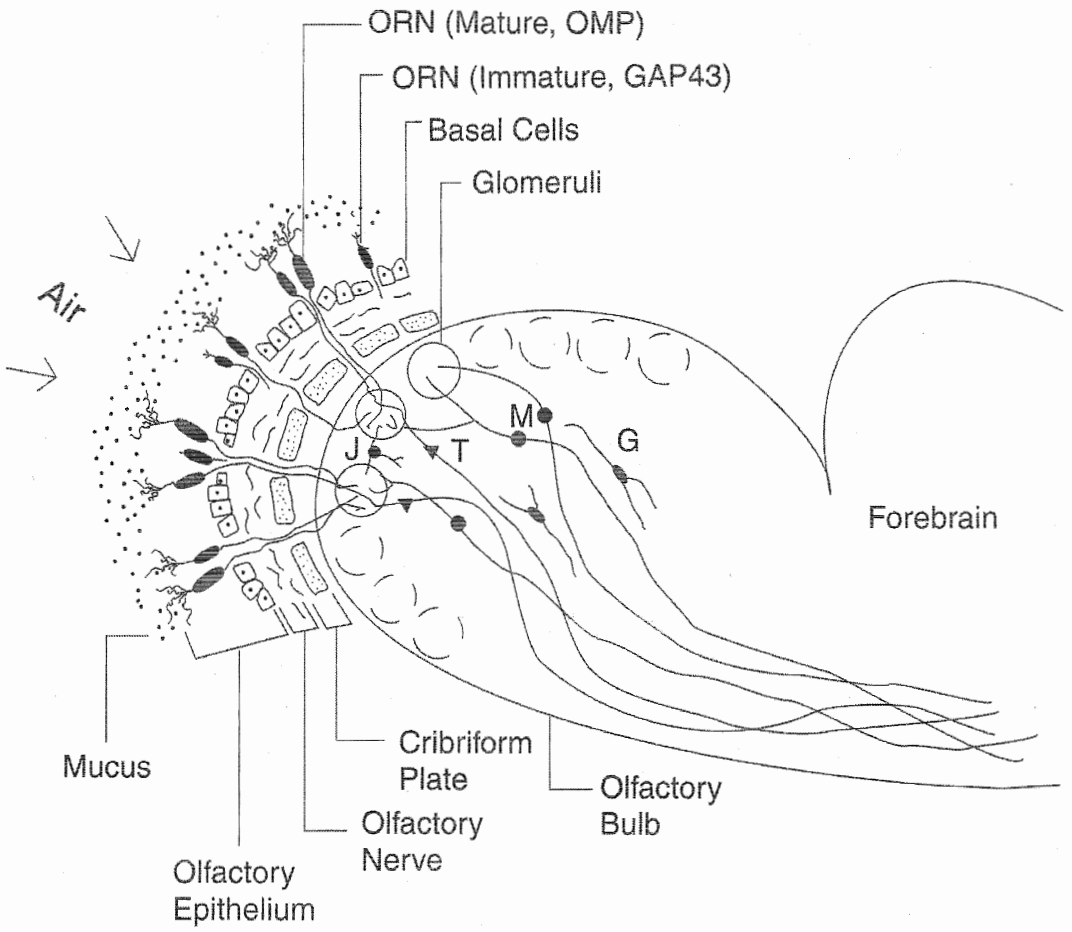


Fig. A- Organization of the olfactory system. Cilia on immature and mature ORNs bind odorants in the air. Axons of mature ORN conduct and synapse with the mitral (M) and tufted (T) cells in the glomeruli of the olfactory bulb. Mitral and tufted cells project their axons through the granule (G) cell layer of the OB and terminate in the forebrain. Basal cells layer the foundation of the olfactory epithelium and divide to develop more immature ORN.

Aims of Study

Specific Aim 1:

Specific aim 1 was to determine the effect of short term estrogen replacement on olfactory nerve maintenance. This specific aim targeted the role of estradiol in an olfactory system which was absent of injury. Specifically, the OE and the OB were examined 3 days after estradiol and placebo pellet implantation. A complete depletion of blood level estrogen was performed by bilaterally ovariectomizing female mice. The OE and OB tissue was examined in both WT and KO mice to determine whether or not apoE genotype affected the olfactory system during estradiol replacement. Furthermore, the goal of this study was to generate critical hypotheses into the role of estradiol replacement in long term maintenance (non-lesion) and during reconstitution of olfactory nerve post injury. This specific aim addressed the following questions:

(a) Does short term estradiol replacement have an effect on the microanatomical appearance of the OE (i.e. thickness)?

(b) Does short term estradiol replacement have an effect on sustainability of ORN in the OE?

(c) Are the synapses of mature ORNs with bulbar neurons affected by short term estradiol replacement?

(d) Are apoE levels in the OE affected by short term estradiol replacement in WT mice?

(e) Are apoE levels in the OB affected by the short estradiol replacement in WT mice?

(f) Does short term estradiol replacement modulate the occurrence of apoE producing astrocytes in the OB?

Results for specific aim 1 will be discussed in chapter 1.

Specific Aim 2:

Specific aim 2 was to determine the effects of long term estrogen replacement on olfactory nerve maintenance. This specific aim also examined the role of estradiol in an olfactory system which was absent of injury. The OE and OB were examined at 0, 3, 7, 21, 42, and 56 days post estradiol replacement in both WT and KO female mice to determine whether or not there was an effect in the olfactory system with estradiol and apoE. Results were critical in identifying the role of long term estradiol replacement on the olfactory system. More importantly, findings indicated whether the absence of estrogen over an extended period of time affected the olfactory system. This specific aim addressed the following questions:

(a) Does long term estradiol replacement have an effect on the microanatomical appearance of the OE (i.e. thickness)?

(b) What effect does long term estradiol replacement have on maturation sustainability in the OE and the OB?

(c) Are the synapses of mature ORNs with bulbar neurons affected by long term estradiol replacement?

(d) Are apoE levels in the OE and the OB effected by the long term estradiol replacement in WT mice?

(e) Does long term estradiol replacement modulate the density of apoE producing

astrocytes in the OB?

Results for specific aim 2 will be discussed in chapters 2 and 4.

Specific Aim 3:

Previous studies have documented that estrogen facilitated axonal growth in animal models and various cell culture systems. The objective of specific aim 3 was to determine if estradiol treatment in ovariectomized WT female mice improved the rate of olfactory nerve recovery following injury. As in specific aim 2, the OE and the OB were examined over the same time course post nerve injury. The following questions were addressed:

(a) Does estradiol treatment affect basal cell proliferation in the OE post lesioning?

(b) Does estradiol treatment promote faster recovery of ORN population post injury?

(c) Does estradiol treatment facilitate axonal growth of ORNs and re-innervation in the OB?

(d) Does estradiol treatment have an effect on apoE expression in the OE and the OB over the time course of repair?

(e) Does estradiol treatment promote synapse development of the newly generated ORNs with bulbar neurons?

(f) Does estradiol treatment contribute to the density of apoE producing astrocytes in the OB?

Results for specific aim 3 will be discussed in chapter 3.

Specific Aim 4:

Cell culture studies have suggested that apoE is required for estrogen to promote neurite outgrowth. Similarly, apoE expression was necessary for increased axonal sprouting post entorhinal cortex lesion due to estradiol treatment. The goal of this aim was to identify whether or not apoE was associated with estradiol's beneficial effects in regeneration post injury. The same parameters were examined in ovariectomized KO female mice and the same questions were addressed as those of specific aim 3.

(a) Does estradiol treatment effect basal cell proliferation in the OE post lesioning?

(b) Does estradiol treatment promote faster recovery of ORN population post injury?

(c) Does estradiol treatment facilitate axonal growth of ORNs and re-innervation in the OB?

(d) Does estradiol treatment promote synapse development of the newly generated ORNs with bulbar neurons?

(e) Does estradiol treatment contribute to the density of astrocytes in the OB?

Results for specific aim 4 will be discussed in chapter 5.

Materials and Methods

Animals

WT C57BL/6J strain and homozygous apoE KO mice were purchased from Jackson Laboratory (Bar Harbor, ME). Mice have been backcrossed on the C57BL/6J strain at least 10 times. WT (apoE+/+) and homozygous KO (apoE-/-) mice were first crossed to generate heterozygous KO mice (apoE+/-). Heterozygous KO offspring were crossed with each other to produce homozygous KO and WT littermates used in this project. ApoE genotyping was verified though PCR and immunoblot. A total of 156 mice were used in this experiment; 78 WT and 78 apoE KO.

Ovariectomy

Four-month-old KO and WT littermates were weighed and ovariectomized. Mice were anesthetized under standard protocol using ketamine (2.4 mg/kg) and xylazine (16 mg/kg) in HCl solution (Sigma-Aldrich, St. Louis, MO). Under sterile operating conditions, ovaries were identified and extracted through bilateral spinal column incisions on the dorsal side. Mice were allowed to recuperate for five days prior to estradiol/pellet placement. Previous studies have shown that five days are essential for depletion of natural cycling estrogen to undetectable levels (63, 64). Animals were randomly selected to receive either replacement with an estradiol pellet (17 β-estradiol, 0.36 mg/pellet, 60 day release, Innovative Research of America, Sarasota, FL) or a placebo pellet. Pellets were placed subcutaneously at the mid-scapular level in the right cheek pouch through an incision made on the dorsal side of the neck. Estradiol pellets release proestrus levels (peak levels) of 17 β-estradiol consistently over 60 days (63-66).

Olfactory Nerve Lesioning

Mice were lesioned five days after OVX and on the same day as pellet implantation. Briefly, mice were anesthetized with ketamine/xylazine as described previously and placed in an immobilizing tube open at one end to expose the nostrils. Aliquots of 50 μL of saline (control) or 50 μL 0.7% Triton X (TX) (Sigma, St. Louis, MO) in saline solution were irrigated into each nostril using a 25 gauge blunt ended needle. Excess solution from bubble formation was expelled by lightly shaking the mice and cleaned with a kimwipe. Approximately 70-80% of the OE was lesioned using this technique (67). Mice were placed under a heat lamp while they recovered and were transferred to animal care facility until time of sacrifice.

BrdU Labeling

Following nasal irrigation, mice were allowed to survive for 0, 3, 7, 14, 21, 42, and 56 days post lesion. Zero day mice did not receive nasal irrigation and were used as a control. A 56 day time course covered the range of degeneration and recovery (68). Also, 56 days coincided with the 60-day estradiol release pellet. Post-irrigated mice received an intraperitoneal injection of Bromodeoxyuridine (BrdU) (50 mg/kg) (Sigma Aldrich, St. Louis, MO) 12 hours prior to sacrifice using a 26 ½ gauge needle and 1 cc syringe. Animals were placed under a heat lamp until time of sacrifice.

Sacrifice and Tissue Preparation

Animals received ketamine/xylazine anesthesia under the protocol stated previously and were perfused transcardially with 4°C 0.1 M PBS solution. The tissue

was fixed by perfusion with freshly prepared 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO) in 0.2 M PBS at 4°C. Following perfusion was the extraction of the nasal turbinates, olfactory bulbs, and brain. The nasal turbinates were immersed in decalcifying solution (Fisher Scientific, Fair Lawn NJ) for one hour and then transferred to 4% paraformaldehyde for another hour. The brain and olfactory bulbs were immersed in 4% paraformaldehyde for two hours. Tissues were transferred to 30% sucrose in PBS for cryoprotection and placed on the shaker at 4°C for 24 hours.

Following tissue fixation, the turbinates were embedded to fill the air spaces between the nasal conchae with gel so that the tissue would not break while sectioning. The canine teeth were excised, and the turbinates were submerged in 4% gelatin/30% sucrose solution in 0.1 M PBS that was cooled to just above room temperature. The tissue was placed in a vacuum for 2 hours and the gel solidified. Tissue was removed from the gel and excess was peeled away. The turbinates were rapidly frozen in dry ice. The olfactory bulbs were separated from the brain and rapidly frozen. Tissue was stored rostral-side up in a foil casing at -80°C.

Stored tissue was mounted using Tissue Freezing Medium (Triangle Biomedical Sciences, Durham, NC) and coronal sections of 12 microns were collected using a Reichert-Jung Frigocut E cryostat. Sections were adhered onto 2 % gelatin/0.5% chromium potassium sulfate pre-subbed slides with 0.1 M PBS and then stored at 4°C until time of staining.

Nissl Staining

Sections were washed in distilled water for 10 minutes and placed in the oven at 37°C for 2 hours. The sections were then defatted with xylene for 30 minutes. Following defatting, the sections were hydrated in 100% ethanol for 10 minutes, 95% ethanol for 10 minutes, and 70% ethanol for 5 minutes. Sections were then washed with distilled water, dabbed, and stained in cresyl violet acetate (Sigma, St. Louis, MO) for 4 minutes. Again, sections were washed with distilled water sent through a series of ethanol (70, 95, and 100%) to remove excess stain and fix staining. Tissue sections were defatted further for 30 minutes in xylene and then coverslipped using permount (Fisher Scientific, Fair Lawn, NJ).

OMP, Gap43, ApoE Immunohistochemistry

Sections were washed in distilled water for 10 minutes and placed in the oven at 37°C for 2 hours. The sections were then washed with 0.1 M PBS for 15 minutes and treated 30 minutes with 0.2% Triton-X 100 to create porous tissues. After triton, the sections were washed with 0.1 M PBS for 5 minutes and treated with 1.0% BSA (Fisher Scientific, Fair Lawn, NJ) for 30 minutes. Sections were washed twice with 0.1 M PBS. Then the tissue was incubated for 20 hours at 4°C with primary antibody in corresponding 4% donkey serum (Biomeda, Foster City, CA) in 0.1 M PBS. Primary antibody dilutions included anti-ApoE (1:1000) (Calbiochem, San Diego, CA), anti-OMP (1:500) (Wako, Richmond, VA), anti-Gap43 (1:500) (Chemicon International, Temecula, CA).

Following primary antibody incubation, sections were washed with 0.1 M PBS for 15 minutes and incubated with secondary antibody. Secondary antibody dilutions

were mixed with corresponding 4% donkey serum in 0.1 M PBS and incubated at room temperature at distinct times and concentrations which included: FITC-donkey anti-goat IgG (1:500) (Jackson ImmunoResearch, West Grove, PA) for 1hour, CY3-AffiniPure donkey anti-goat IgG for 1.5 hour (1:250) (Jackson ImmunoResearch, West Grove, PA) and Alexa-donkey anti-rabbit IgG (1:100) (Invitrogen, Eugene, OR) for 3 hours. Finally, sections were washed with 1.0 M PBS for 15 minutes and coverslipped with anti-photobleach Vectashield mounting media (Vector Laboratories, Burlingame, CA).

Synaptophysin and GFAP Immunohistochemistry

Sections were washed in distilled water for 10 minutes and placed in the oven at 50°C for 1 hour. Tissues were then washed with 1.0 M PBS for 10 minutes followed by 0.2% Triton-X 100 for 30 minutes. Sections were rinsed twice with 0.1 M PBS and incubated with primary antibody in 4% corresponding serum (donkey or goat) in 1.0 M PBS for 20 hours at 4°C. Primary antibodies included rabbit anti-SYN (1:500) (Cell Marque Corporation, Rocklin, CA) and mouse anti-GFAP (1:500) (Accurate Chemical and Scientific Corporation, Westbury, NY).

Following primary antibody incubation, sections were washed with 0.1 M PBS for 10 minutes and incubated with secondary antibody for 1.5 hours at room temperature. Secondary antibodies were prepared in 4 % serum (donkey or goat) in 1.0 M PBS and included FITC-goat anti-rabbit IgG (1:200) (Sternberger Monoclonals, Baltimore, MD) and TRITC-donkey anti-mouse IgG (1:500) (Jackson ImmunoResearch, West Grove, PA). Sections were washed with 1.0 M PBS for 10 minutes and coverslipped with anti-photobleach Vectashield mounting media.

BrdU Immunohistochemistry

Sections were washed in distilled water for 10 minutes and placed in the oven at 37°C for 2 hours. The sections were then washed with 0.1 M PBS for 15 minutes and treated 30 minutes with 0.2% Triton-X 100 to create porous tissues. After triton, the sections were washed with 0.1 M PBS for 5 minutes and treated with 2 N HCl for 1 hour. Following HCl, sections were washed with Hank's Balanced Salt Solution (Invitrogen, Grand Island, NY) for 10 minutes. Then sections were treated with 1.0% BSA (Fisher Scientific, Fair Lawn, NJ) for 30 minutes. Sections were washed twice with 0.1 M PBS. Then the tissue was incubated for 20 hours at 4°C with rat anti-BrdU (1:500) (Accurate Chemical and Scientific Corporation, Westbury, NY) in corresponding 4% goat serum in 0.1 M PBS.

Following primary incubation, the sections were rinsed twice with 1.0 M PBS for 5 minutes. A 1:250 concentration of CY3-goat anti-rat IgG (Jackson ImmunoResearch, West Grove, PA) in 4% goat serum in PBS was applied to the sections and incubated at room temperature for 1.5 hours. Sections were washed 3 times for 5 minutes with 1.0 M PBS and coverslipped with anti-photobleach Vectashield mounting media.

Microscopy and Imaging

Stained sections were examined using an Olympus BX-50 microscope. Filterless light was used to observe CV stained tissue and fluorescent microscopy was observed using the appropriate filters for TRITC, FITC, and CY3 conjugated secondary antibodies. Images were captured using a Pixera Digital Camera (Pixera, Los Gatos, CA) and saved as high resolution TIF files. Nasal epithelium tissue images were captured from the

designated study area in the olfactory epithelium on the septum (Figure B). Olfactory bulb images were captured from the designated study area on the medial side from the glomerular layer under high magnification (600X/1000X) (Figure C). Image analysis was performed using Scion Image software (Scion Image, Frederick, MD). Specificity was determined by comparing stain in the presence of antibodies with that of just serum.

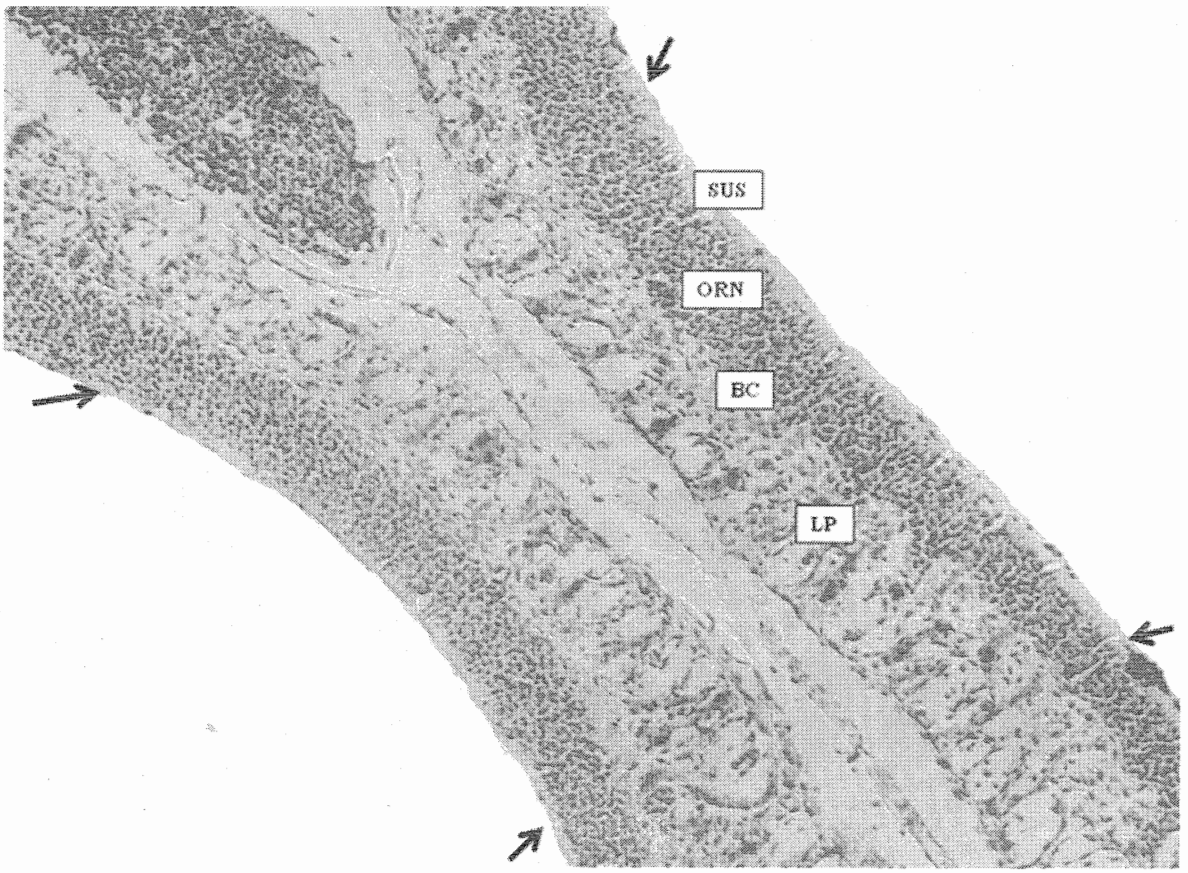


Fig. B- Cresyl violet staining of the nasal septum. The olfactory system was investigated on both sides of the septum from areas designated between the arrows. Labeled areas: Sustentacular cells (SUS), Olfactory Receptor Neurons (ORN), Basal Cells (BC), and Lamina Propria (LP).

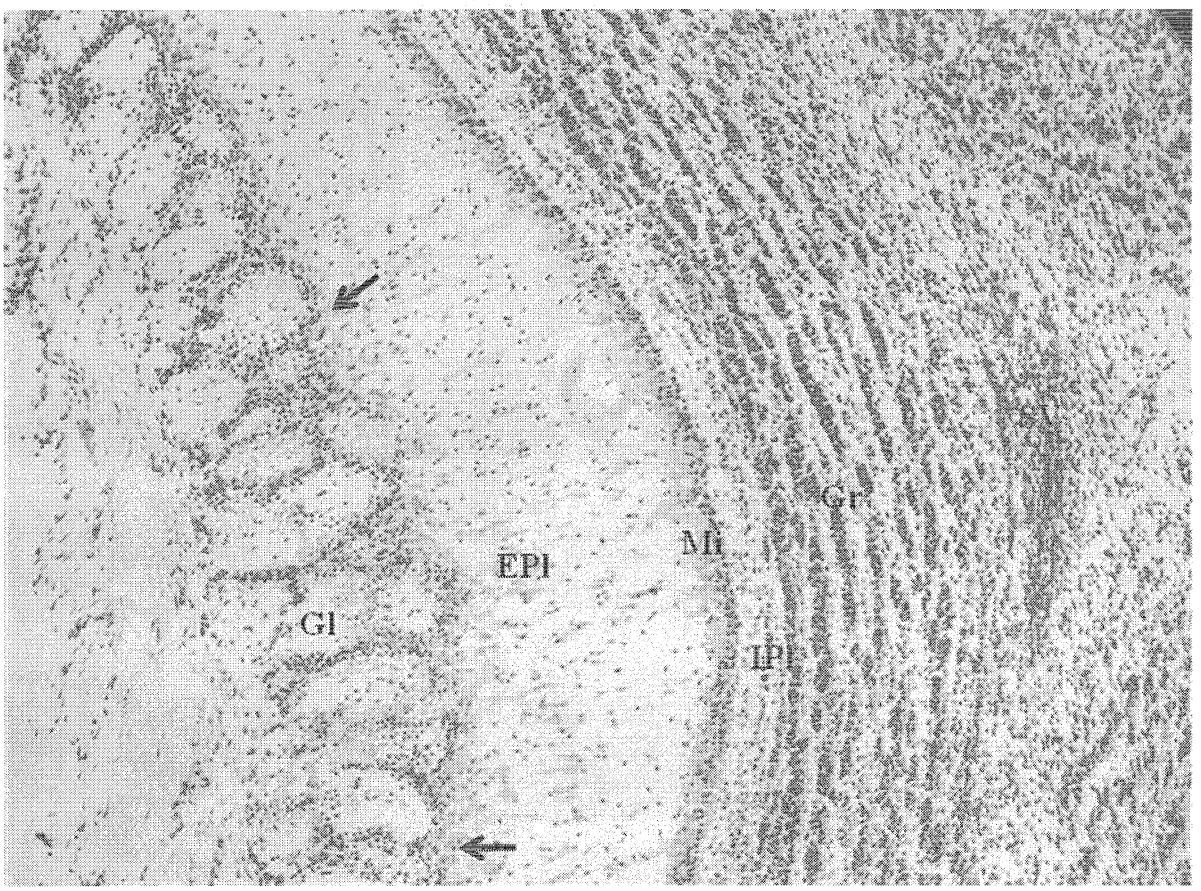


Fig. C- Cresyl violet staining of the olfactory bulb. The OB was investigated in the glomerular layer area designated between the arrows. Labeled areas: Glomerulus (Gl), External Plexiform Layer (EPl), Mitral Cell Layer (Mi), and Internal Plexiform Layer (IPl), Granule Cell Layer (Gr), and Subventricular Zone (SV).

Maintenance and Regeneration in the OE

Morphological thickness of the olfactory epithelium 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. Analysis was performed to compare the effect of short term and long term estradiol treatment within WT and KO genotypes. In addition, analysis of thickness was performed to assess the

progress of regeneration after nerve lesioning between estradiol /placebo treatment in WT and KO mice.

Quantification of OMP and Gap43 in the OE

The number of mature neurons was determined by labeling positively stained OMP neurons in the study region of the olfactory epithelium septum. Images of 600X magnification were analyzed in measurements of 100 μm utilizing Scion Image calibration. Data was expressed as the number of OMP positive cells per 100 μm length.

Similarly, the number of immature neurons was determined by labeling positively stained Gap43 cells within the study region. Quantification was performed through calibration of the stage micrometer and ocular micrometer under 600X magnification. The number of Gap43 positive olfactory receptor neurons was determined in 100 μm length.

Quantification of BrdU in the OE

The total number of dividing basal cells was quantified within a total of 1 mm length. Labeled nuclei were analyzed under 1000X magnification. Data was expressed as the number of BrdU positive cells in 1 mm in the study region of the septum.

Analysis of ApoE, OMP, and SYN in the OB

Images were taken from the medial edge in the glomerular layer between the olfactory nerve tract and the internal plexiform layer of the olfactory bulb under 600X magnification. Images were saved as high resolution TIF files and analyzed in Scion Image. Differences in immunoreactivity were determined from a 2-3 glomeruli area by

blinding the slides and scoring for staining intensity. SYN and OMP compartmentalizion within the glomerulus was compared and diffuse ApoE in the surrounding area was determined.

Quantification of GFAP in the OB

Images were taken in the same area of those taken from SYN, OMP, and ApoE. A 5 by 5 grid box (125 μm^2 area) was placed over the image, and astrocyte processes were marked with a dot if they crossed the grid. Below is an example of quantification of process density on a single image (Figure D).

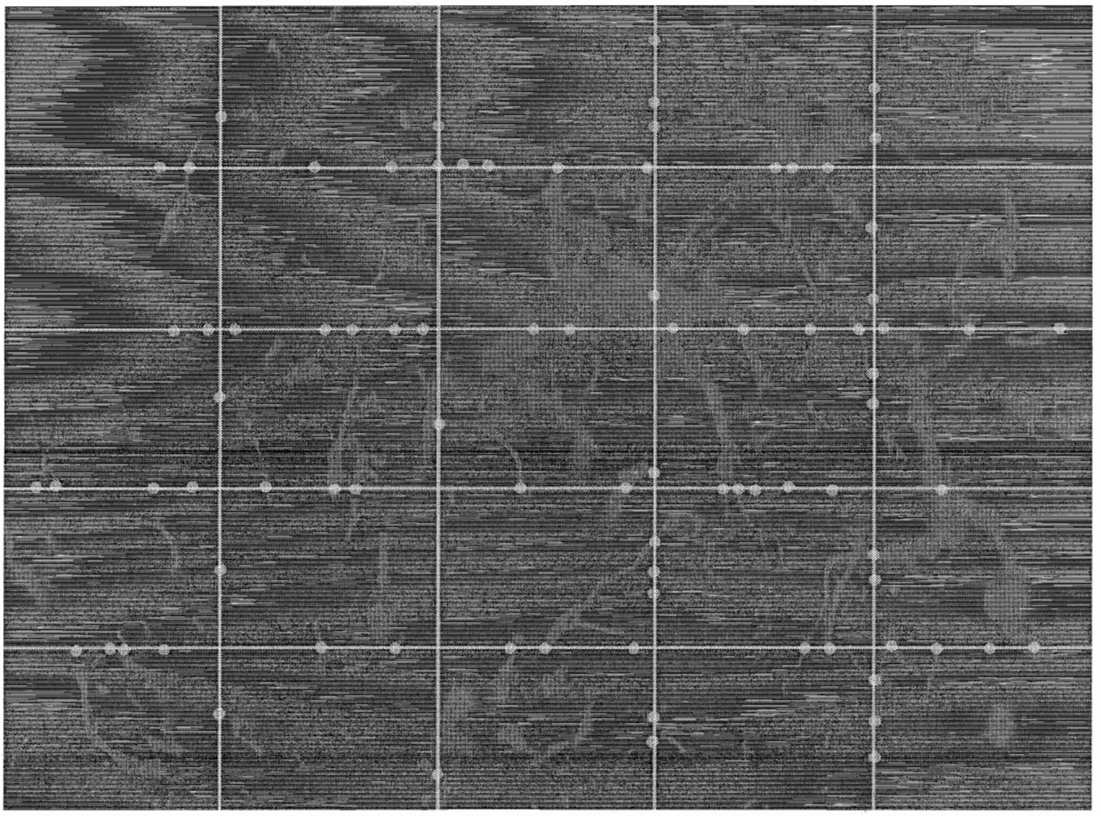


Fig. D- GFAP staining of the OB with overlying Grid.

Statistical Analysis

All quantification procedures were repeated 3 times for each experiment. 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.

Chapter 1: Short Term Estrogen Replacement in WT and KO Normal Mice

An assessment of short term estrogen replacement was conducted in order to determine the initial effects of estrogen in the olfactory system post OVX. Experiments performed on WT and KO mice examined interactions of estradiol with apoE. This experiment was performed on normal mice without induced nerve injury.

General results from this experiment established a foundation for future experiments. First, results from this study were important in developing hypotheses for estradiol's function in the olfactory system over long periods of time. Second, a fundamental understanding of short term estrogen replacement established whether or not estradiol had neuroprotective effects on ORNs. Lastly, having a baseline of olfactory nerve maintenance in the absence of injury helped formulate hypotheses about apoE and estrogen treatment during olfactory nerve recovery.

For this study, a detailed examination of the OE and the OB was performed 3 days after estradiol or placebo pellet implantation. An analysis of morphoanatomic features in the OE was performed. This included quantification of thickness, maturation, and apoE. Furthermore, immunohistochemical analysis of the glomerular layer of the OB was performed. Immunostaining in the OB included apoE, OMP, SYN, and GFAP.

These parameters helped identify early nerve preservation of the olfactory system post OVX. Investigation of OE thickness determined whether or not ORN turnover was affected in the OE, and analysis of OMP identified which ORNs were affected. ApoE immunohistochemisty in the OE determined whether or not estradiol played a regulating role. Furthermore, examination the glomeruli in the OB tested the effects of estradiol on

synaptic activity at the terminal end of ORNs. SYN immunostaining identified how many ORN/bulbar neuron synapses were present. OMP immunostaining isolated if synaptic signals were transmitted from mature ORN to the mitral and tufted cells. Finally, quantification of astrocytes and the occurrence of apoE in the OB established measures of facilitation.

I expected that short term estrogen treatment would be beneficial in only WT mice. WT mice treated with estradiol would have a thicker OE containing more ORNs. I also expected that apoE would be greater in estradiol treated mice in both the OE and the OB. OMP, SYN, and GFAP would all be greater in WT estradiol treated mice. WT placebo treated, KO placebo treated, and KO estradiol treated mice would have no observable differences between them.

Results:

Estradiol treated mice had a thicker OE compared to placebo treated mice (Figure 1). Analysis of variance confirmed a significant difference between estradiol and placebo treatment ($F_{1,1}$=10.17, p=0.013). This interaction was independent of the presence of apoE ($F_{1,1}$=0.172, p=0.689). Although expected from previous studies, estradiol did not increase apoE expression in the OE of WT mice. However, apoE levels were distinctly greater in the OB (Figure 2).

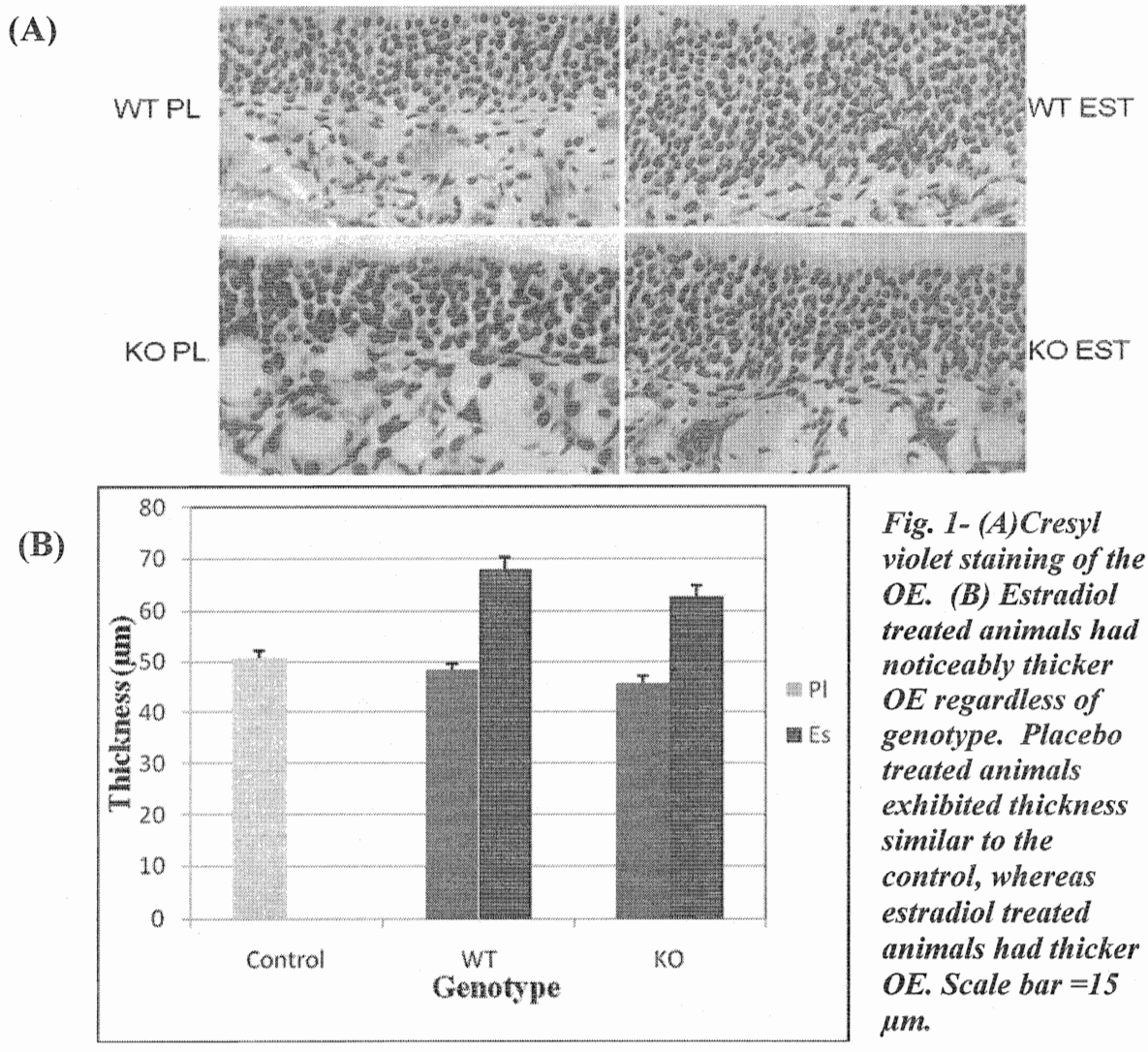


Fig. 1- (A)Cresyl violet staining of the OE. (B) Estradiol treated animals had noticeably thicker OE regardless of genotype. Placebo treated animals exhibited thickness similar to the control, whereas estradiol treated animals had thicker OE. Scale bar =15 µm.

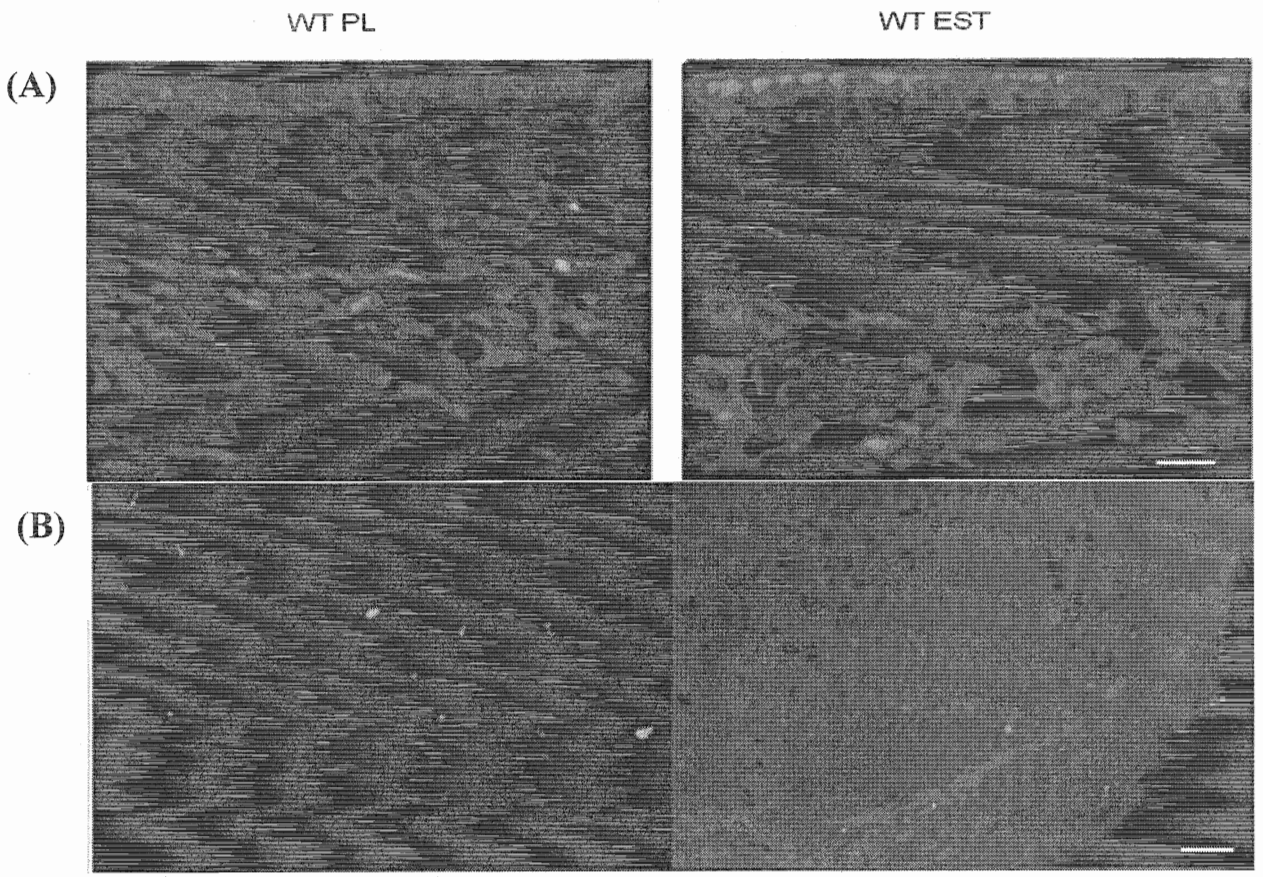
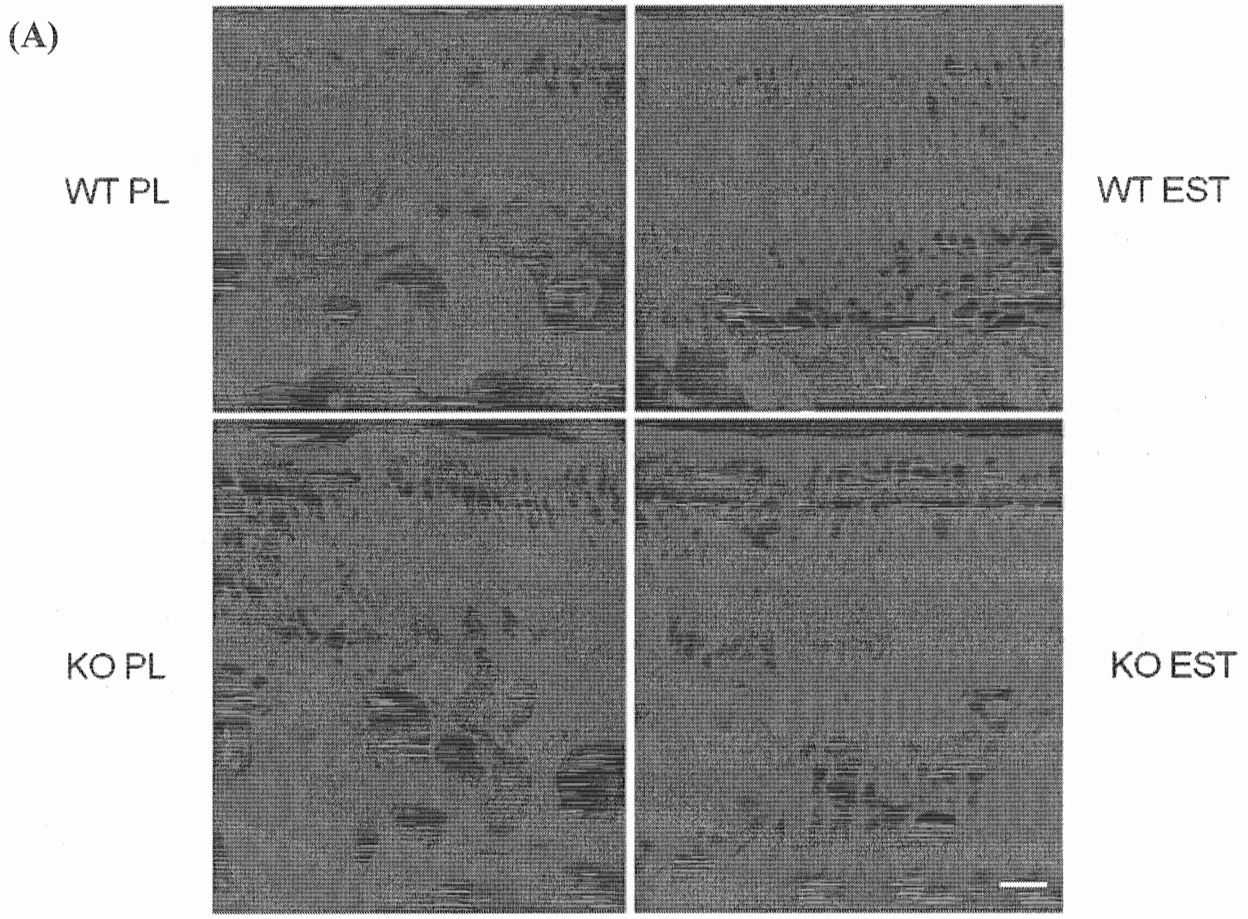


Fig. 2- (A)ApoE expression in the OE and OB of WT mice. No significant difference of immunoreactivity was observed between estradiol and placebo treated mice in the OE. (B) More apoE was present in the OB of estradiol treated mice. Scale bar= 15 μm.

Maturation also varied between estradiol and placebo treatment. More OMP^+ ORNs were observed in estradiol treated mice (Figure 3). An ANOVA confirmed a significant difference between treatments ($F_{1,1}$= 71.46, $p<0.001$). There was also a genotype effect on OMP. Overall, WT mice had more OMP^+ cells than that of KO mice ($F_{1,1}$= 9.93, p=0.014). There was a slight loss of maturation in KO mice treated with placebo pellet. OMP immunoreactivity in the bulb was consistent with observations in the OE. Examination of the glomerular layer showed more immunoreactivity in estradiol treated mice (Figure 4).



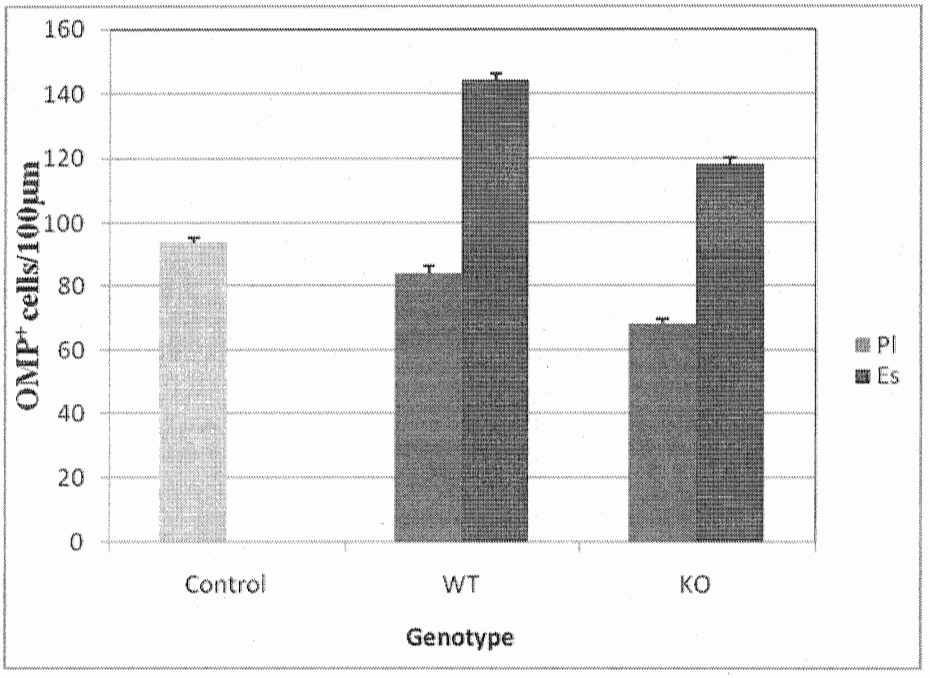


Fig. 3- (A) OMP labeled cells in the OE. (B) The number of OMP$^+$ ORNs nearly doubled in estradiol treated compared to placebo treated. WT mice overall had slightly more OMP cells when compared to KO. Scale bar= 15 µm.

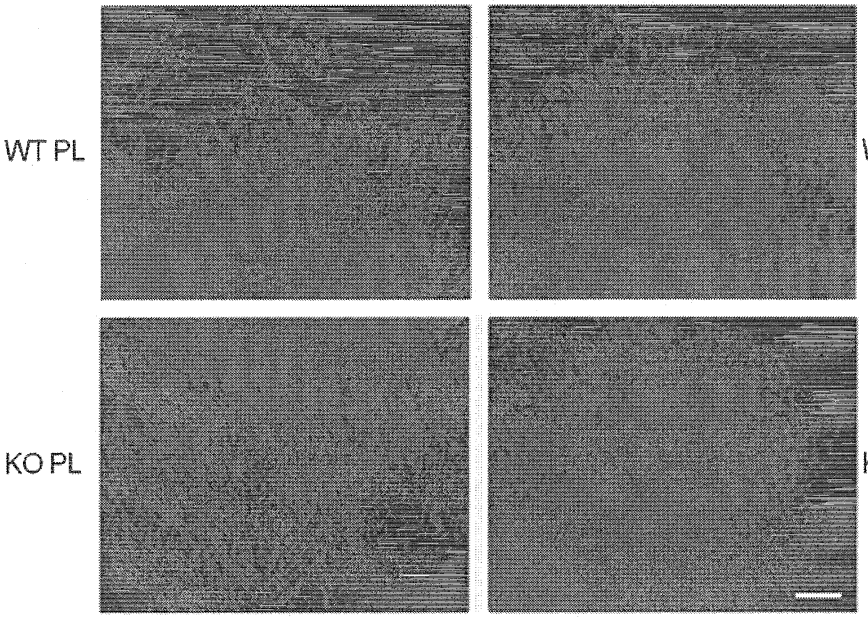


Fig. 4- OMP staining in the glomerular layer of the OB in WT and KO. More OMP was observed in estradiol treated compared to that of placebo treated mice. Scale bar=15μm.

Synaptophysin immunoreactivity was comparable to that of OMP in the OB. There was a noticeable increase in SYN in WT mice treated with estradiol. WT placebo treated mice had slightly less than WT estradiol treated. There was a visible decrease in KO placebo treated mice (Figure 5).

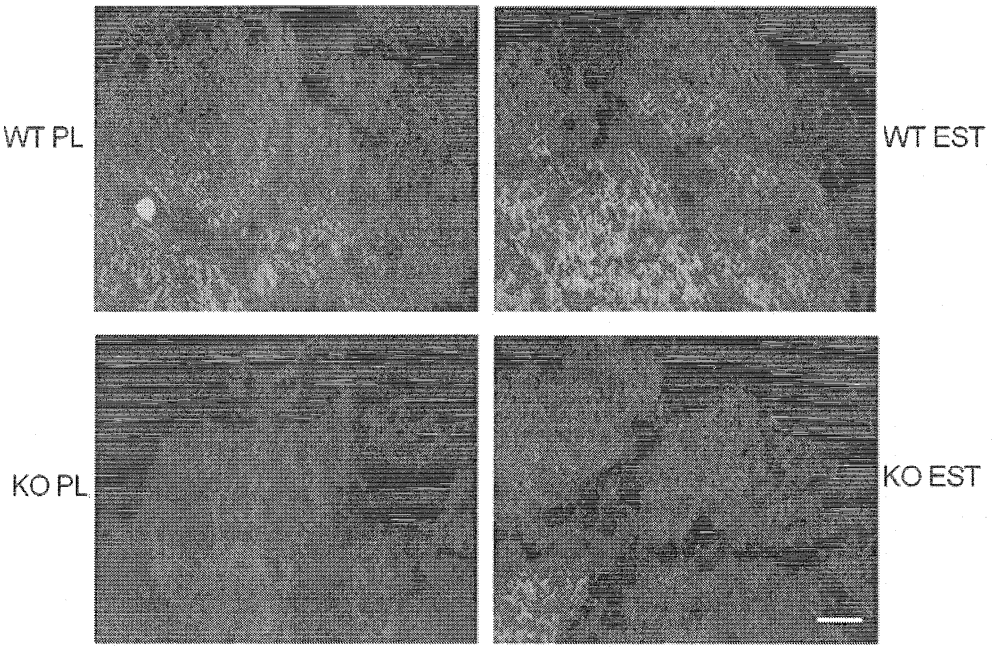


Fig. 5- SYN staining in the glomerular layer of the OB in WT and KO. The most SYN was observed in WT estradiol treated and the least in placebo treated KO mice. Scale bar=15μm.

Astrocyte process density in the glomerular layer was quantified from GFAP immunoreactivity (Figure 6). Estradiol treated mice had more astrocytes compared to placebo treated mice ($F_{1,1}$=24.46, p<0.001). This interaction was independent of genotype ($F_{1,1}$=1.40, p=0.271).

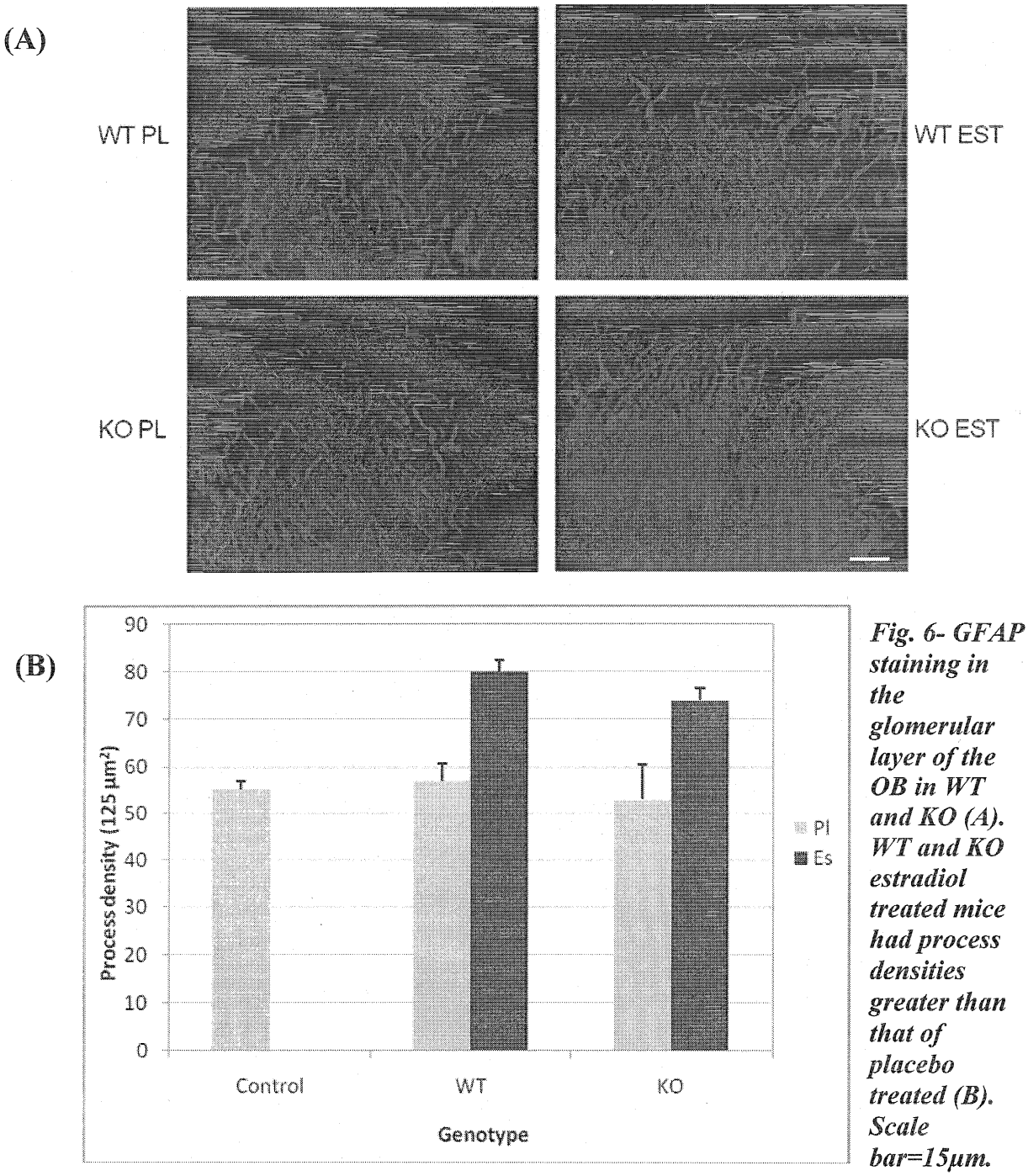


Fig. 6- GFAP staining in the glomerular layer of the OB in WT and KO (A). WT and KO estradiol treated mice had process densities greater than that of placebo treated (B). Scale bar=15µm.

Discussion:

A thicker OE was observed in estradiol treated mice. However, a thicker OE was not solely dependent on apoE since KO mice treated with estradiol also had a thicker OE. In placebo treated mice, the OE was thin but still similar to the thickness of the OE in control mice. Therefore, it appears that estradiol treatment increases the thickness of the OE while not having estradiol did not result in a weakening effect.

Estradiol treated OE was thicker because it contained more mature ORNs. This result signified that estradiol treatment may have promoted immature neurons to develop into their mature state. A greater amount of OMP in the OE led to a greater amount of OMP immunoreactivity in the OB. Furthermore, the most SYN observed in the glomeruli was in estradiol treated WT mice and the least in KO placebo treated mice. This was most likely due to higher and lower OMP immunoreactivity, respectively.

The mechanism of increased maturation patterns was most likely influenced by activity in the OB. When apoE levels in the OE were examined, no difference was observed between WT placebo treated mice and WT estradiol treated mice. However, there was a difference in apoE immunoreactivity in the OB of WT mice. WT placebo treated mice had almost no apoE present in the OB, and estradiol treated mice had a considerable amount. ApoE could have induced axonal growth of immature neurons to the glomeruli of the OB. Immature ORNs developed into mature ORNs when synapses were formed with the mitral and tufted cells. This led to increased OMP and synaptophysin in the OB. ApoE was likely transported and released by astrocytes since estradiol treated mice had significantly more astrocytes.

If apoE was the sole link to estrogen's short term effect, then there should be no differences between estradiol and placebo treatments in KO mice. To explain this phenomenon, the OVX effect is considered. The ovaries were bilaterally removed and blood level estrogen was depleted in all animals for 5 days. During these 5 days, natural cycling estrogen was progressively approaching complete depletion. The olfactory nervous system responded to estrogen depletion as a target site of injury. Then upon estradiol pellet implementation an influx of estrogen was released back into the system. This influx increased astrocytes in the OB. Since the astrocytes did not regulate apoE levels in KO mice, it is likely that they contributed to metabolic support via an apoE independent mechanism. But in the end, apoE released by astrocytes in WT mice treated with estradiol enhanced any effects evident in KO estradiol treated mice.

Chapter 2: Estrogen in WT Normal Mice: Time Course Study

An evaluation of long term estrogen replacement was performed over a period of 56 days. The olfactory system was studied at series of time points which included 0, 3, 7, 21, 42, and 56 days. These time points were chosen because any changes in the OE or the OB could be observed consistently throughout the time course and will correspond with time points during repair (79). Also, the estradiol pellet maintained a constant proestrus level of release up to 60 days.

This study was a follow up to short term estrogen treatment, but only focused on WT mice. A clear understanding of how estrogen or lack of estrogen affects the olfactory system over time was warranted before injury studies. Results from this study can assess whether or not there was any consequence for taking estrogen out of normal mice. Results developed hypotheses regarding the neuroprotective effects of estradiol addition post OVX and how estradiol treatment would influence olfactory nerve repair.

Again, the OE was examined for thickness and maturation patterns. A close examination of apoE levels were performed in the OE and the OB. Maturation, synaptogenesis, and occurrence of astrocytes were assessed in the OB over the time course (0-56 days).

Thickness assessment tracked any atrophic or thickening effects due to either estradiol or placebo treatment. OMP quantification in the OE provided answers to whether thickness outcomes were due to mature ORN turnover. Additionally, examination of apoE levels in the OB and OE investigated whether or not any correlations or fluctuations existed among treatments. Lastly, OMP, SYN, and GFAP studies in the OB provided possible mechanisms for treatment outcomes in the OE.

Based on results from short term estrogen treatment, I expected that estradiol treated mice would maintain a thicker OE, and placebo treated mice would experience a reduction in thickness. Further, estradiol would be vital in maintaining mature ORNs. Therefore, placebo treated mice would experience cumulative neuronal death over time. I anticipated few, if any, mature ORN in placebo treated mice by the end of the time course. I, thus, anticipated there would be significantly less OMP and SYN immunoreactivity in the OB of placebo treated mice. Also, a reduction of apoE levels would be present in the OE and OB of placebo treated mice. Less apoE in the OB would mean a reduction in the amount of astrocytes in placebo treated mice. Conversely, estradiol treated mice would have higher astrocytes and more apoE.

Results:

Estradiol treated mice had a thicker OE compared to that of the placebo treated mice over time. The placebo treated mice had a thinner OE from the beginning of the time course and maintained the same thinness through each time point, ~50 μm ($F_{1,4}$=53.19, $p<0.001$). From 3 days, there was a slight thickness reduction in the estradiol group until 21 days post treatment. After 21 days, estradiol treated mice OE thickened to levels around 65 μm, that which was observed at 3 days (Figures 7 & 8).

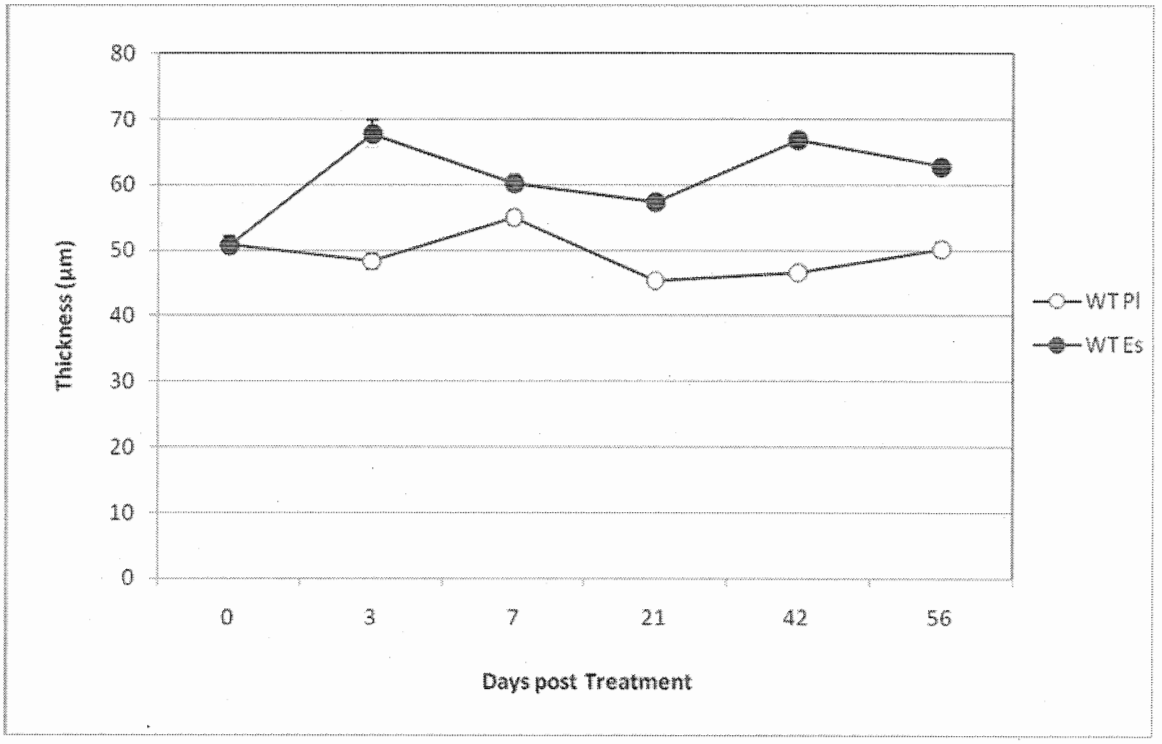


Fig. 7- Graph of OE thickness in WT normal mice over a time course of 56 days. Estradiol treated mice had thicker OE compared to the OE of placebo treated mice. There was an indicative decrease in OE thickness in the estradiol group at 7 and 21 days and an increase again at 42 days.

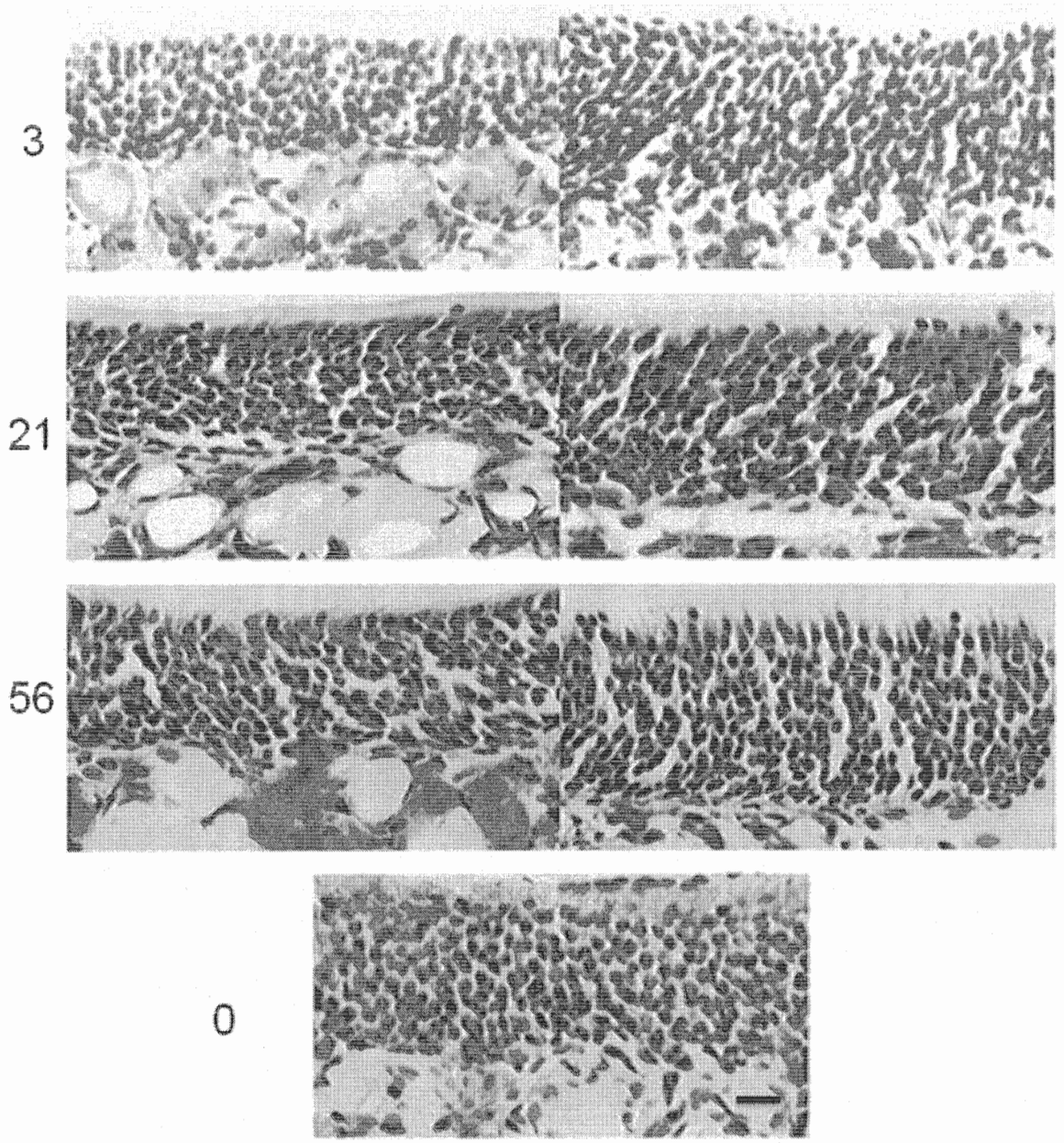


Fig. 8- Cresyl violet staining of the OE in WT normal mice. Pictures displayed were at time points of 0, 3, 21, 56 days post treatment. Thicker OE was observed in estradiol treated mice throughout the 56 day period, whereas thinner OE was observed in placebo treated. Placebo treated OE was comparable to 0 day thickness. Scale bar=15 μm.

Examination of OMP in the OE corresponded with thickness analysis. Placebo treated mice had less OMP^+ cells throughout the time course ($F_{1,4}=174.3$, $p<0.001$). Furthermore, there was a significant spike then decline in OMP^+ cells from 3 to 7 days in

estradiol treated mice. This was followed by an initial recovery at 21 days ($F_{1,4}$=6.692, $p<0.001$). The OE of placebo treated mice maintained ~80 OMP^+ cells per 100 μm with the exception of a small decline at 42 days (Figures 9 & 10).

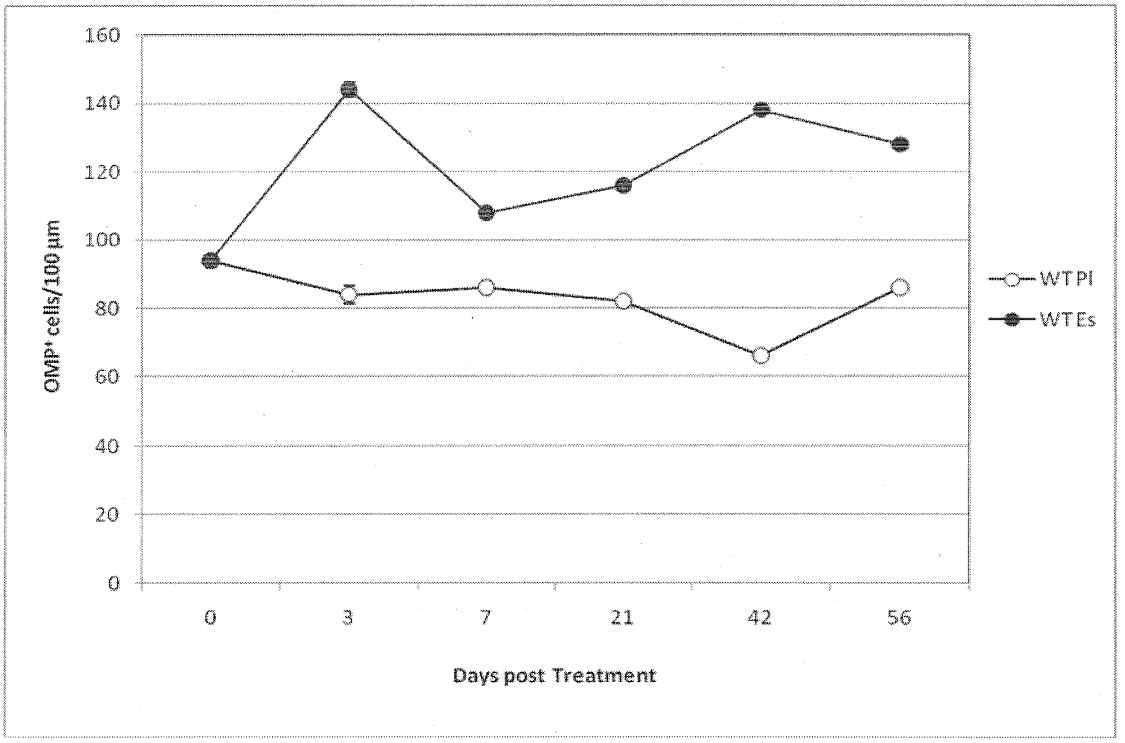


Fig. 9- Graph of OMP in the OE in WT normal mice with maturation trends graphed over a period of 56 days. There was a significant peak at 3 days in estradiol treated mice followed by a drop at 7 and gradual recovery from 21 to 56 days. Placebo treated animals had less OMP^+ cells than estradiol treated at all time points. There was less variation in placebo treated mice having only a small decline in OMP at 42 days.

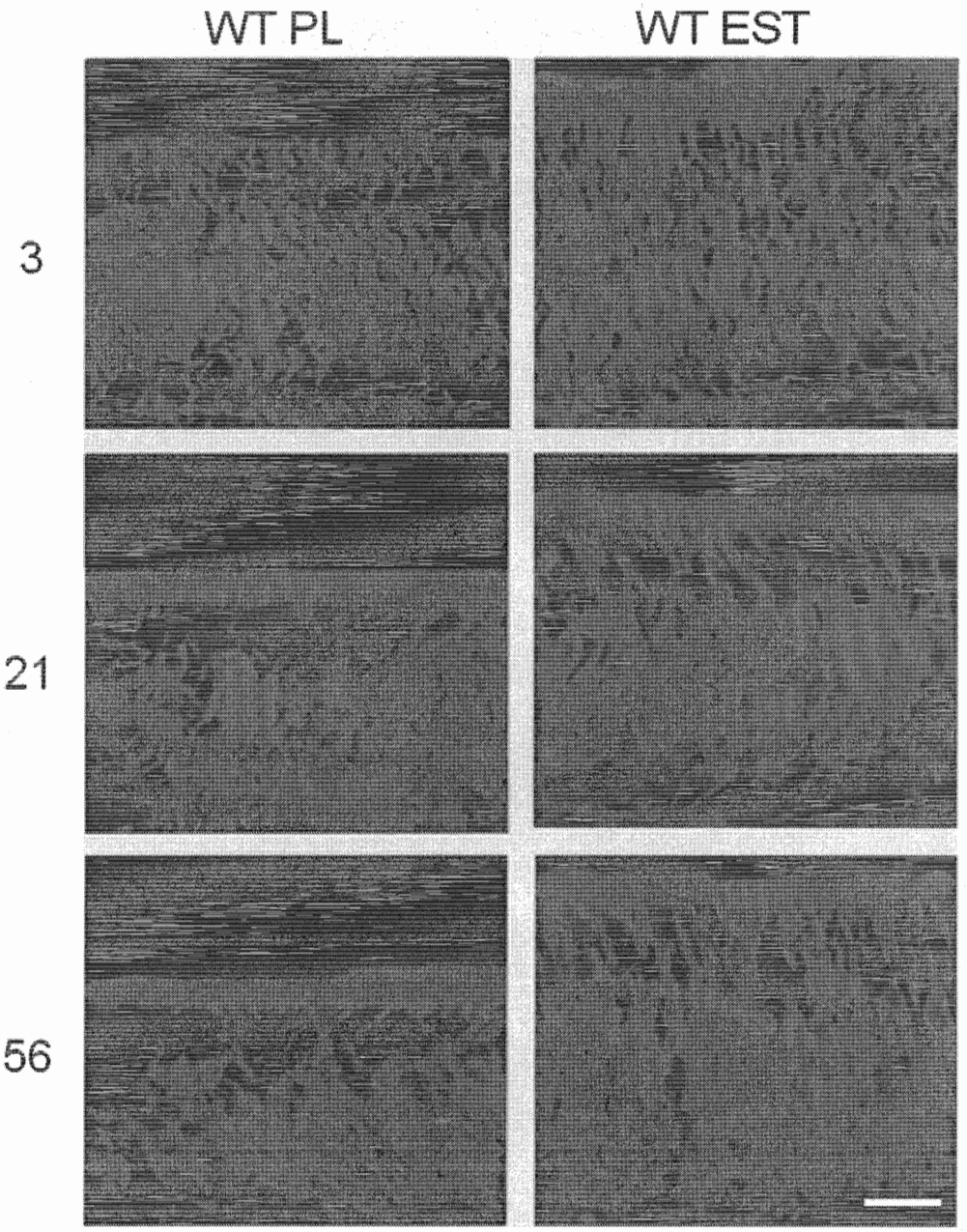


Fig. 10- OMP labeled cells in the OE in WT normal mice. Pictures displayed were at time points of 3, 21, 56 days post treatment. More OMP^{+} cells were observed in estradiol treated mice throughout the 56 day period compared to placebo treated. Scale bar=15 μm.

There was no observable difference in apoE levels in the OE among treatments throughout the time course. However, there was a greater amount of apoE present in the OE at the end of the time course than at the beginning. Still at 56 days, apoE levels were

comparable between the estradiol and placebo treatments (Figure 11). This result suggested that apoE increased in analogous quantities over the time course post OVX for both treatments.

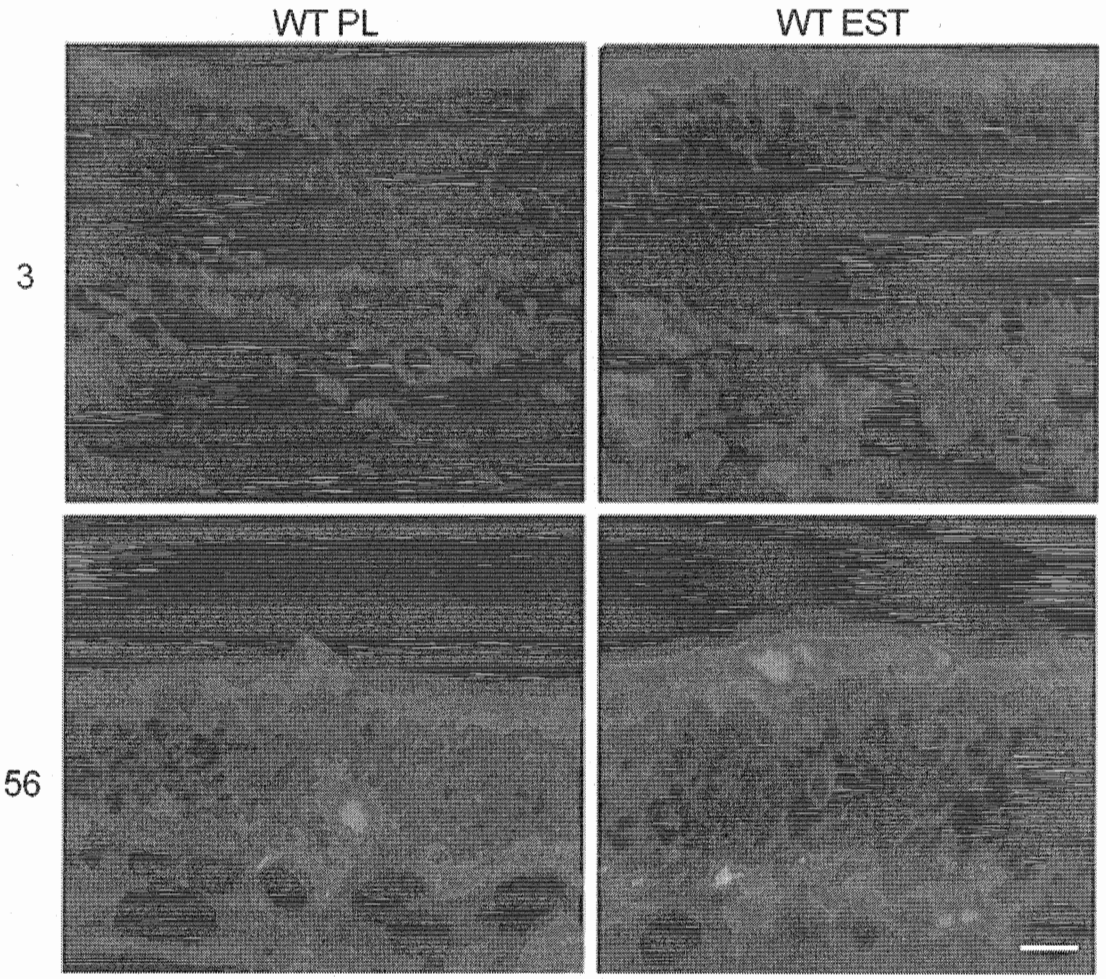


Fig. 11- ApoE immunostaining in the OE in WT normal mice. There was no observed difference between treatments over time however there was more apoE present at 56 days. ApoE was consistent throughout the microanatomy of the OE from the lamina propria to the heads of the sustentacular cells. It was indicative that apoE increased at each time point post OVX. Scale bar=15 μm.

ApoE immunoreactivity in the OB was different between treatments and fluctuated over the time course in placebo treated mice. Overall, estradiol maintained high levels of apoE at each point in the time course. Placebo treatment exhibited low

levels at 3 days, followed by an increase at days 7 and 21. ApoE then decreased at 42 days and increased again at 56 days (Figure 12).

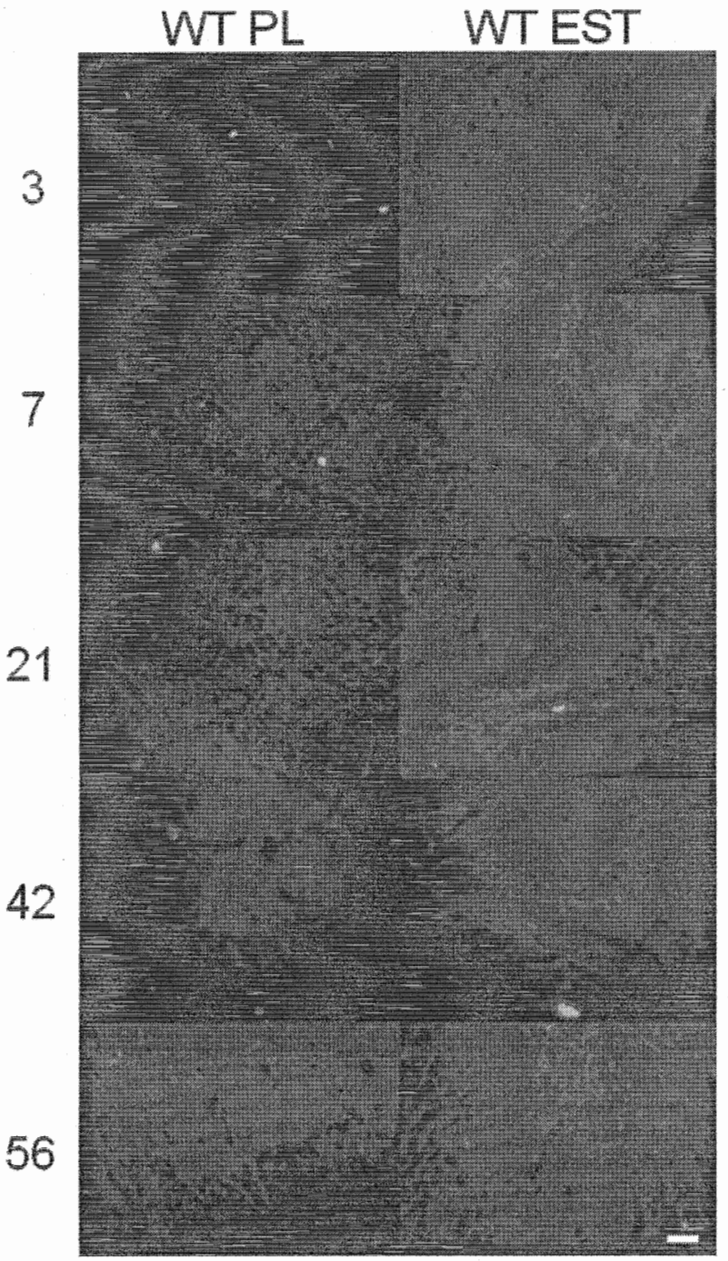


Fig. 12- ApoE immunostaining in the glomerular layer of the OB in WT normal mice. There was more apoE immunoreactivity present in estradiol treated mice at each time point, and levels did not fluctuate over time. Placebo treated mice exhibited low level fluctuation over the time course. Levels were low at 3 and 42 days and higher at 7, 21, and 56 days. Scale bar=15 μm.

OMP in the glomerular layer of the OB paralleled OMP results found in the OE. More OMP immunoreactivity was observed in estradiol treated mice. OMP was greatest at the beginning and end of the time course in estradiol treated, whereas OMP was

reduced across each time point in placebo treated (Figure 13). Synaptophysin immunoreactivity in the OB was equivalent to that of OMP (Figure 14).

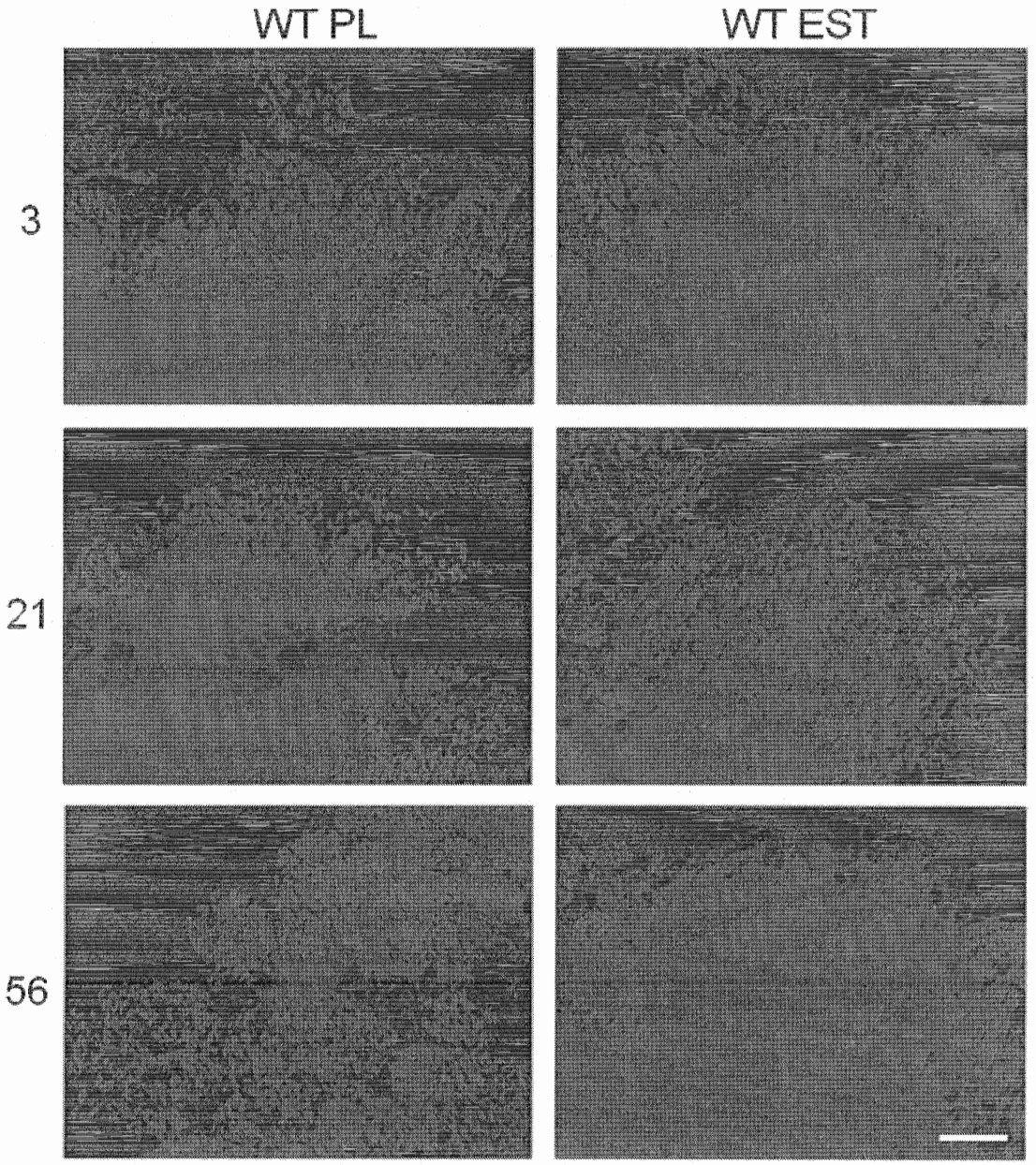


Fig. 13- OMP immunoreactivity in the glomerular layer of the OB of WT normal mice. The most OMP was observed in estradiol treated at 3 and 56 days. Scale bar=15 μm.

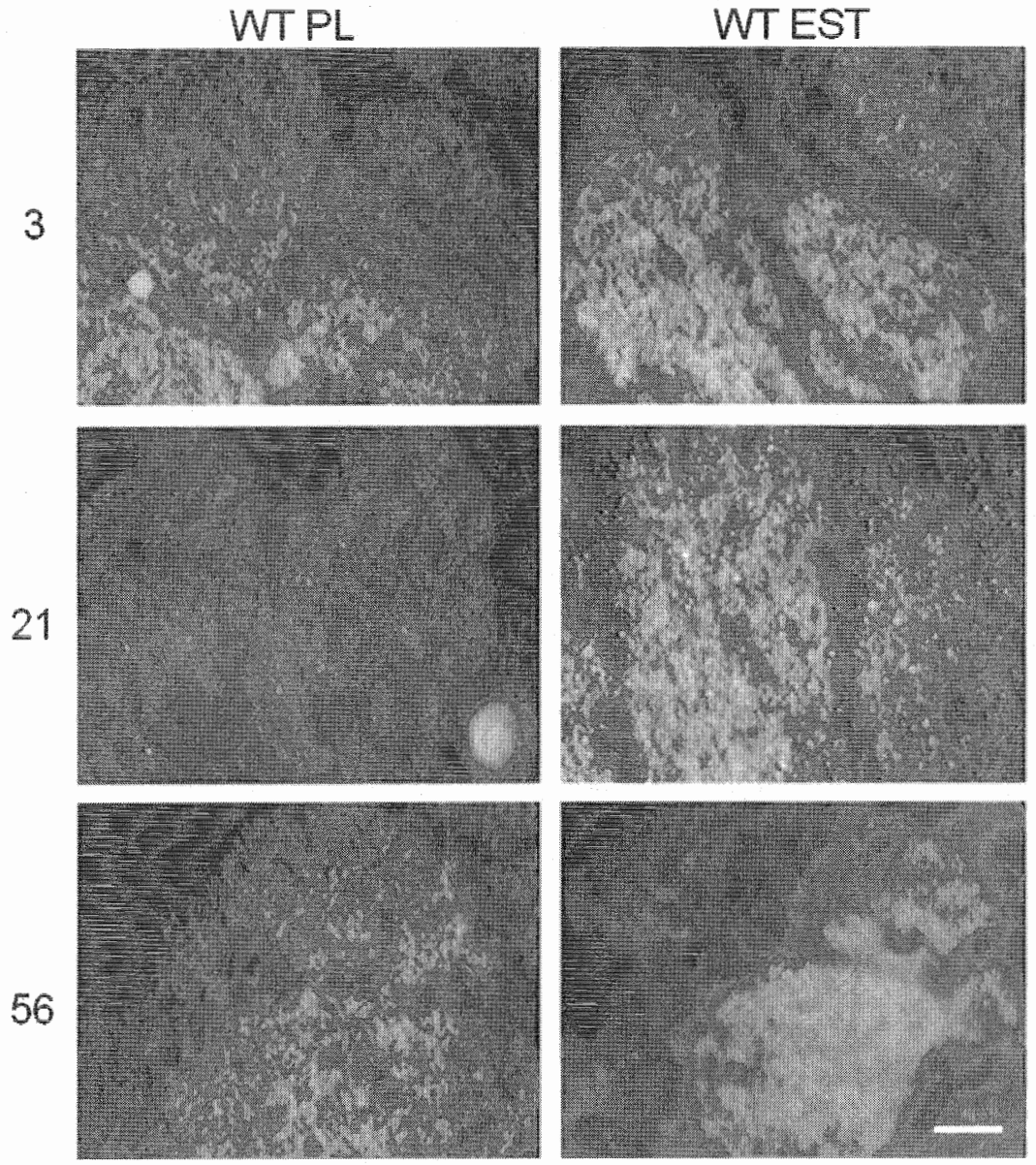


Fig. 14- SYN immunoreactivity in the glomerular layer of the OB of WT normal mice. SYN was higher in estradiol treated mice throughout the time course of study. SYN levels were observed to be the lowest at 21 days in placebo treated. Scale bar=15 μm.

Estradiol increased astrocyte occurrence in the OB, and more GFAP immunoreactivity was observed throughout the glomerular layer (Figures 15 & 16). Astrocyte process density was significantly greater in estradiol treated mice compared to placebo treated mice ($F_{1,4}$=73.68, $p<0.001$). The prevalence of astrocytes did not change

between time points in placebo treated mice and remained higher after 3 days in estradiol treated mice ($F_{1,4}=0.649$, $p=0.634$).

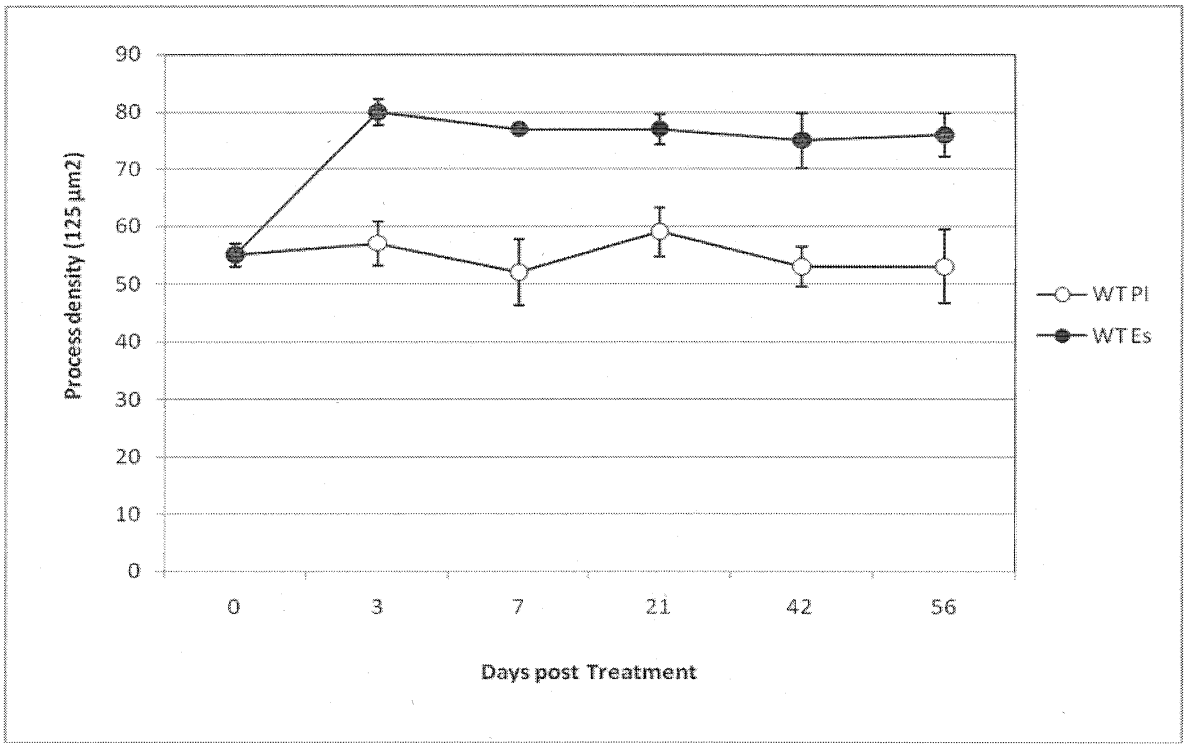


Fig. 15- Graph of astrocyte process density in the glomerular layer of the OB. Estradiol treated mice had more astrocytes present through the time course than that of placebo treated mice. The number of astrocytes did not fluctuate over the course of time in either treatment after 3 days.

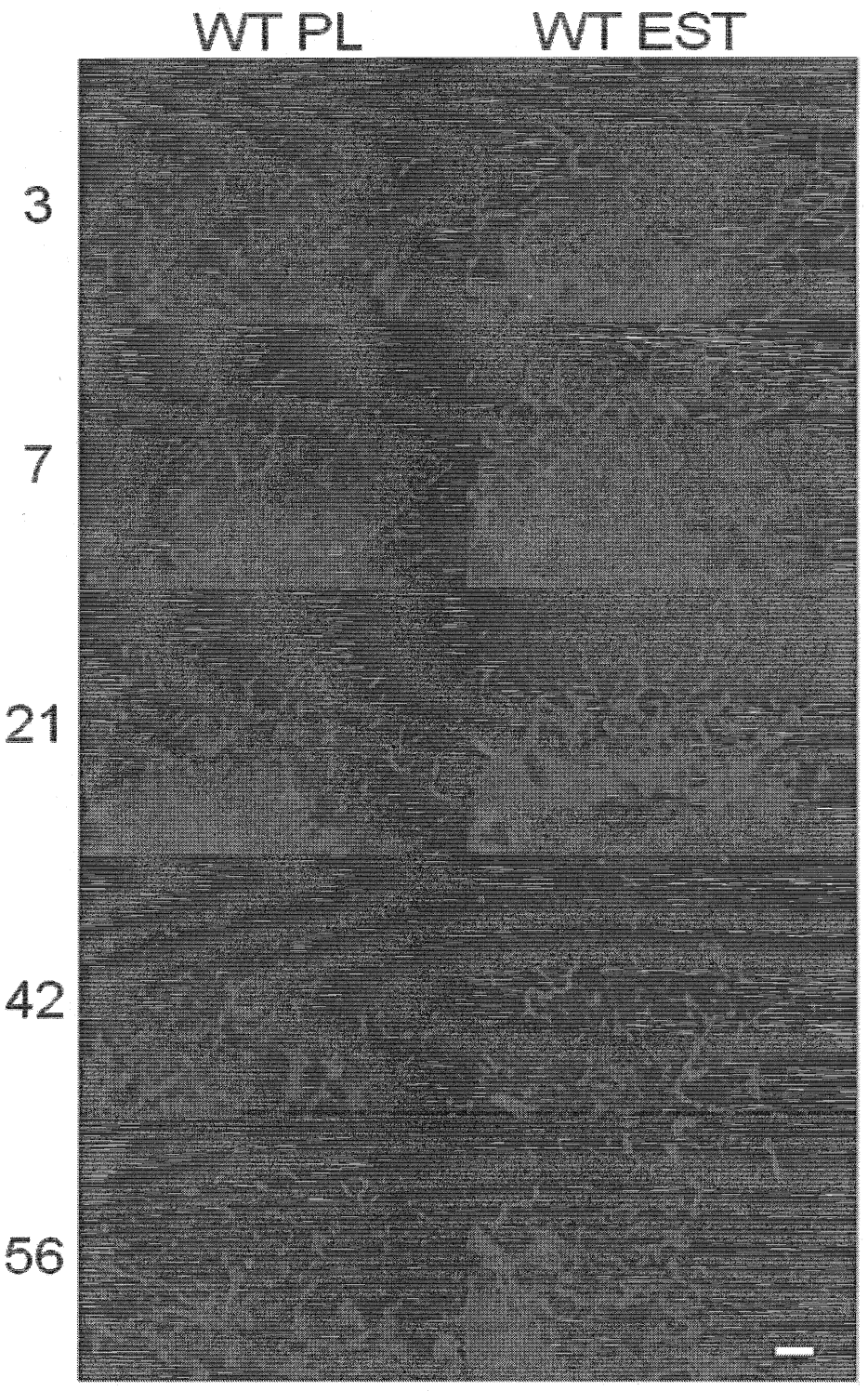


Fig. 16- GFAP assessment in the OB. Immunohistochemical analysis showed astrocyte processes throughout the glomerular layer. Estradiol treated mice had more astrocytes than placebo treated at each time point. Scale bar=15 μm.

Discussion:

WT mice treated with estradiol maintained a thicker OE throughout long term estrogen replacement. The largest spike in thickness was from 0 to 3 days. After this initial spike, the OE began to shrink until increasing again at later time points. Thickness results were contingent upon maturation patterns in the OE. There was a large increase in the number of mature ORNs in the OE at 3 days. Due to a possible overcompensation there was a dramatic decrease at 7 days. The mature ORNs reached the site of synapse but did not continue to transmit information, their synapses were pruned, and they underwent apoptosis. Then from 7 days onward the number of mature ORNs began to subtly increase again to higher amounts.

Taking away estrogen did not have an atrophic effect on WT mice. Placebo treated mice maintained relatively the same OE thickness and maturation throughout the time course. The only decrease in mature ORNs was at 42 days. At 56 days, maturation was re-established which suggested long term estrogen depletion was not detrimental to normal ORN turnover in the OE.

Furthermore, an assessment of apoE in the OE demonstrated that apoE increased in both placebo and estradiol treated mice over time; there was no difference in immunoreactivity between treatments. One explanation is that apoE levels increased, post OVX.

Moreover, there were significant variations in apoE between treatments in the OB. Estradiol treatment was responsible for keeping apoE levels consistently high throughout the time course. Accordingly, placebo treated mice had low level fluctuation

of apoE in the OB. Astrocytes may be responsible for apoE differences since occurrence was substantially greater in estradiol treated mice.

Overall, long term estrogen treatment was beneficial but not necessary for olfactory nerve function. Estradiol's benefits were primarily designated to advancement of nerve maturation. Estradiol was vital in maintaining and escalating maturation in the olfactory system via apoE in the OB. Estradiol treatment increased the prevalence of astrocytes in the OB, which consequently transported more apoE. More apoE in the glomerular and olfactory nerve tract layer led to an increase in the amount of mature ORN synapsing with the mitral and tufted cells.

Chapter 3: Estrogen in WT Injured Mice

WT mice underwent olfactory nerve lesion and received either estradiol or placebo replacement. An evaluation of repair was conducted over a 56 day period. The purpose of this study was to determine whether or not estradiol treatment affected the rate of olfactory nerve recovery. Reconstitution of the olfactory system was studied at 0, 3, 7, 21, 42 and 56 days. These time points corresponded with previous studies in WT normal mice. Also, estradiol pellet maintained a constant proestrus level of release for up to 60 days.

The same immunohistochemistry was performed in the OE and the OB in this experiment with a few additions. Thickness was analyzed in the OE through cresyl violet staining. This was followed by a complete assessment of cells in the OE. First, cell division was quantified through BrdU labeling. Second, ORN development and maturation was analyzed by Gap43 and OMP, respectively. Third, ApoE levels were observed in the OE and OB over time. Lastly, OMP, SYN, and GFAP were assessed to determine activity in the OB during ORN re-establishment.

The immunohistochemistry performed in this experiment uncovered the time course of estradiol's effect. Thickness data initially revealed if and when differences in recovery patterns occurred between placebo and estradiol treated mice. Examination of BrdU, Gap43, and OMP pinpointed which OE cells benefited by having estrogen and at what point(s) during recovery. Additionally, apoE expression in the OE suggested

whether or not estrogen and apoE were linked in repair. GFAP in the OB revealed whether astrocyte occurrence coincided with apoE fluctuations during repair. Finally, OMP and SYN immunoreactivity disclosed any differences in mature ORN re-innervation and synaptogenesis between estradiol and placebo treatments.

Based on short term and long term estrogen results in normal mice, I expected that estradiol treated mice would have a thicker OE from 3 days onward. I hypothesized that placebo treated mice would still have sufficient recovery but at a slower rate, because estradiol would increase the amount of cell division early on, leading to more immature ORN and more mature ORN development at earlier time points during repair. I considered, apoE in the OE would be influential in facilitating estradiol's effect, and I predicted more apoE in estradiol treated mice. Furthermore, estradiol treated mice would have a greater amount of OMP immunoreactivity in the OB due to more OMP in the OE at all time points in repair. Synaptogenesis would be greater at earlier points in repair in estradiol treated mice via apoE promotion of synaptic branching. ApoE in the OB will be transported by astrocytes, and astrocyte occurrence will be greater at earlier time points in nerve repair.

Results:

An analysis of thickness showed a significant interaction between time points and treatment during recovery. Recovery was slower and less efficient with placebo treatment compared to estradiol treatment ($F_{1,4}$=3.17, p=0.036). Separation occurred at 21 days, which suggested a delay in estradiol's developmental effect at days 3 and 7 (Figures 17 & 18).

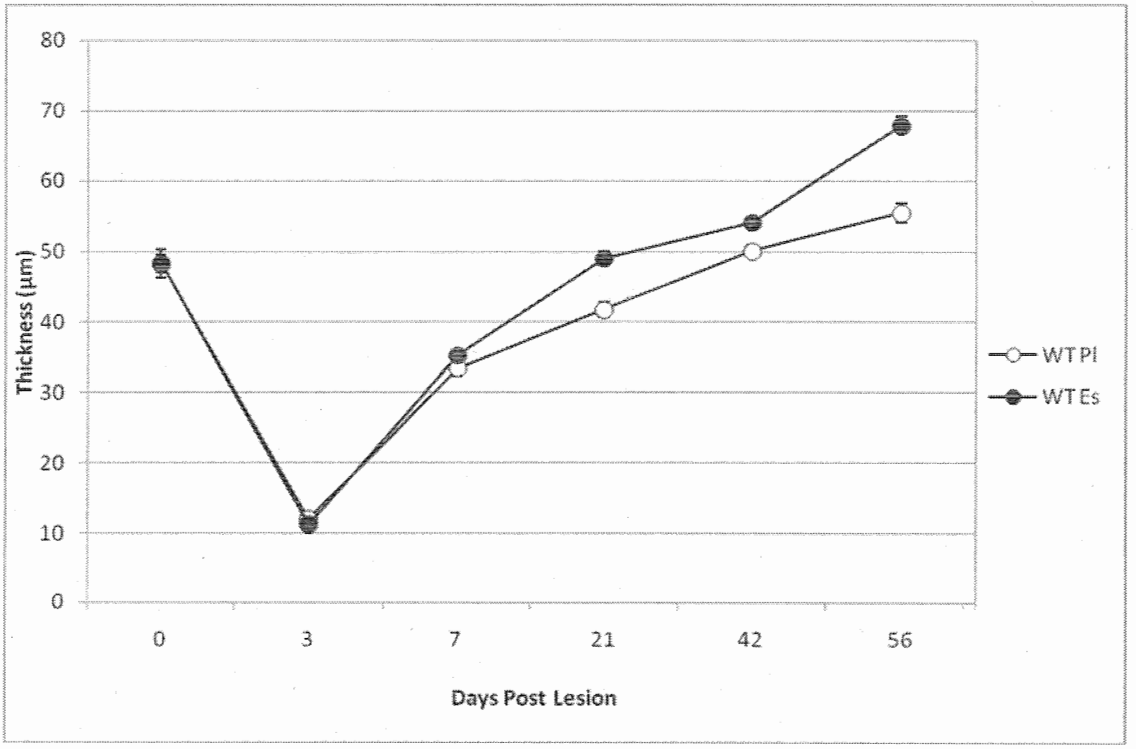


Fig. 17- Graph of OE thickness in WT mice over 56 days. Recovery was more efficient in estradiol treated mice. Estradiol's effect on thickness happened after the 7 day time point. Separation of thickness between treatments at 21, 42, and 56 days suggested a faster rate of recovery in estradiol treated mice.

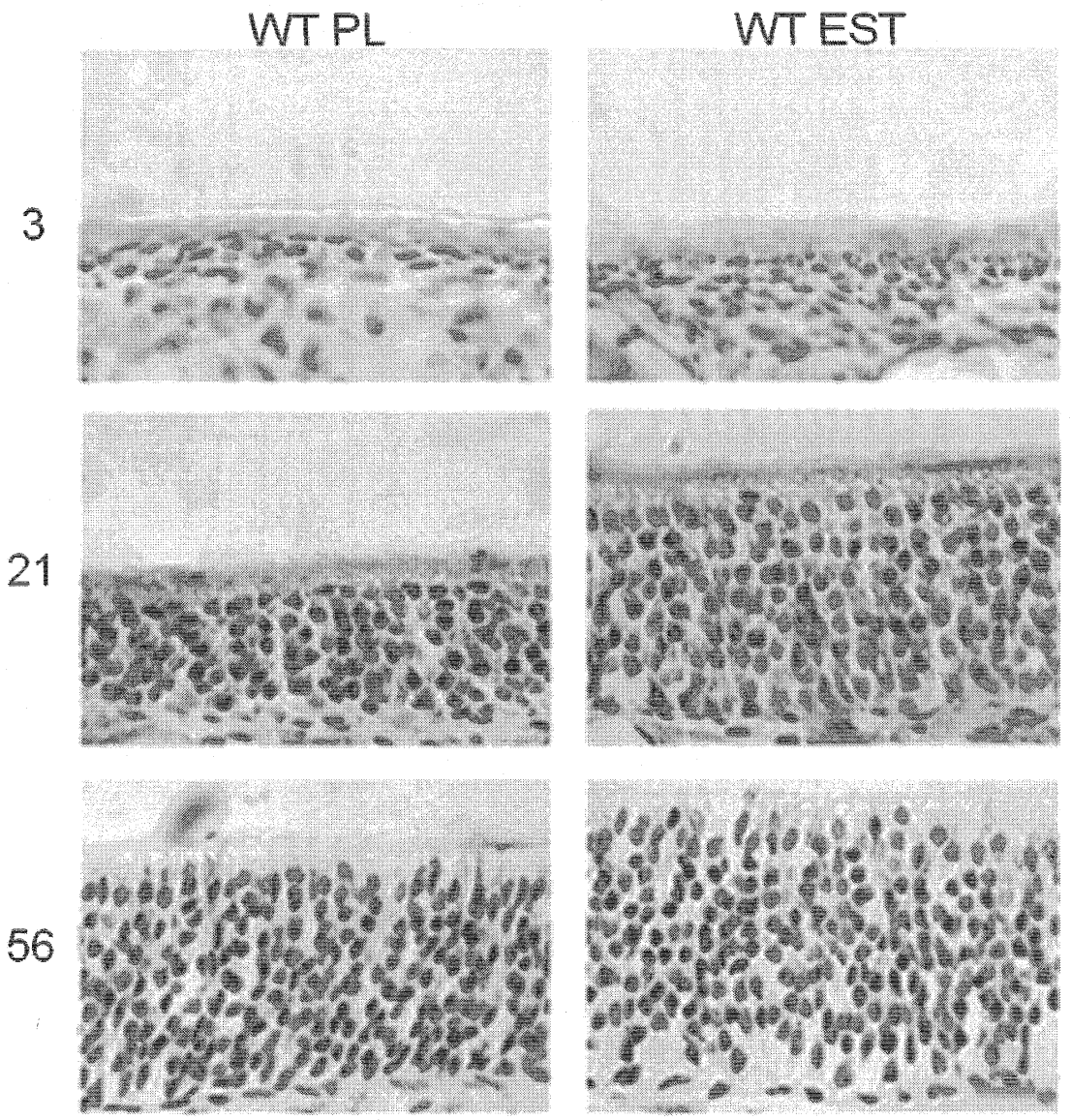


Fig. 18- Cresyl violet staining in the OE of WT mice over 56 days. There was no significant effect between treatments at 3 days. At 21 days there was a noticeable difference in thickness. This trend continued through 56 days. Scale bar=15 μm.

ApoE levels in the OE were comparable between treatments. Both treatments had apoE levels increase throughout the time course. The only observable difference was at 56 days, where apoE immunoreactivity was slightly more in estradiol treated mice (Figure 19).

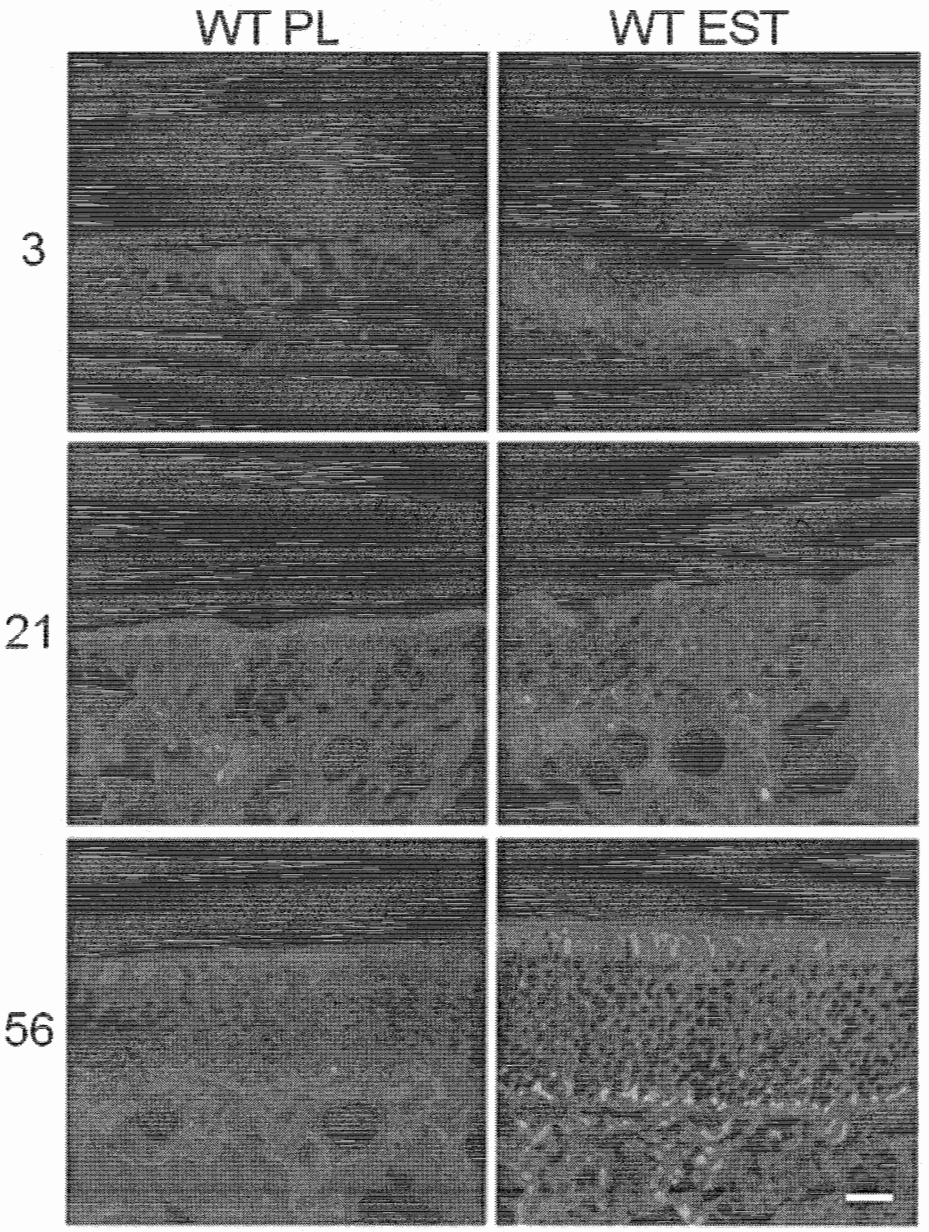


Fig. 19- ApoE staining in the OE. No difference was observed between treatments at 3 days. There was an increase in both groups at 21 days, where apoE seemed to peak. At 56 days, apoE immunoreactivity was still prevalent in both treatments but more so in estradiol treated. Scale bar=15 μm.

Cell division in the OE was examined through BrdU labeling. BrdU localization was distinguished on patches in the nasal septum of the OE and limited to the basal cell layer above the lamina propria (Figure 21). A significant difference of cell division was

found between estradiol and placebo treated mice ($F_{1,4}$=8.80, p=0.008). Examination of $BrdU^+$ cells showed a peak at 3 days after lesioning in both placebo and estradiol treated mice. Estradiol treated mice peaked at about 142 $BrdU^+$ cells per millimeter, whereas placebo treated peaked at about 95 $BrdU^+$ cells per millimeter. Cell division remained high in both groups at 7 days with estradiol having significantly more $BrdU^+$ cells ($F_{1,4}$=4.084, p=0.014). At time points thereafter, neurogenesis was invariable between treatments (Figure 20).

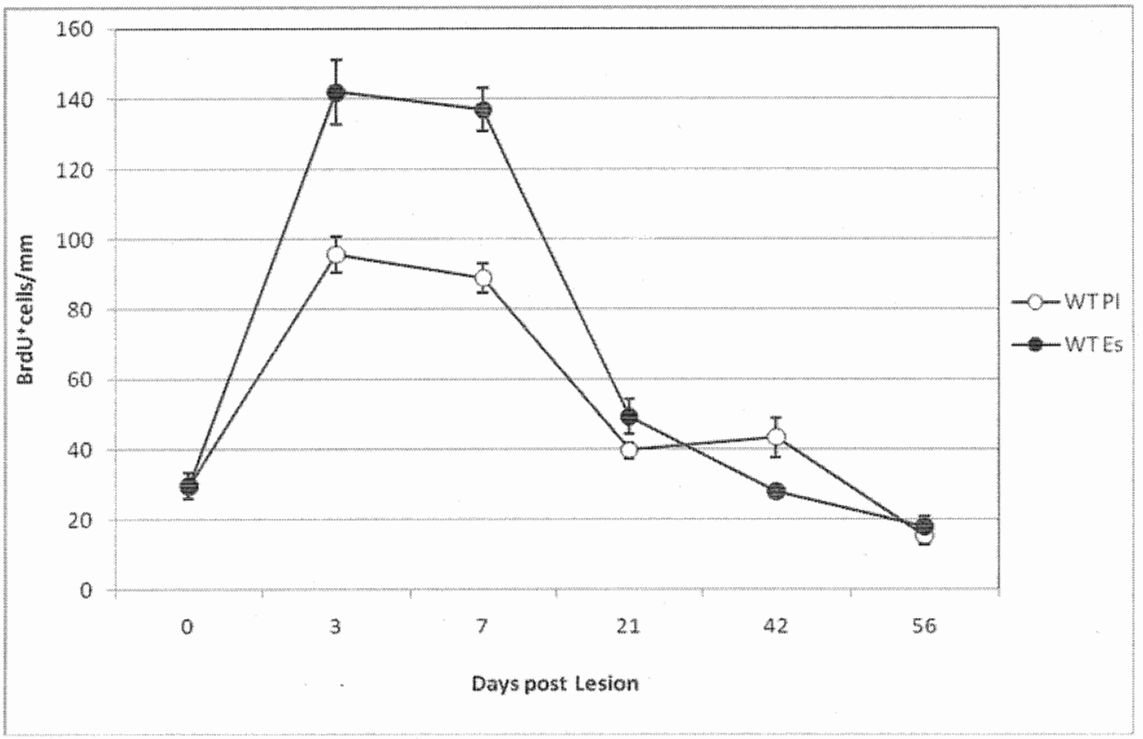


Fig. 20- Graph of cell division in the basal cell layer of the OE. A dramatic peak in $BrdU^+$ cells was seen in both treatments at 3 days post lesion. Cell division remained high at 7 days for both groups. Overall, estradiol treated had higher cell proliferation at 3 and 7 days and comparable numbers at later time points.

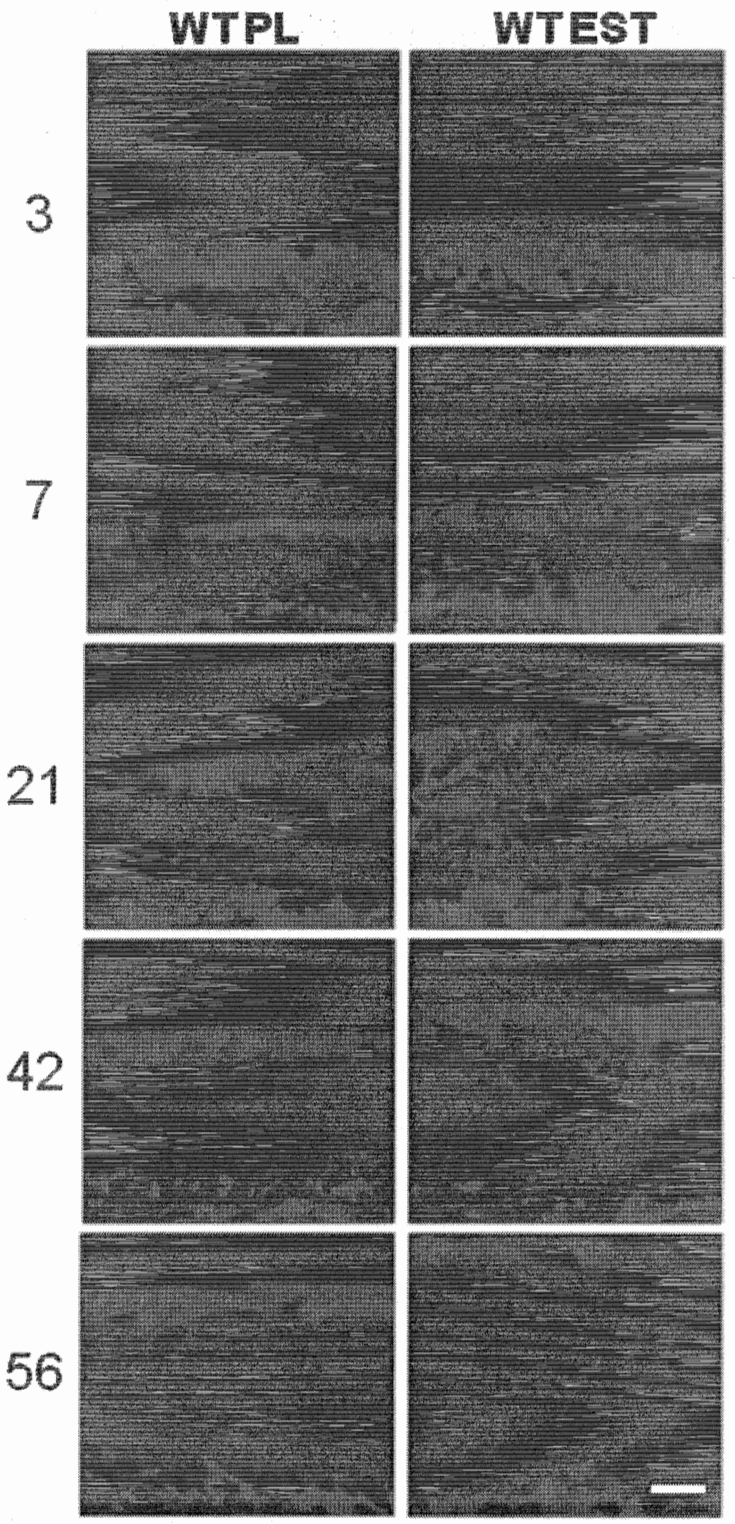


Fig. 21- BrdU labeled cells in the basal cell layer of the OE. Cell division was highest 3 days after lesion in estradiol treated mice and remained higher than placebo treated mice at 7 days. BrdU was comparable between treatments at later time points. Scale bar=15 μm

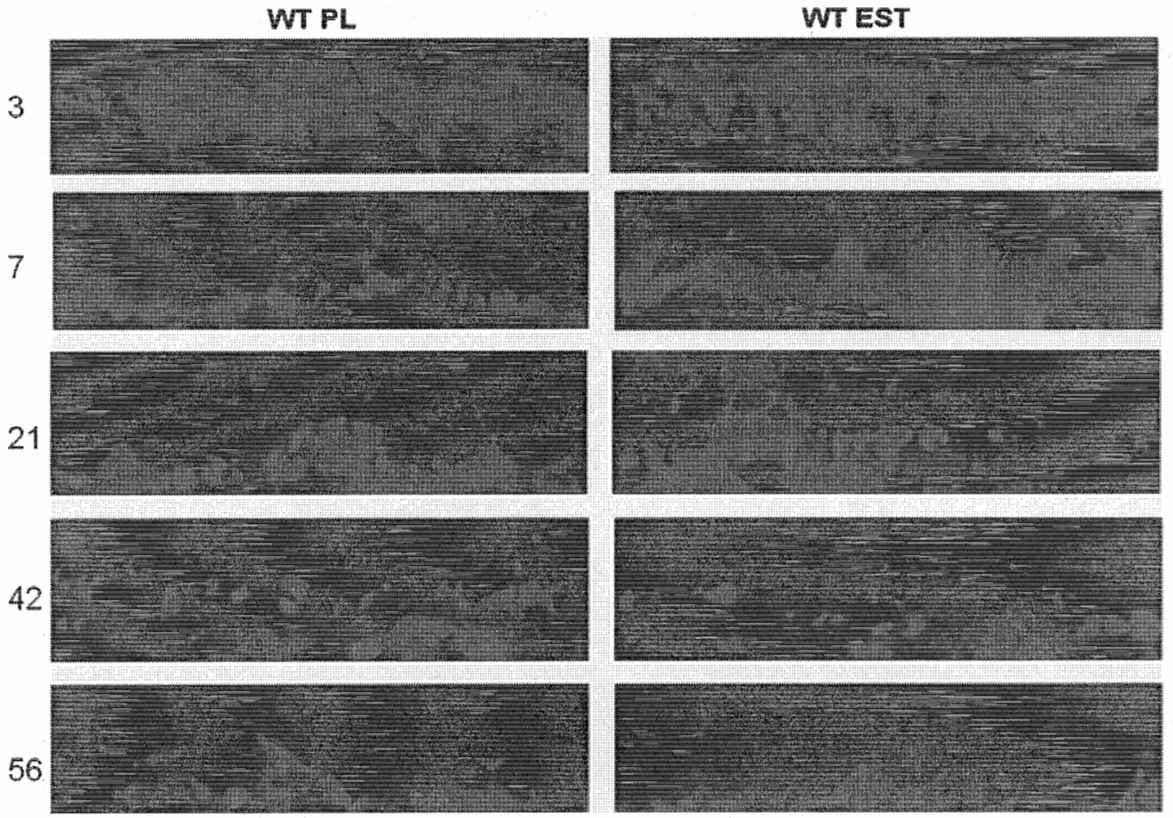


Fig. 21 MAGNIFIED- Scale bar=15 μm

Immature ORN restoration was examined through Gap43 immunoreactivity. $Gap43^+$ cells were present in between the basal cell and mature neuronal layer in the OE and present in various patches throughout the nasal septum during repair (Figure 23). Interaction of estradiol and apoE produced more immature neurons in a shorter amount of time ($F_{1,4}$=34.22, $p<0.001$). At 7 days, estradiol treated mice experienced a peak of 43 $Gap43^+$ cells per 100 μm and sustained nearly the same amount of $Gap43^+$ cells at 21 days. Then the number of $Gap43^+$ cells fell at 42 and 56 days. The placebo treated mice experienced a delay in immature ORN reconstitution compared to estradiol treated mice. Gap43 continued to fall in placebo treated at 7 days and then peaked at 33 $Gap43^+$ cells per 100 μm at 21 days. The number then decreased at 42 and 56 days (Figure 22).

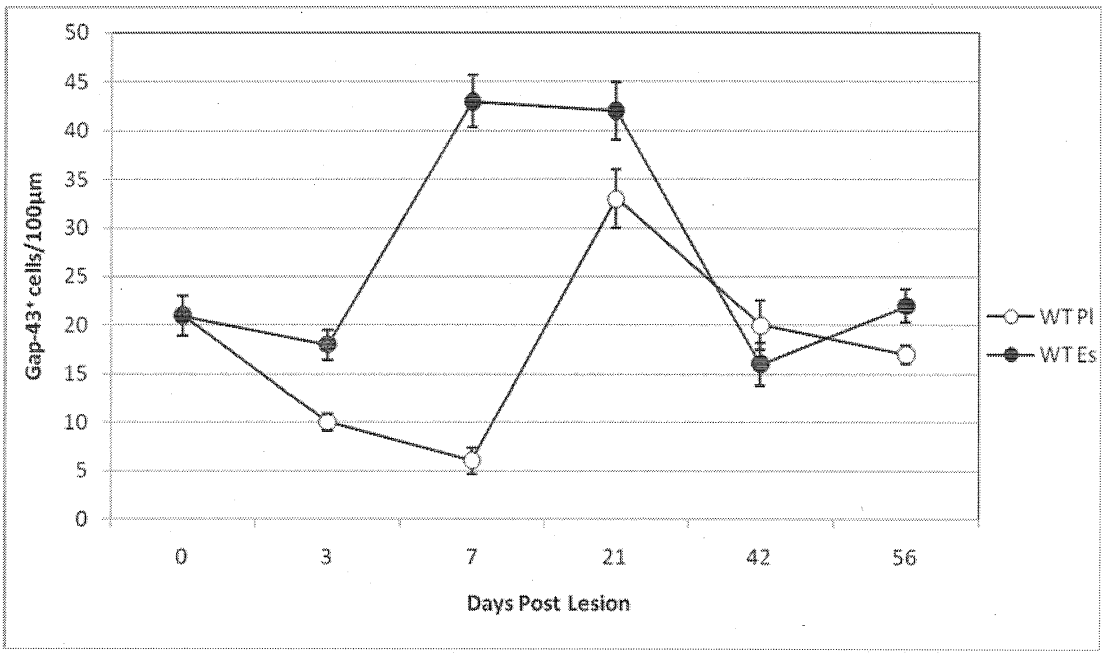


Fig. 22- Immature ORN reconstitution in the OE. Estradiol treated mice had more $Gap43^+$ cells than placebo treated mice at all time points except 42 days. Estradiol treated peaked at 7 days and remained higher on 21 days before dropping on 42 and 56 days. Gap43 in placebo treated mice fell until replenishment at 21 days and falling at 42 and 56 days.

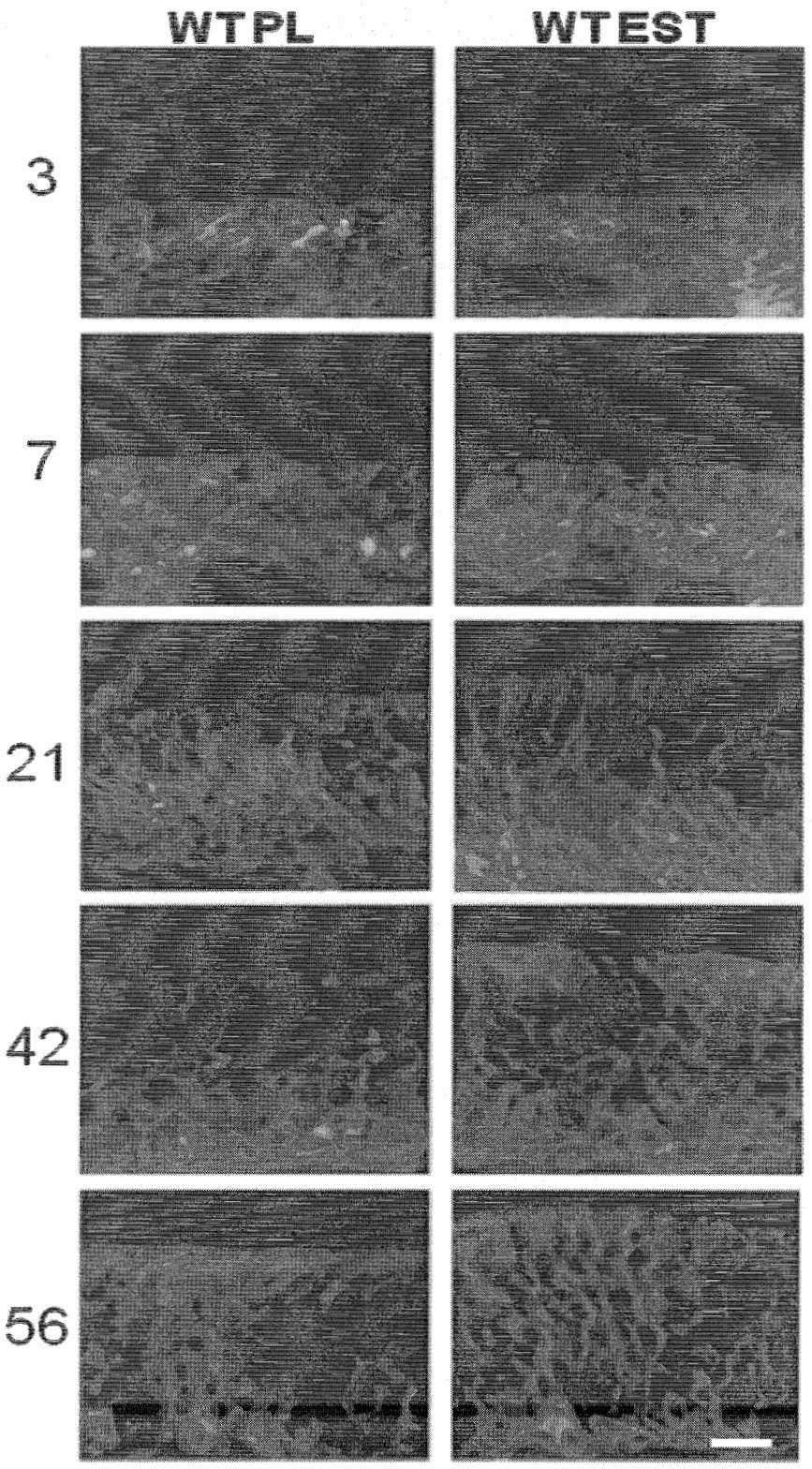


Fig. 23-Gap43 labeled cells in the OE. Gap43^{+} cells were evident 3 days after lesion in both treatments. The most Gap43^{+} ORNs were observed at 7 and 21 days post injury/treatment in estradiol treated mice. Scale bar=15 µm

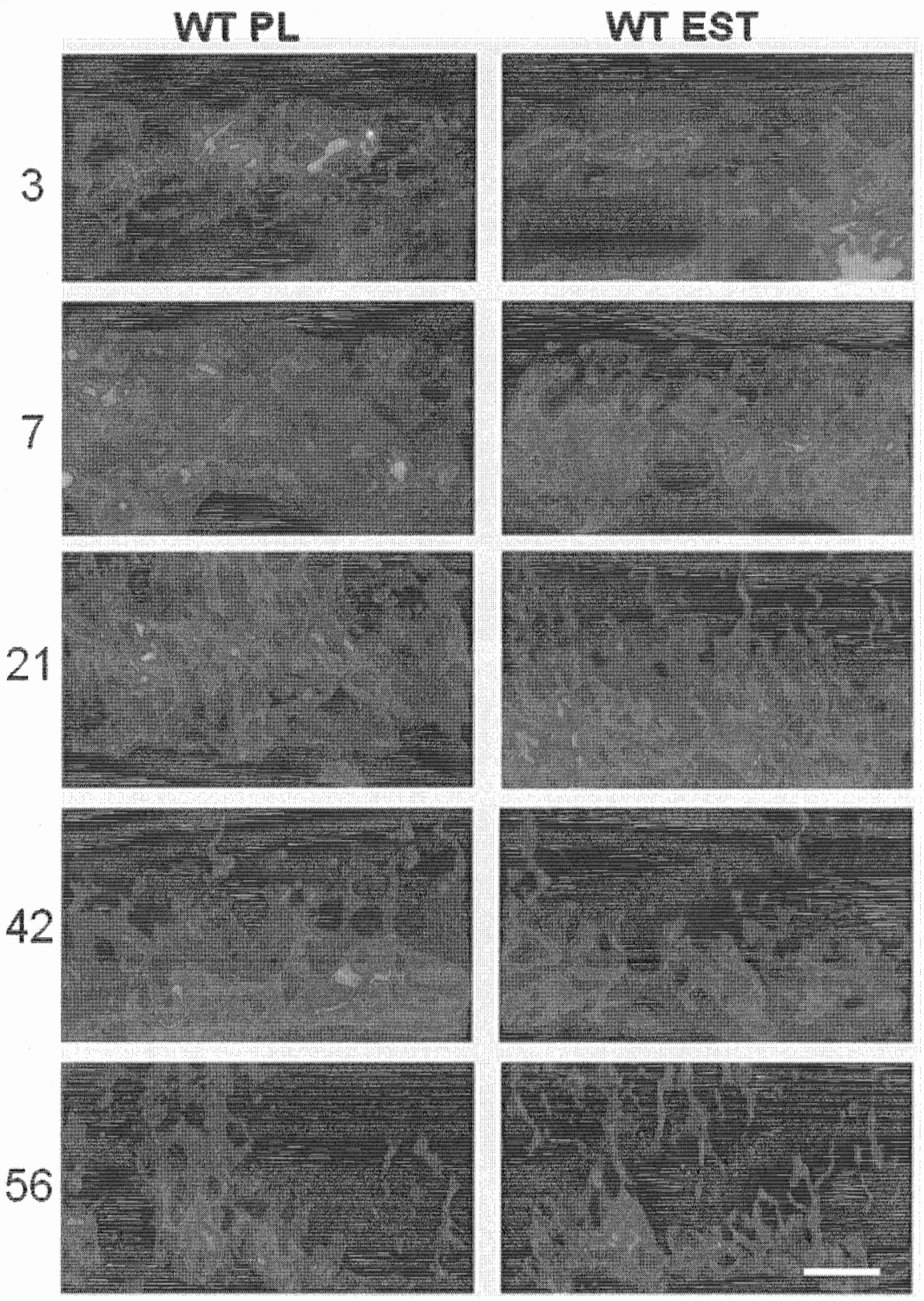


Fig. 23 MAGNIFIED - Scale bar=15 μm

Estradiol treatment stimulated a greater rate of OMP recovery after injury. Immunohistochemical analysis of OMP showed that ORNs reached maturity faster and in greater numbers than placebo treated ORNs from 7 days onward (Figure 24 & 25). An analysis of variance indicated a significant difference in OMP recovery between treatments ($F_{1,4}$=30.12 , $p<0.001$). It was also observed that estradiol treated animals had around 130 OMP^+ cells and placebo treated had 90 OMP^+ cells at 56 days. This was the same number observed at 56 days in WT normal mice which indicated both treatments reached full recovery potential (refer back to Figure 9).

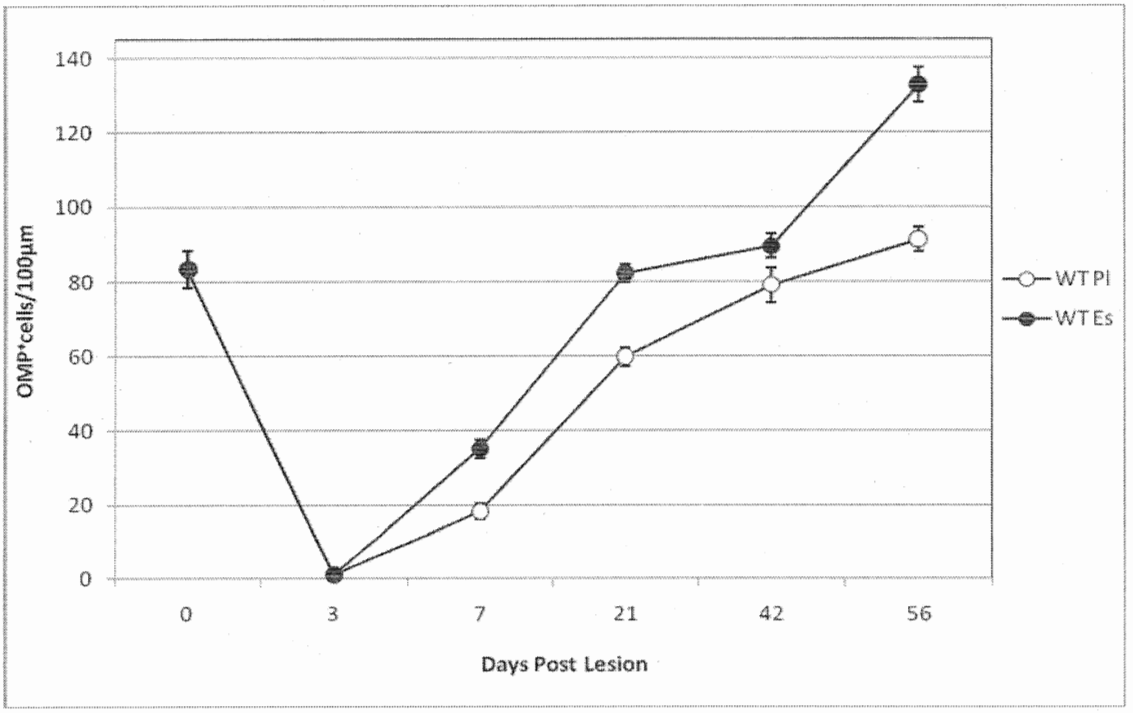


Fig. 24- Graph of OMP in the OE. Few OMP^+ cells were quantified in the OE 3 days after lesion in both treatments. At 7 days estradiol treated mice started to show faster maturation. This pattern was sustained throughout the rest of the time course. At the end of the time course estradiol treated mice had more OMP^+ cells than at 0 days, whereas placebo treated numbers were similar.

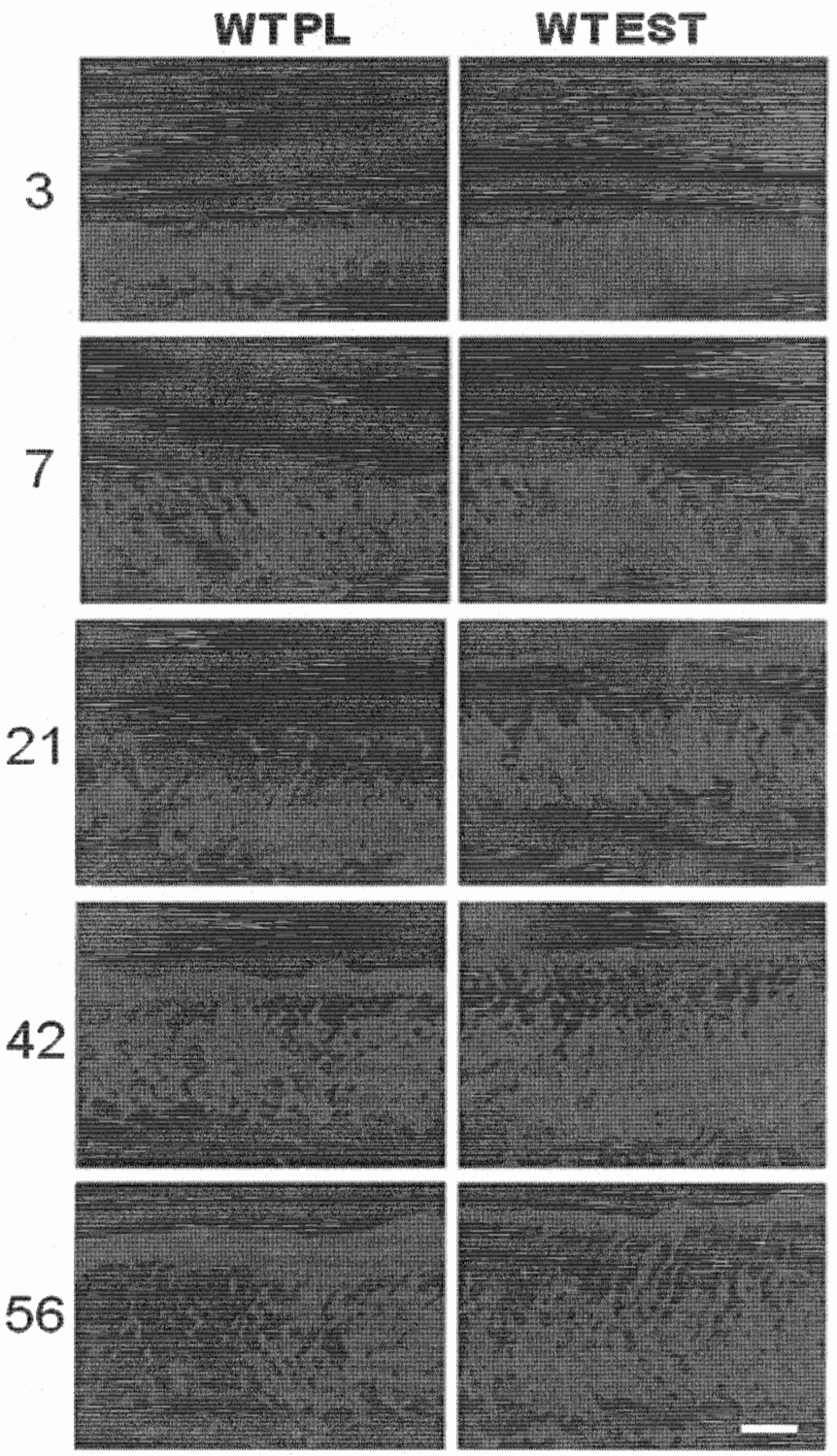


Fig. 25-OMP labeled cells in the OE. Few OMP$^+$ cells were observed at 3 days in either treatment and more OMP$^+$ cells were observed in estradiol treated mice from 7 to 56 days. Scale bar=15 μm

Estradiol treated mice had greater apoE immunoreactivity in the OB post lesion compared to placebo treated mice. In estradiol treated, apoE was noticeably the greatest at 3 and 7 days followed by a drop at 21 and 42; apoE then increased at 56 days. In placebo treated, apoE was also dispersed throughout the OB with comparable levels at 3 and 7 days. There was a slight decrease at 21 days and a noticeable decrease at 42 days. ApoE levels increased slightly at 56 days (Figure 26).

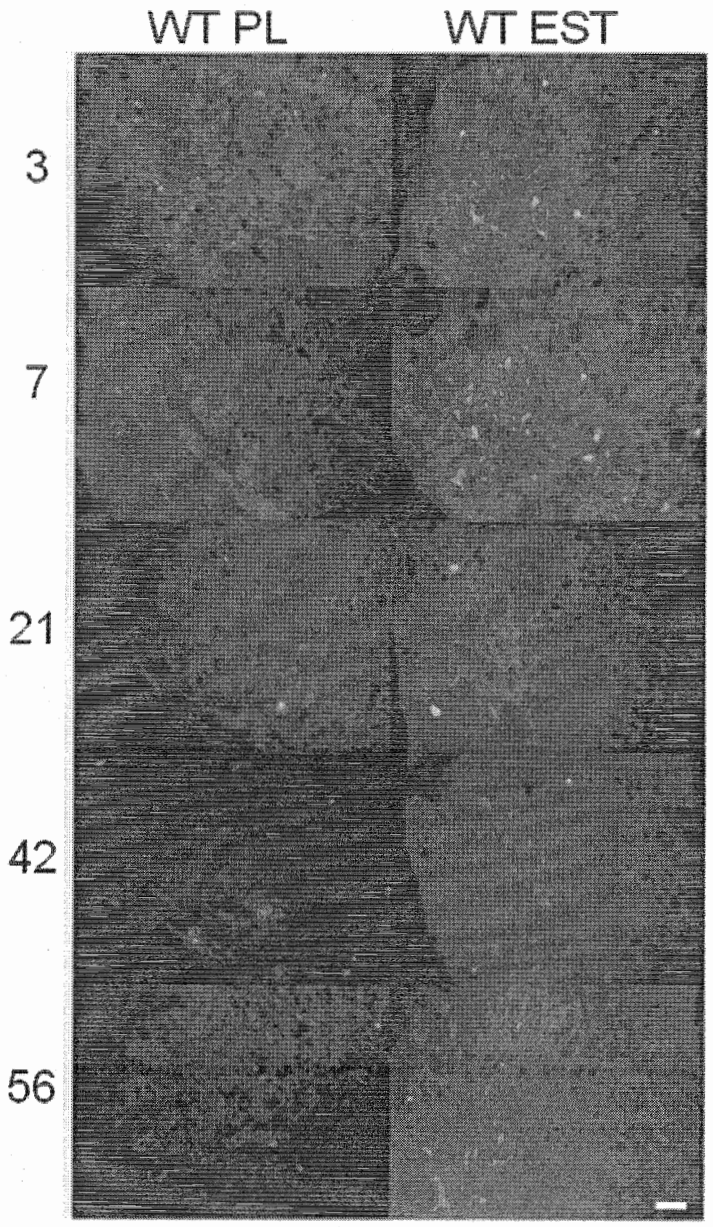


Fig. 26- ApoE staining in the glomerular layer of the OB. More immunoreactivity was present in estradiol treated mice at each time point, with the highest levels at 3, 7, and 56 days. ApoE levels in placebo were greater at 3 and 7 days with the lowest at 42 days. Scale bar=15 μm.

Only slight differences in OMP immunoreactivity in the OB were observed between estradiol and placebo treated mice during repair. No considerable difference was observed between treatments at 3 and 21 days, however estradiol treated mice had more OMP at 56 days (Figure 27).

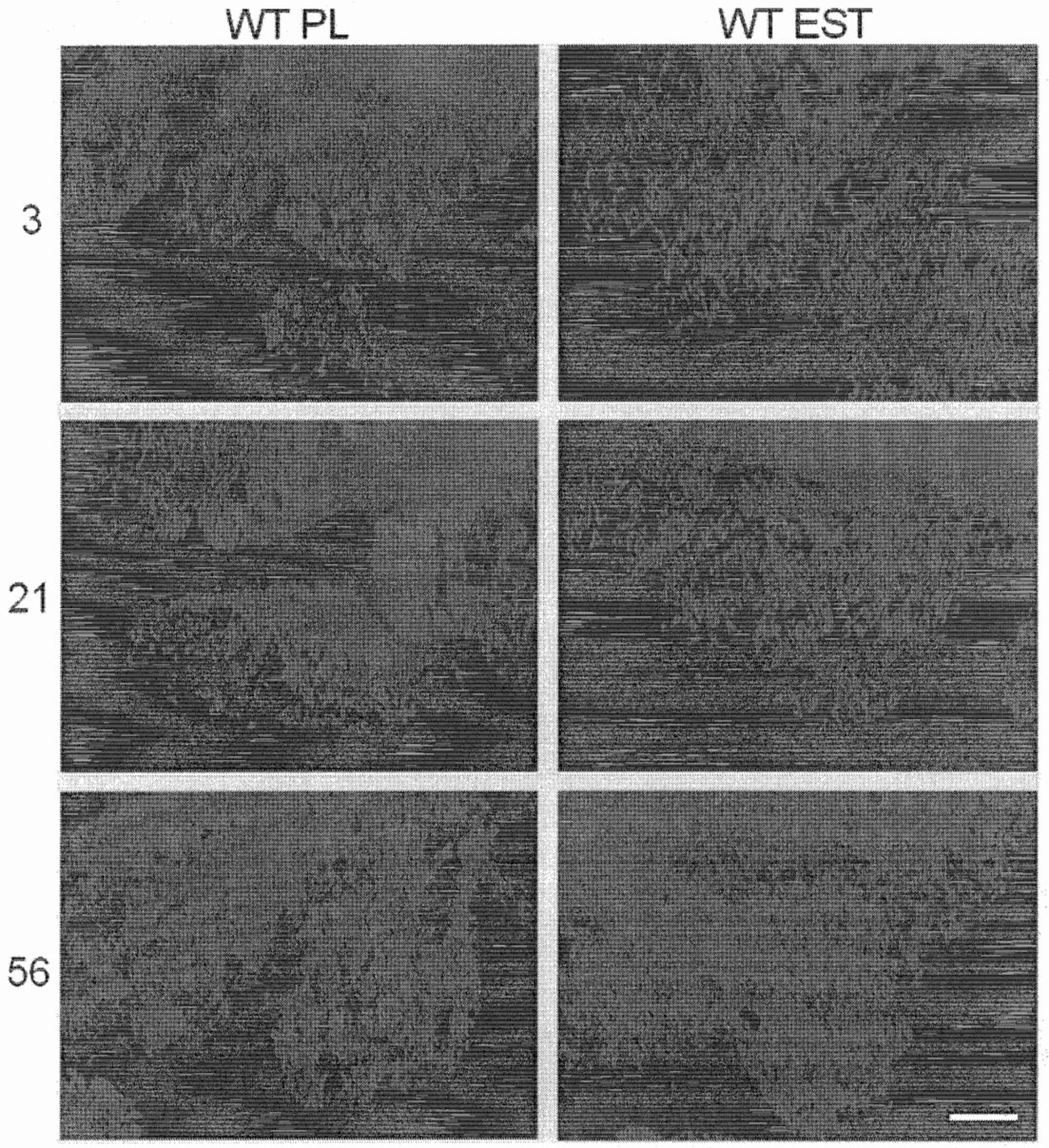


Fig. 27- OMP immunoreactivity in the glomerular layer of the OB. OMP was diffuse in the glomeruli of both treatments at 3 days with slightly more emerging by 21 days. No difference between treatments was observed until 56 days. At 56 days, glomerular re-innervation of OMP was greater in estradiol treated. Scale bar=15 μm.

Synaptophysin immunoreactivity in the OB was greater in estradiol at later time points. No difference between treatments was observed at 3 days. Slightly more SYN was observed in the glomerular layer of estradiol at 21 days and 56 days (Figure 28).

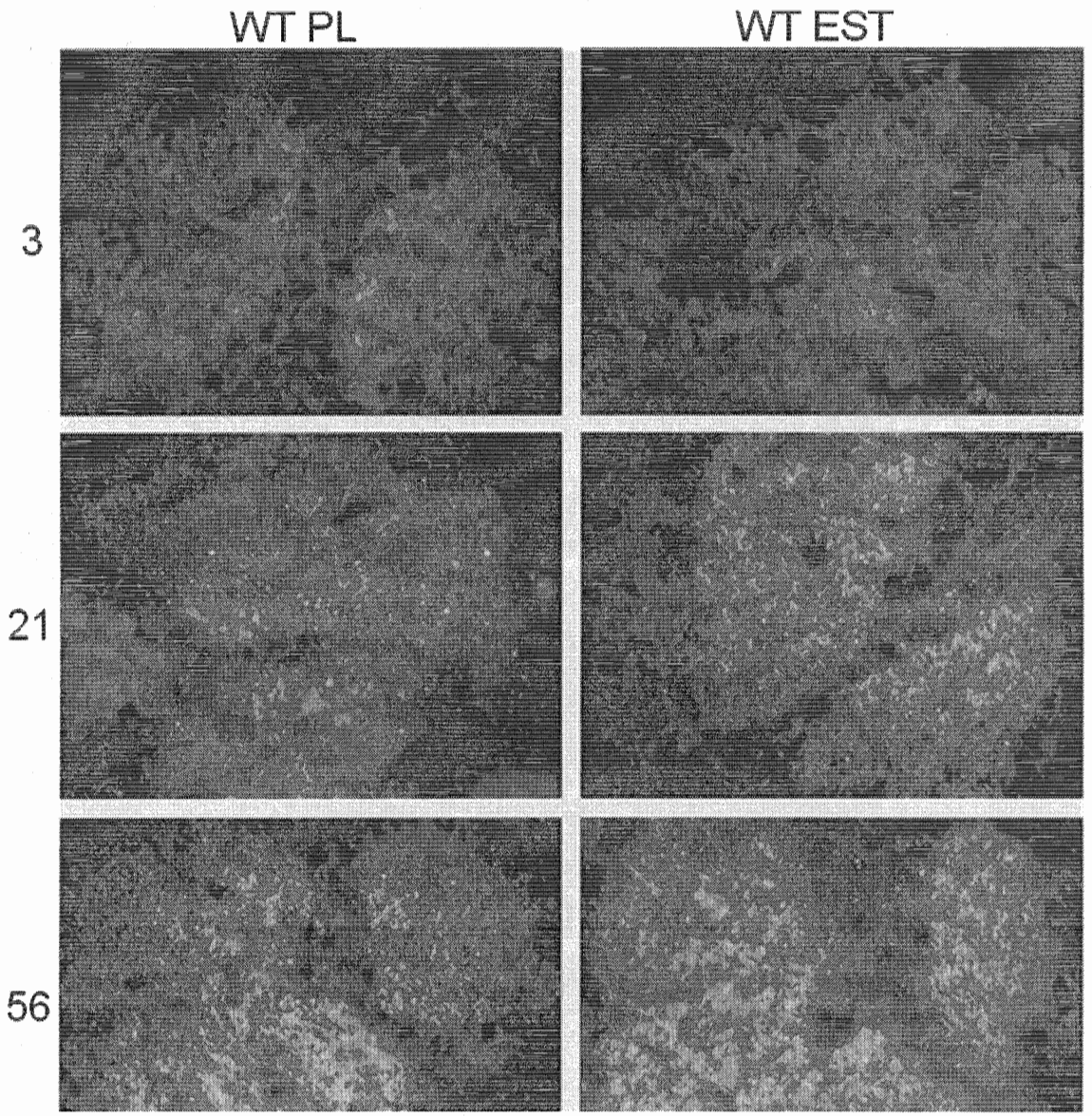


Fig. 28- SYN immunoreactivity in the glomerular layer of the OB. SYN was effectively nonexistent 3 days after lesion in both estradiol and placebo treated mice. At 21 days SYN was more complete in both treatments but more so in estradiol treated. At 56 days, SYN levels recovered more so with estradiol treatment. Scale bar=15 μm.

Astrocyte occurrence was dissimilar between treatments at earlier time points in repair ($F_{1,4}$=6.391, p=0.002). Both treatments experienced an increase in astrocytes upon lesion with process density relatively greater in estradiol treated ($F_{1,4}$=24.38, p<0.001). Peaks of 135 processes per 125 μm^2 in estradiol and 105 processes per 125 μm^2 in placebo occurred at 7 days before both dropping to 55 processes per 125 μm^2 at 21 days. Then both placebo and estradiol treated rebound to densities ~110 processes per 125 μm^2 at 42 and 56 days (Figure 29 & 30).

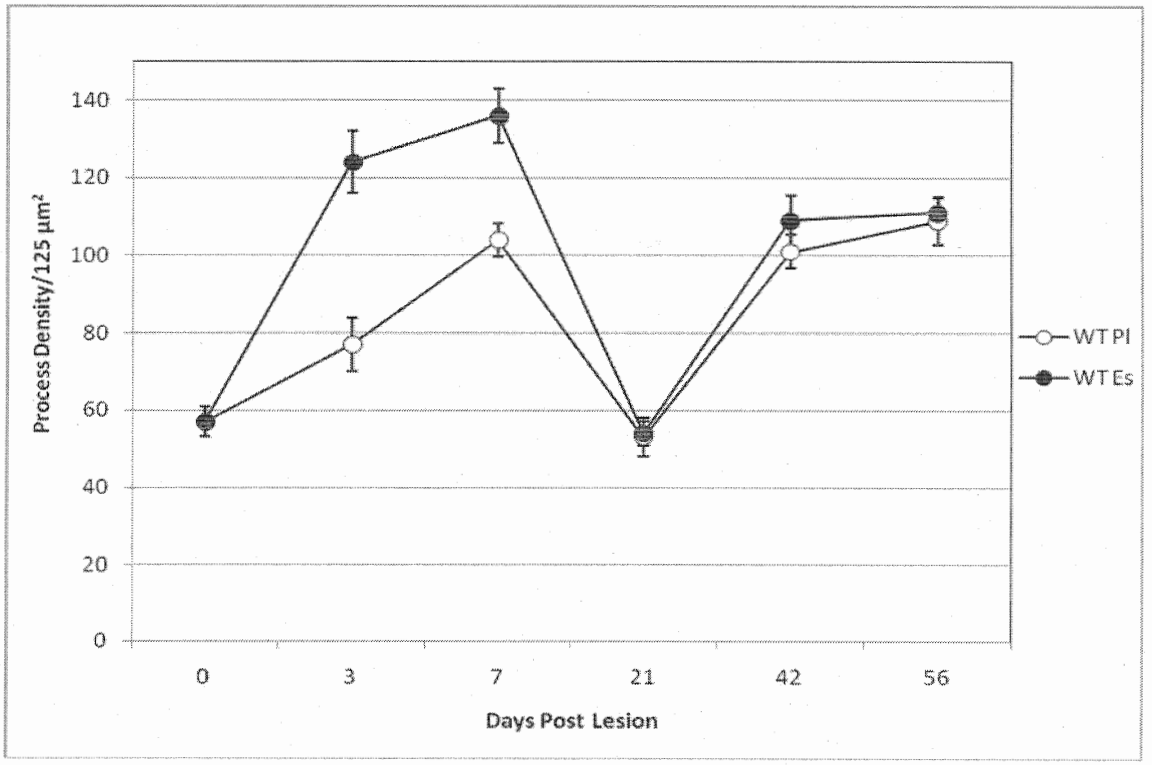


Fig. 29- Graph of astrocyte process density in the glomerular layer of the OB. Estradiol treated mice had a more predominant spike in astrocytes at 3 and 7 days post lesion. Both treatments dropped to 0 day levels at 21 days. An equivalent recuperation was evident at 42 and 56 days in both treatments.

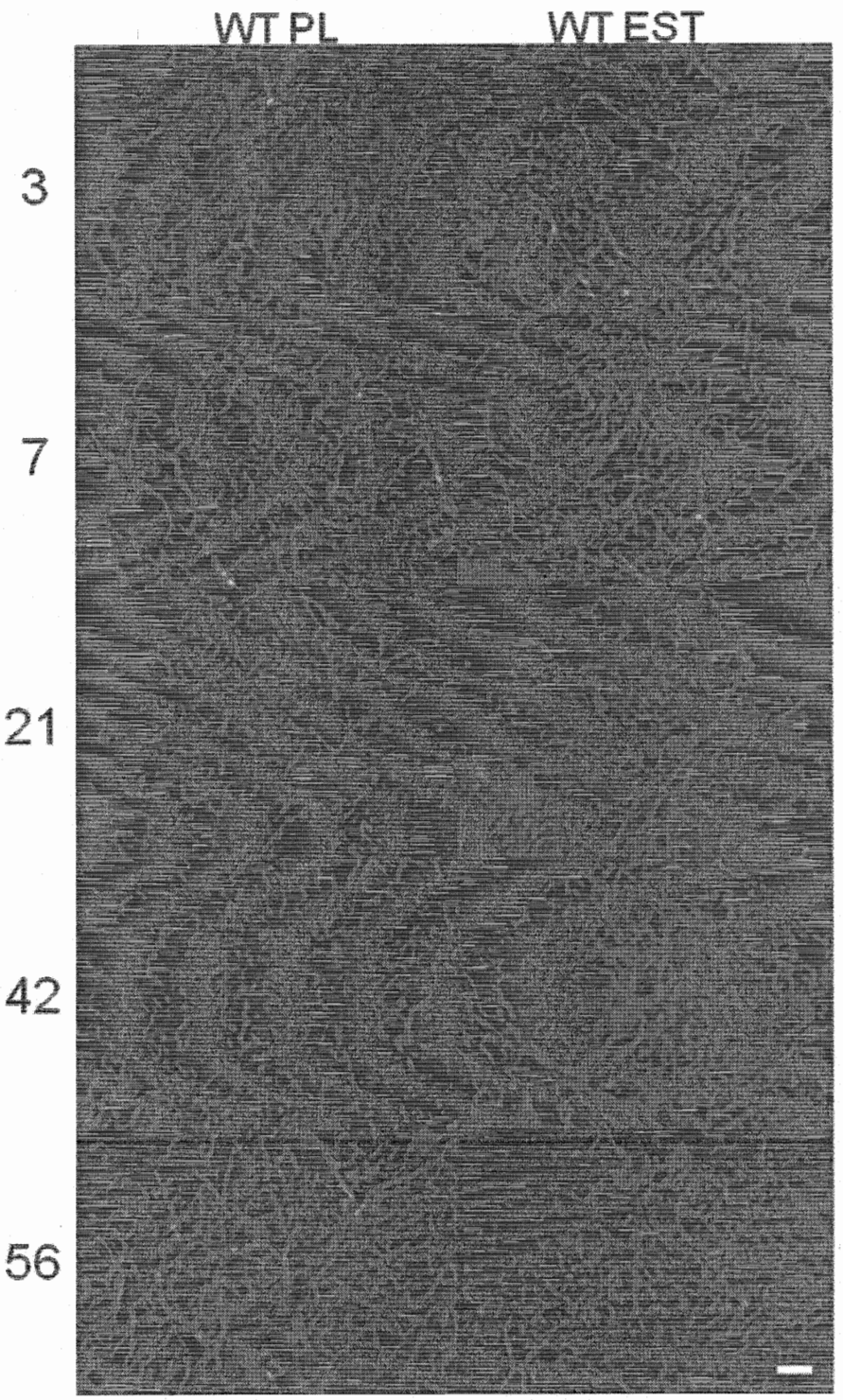


Fig. 30- GFAP assessment in the OB. Immunohistochemical analysis showed astrocyte processes throughout the glomerular layer. Estradiol treated mice had more astrocytes than placebo treated after mice 3 and 7 days. GFAP decreased in both treatments at 21 days and then increased in equal amounts at 42 and 56 days. Scale bar=15 µm.

Discussion:

Estradiol promoted a faster rate and greater amount of recovery over the time course. A difference in thickness between estradiol and placebo treated was not evident until 21 days. A difference in thickness at this time point was due to higher basal cell division yielding more immature and mature ORN development early on in repair. Estradiol treated mice had about 65 percent more dividing cells at 3 and 7 days compared to placebo treated mice. Consequently, a large emergence of immature ORN was observed from 3 to 21 days in estradiol treated. Placebo treated mice experienced a delay in development of immature ORN. The amount of immature ORN in placebo treated mice increased from 7 to 21 days and did not nearly peak at levels observed in estradiol treated mice. More immature ORN in estradiol treated mice yielded more mature ORN through the rest of the time course.

Estradiol was beneficial but not necessary for OE reconstitution. WT placebo and estradiol treated mice both achieved maximum repair potential. At the end of the time course, estradiol treated mice had a significantly thicker OE with more mature ORNs than that at 0 days. The final thickness and OMP quantities were equivalent to those observed at 56 days in WT normal mice with estradiol in the previous study. At 56 days, placebo treated mice had the same thickness and mature ORNs as that at 0 days. These numbers were equal to those observed previously in WT normal mice treated with placebo pellet. If it were possible to study time points beyond 56 days, then a plateau effect would most likely be observed in both treatments.

It was unclear whether or not the recovery differences early on in the OE were due to apoE occurrence in the OE. No differences in apoE were observed at 3 days. This

result suggests that estradiol may have increased the amount of cell division independent of apoE in the OE. Also, there was no difference in apoE between treatments at 21 days. Therefore, apoE did not play a vital role in immature ORN differences. The only observable difference was at 56 days where estradiol treated mice had more apoE. More apoE at 56 days could have contributed to maintaining the large separation of mature ORNs between treatments at 56 days.

Estradiol treatment had more of an effect on apoE fluctuations in the OB and was influential in the transport of apoE to the OB upon injury. ApoE was more prevalent in the OB at times when it needed to be. At earlier time points more apoE was needed for OMP and synapse recovery. As newly developed immature ORN reached the glomeruli of the OB they formed a synapse with the mitral and tufted cells (at this point considered mature ORNs). ApoE was vital in growing immature ORNs' axons, sustaining ORN synapse, and promoting synaptic branching between neurons as evidenced by OMP and SYN immunoreactivity in the OB. Furthermore, the drop in apoE at 21 and 42 days was most likely because the first group of immature ORNs had developed to mature ORNs at these time points and these synapses were preserved in the glomeruli. But there was a substantial increase in apoE in estradiol treated mice and a slight increase in placebo treated mice at 56 days. ApoE most likely increased to promote synaptic branching in the large influx of mature ORN which developed in the OE from 42 to 56 days.

The prevalence of astrocytes in the OB was concurrent with apoE levels. The increase in astrocytes from 0 to 7 days in both treatments was due to a response to transport apoE to the site of repair. At 21 days both treatments experienced a steep decline in the amount of astrocytes to 0 day amounts. This result was most likely

because thickness and cell division had been relatively restored to 0 day levels. The first sequence of immature ORNs had restored mature ORNs to around 0 day amounts. Therefore, the OB responded as if the olfactory nerve was completely repaired and there was no need for apoE transport. Consequently apoE levels declined. Then at 42 and 56 days, there was a recurrence of astrocytes because mature ORN were still innervating the OB and there was a need to uphold synaptic plasticity between ORNs and bulbar neurons. This was evident by the increase in SYN immunoreactivity at 56 days.

Taken as a whole, estradiol was beneficial in the OE during cell division and in the OB during ORNs axonal growth and synaptogenesis. Estradiol mainly modulated apoE levels to sustain and enhance maturation. Without estradiol, the olfactory nerve still recovered but with less efficiency.

Chapter 4: Estrogen in KO Normal Mice: An Analysis Over Time Compared With WT Normal Mice

Long term estrogen replacement was examined in KO mice in the absence of injury. This experiment was performed the same as in WT normal mice. The OE was examined at 0, 3, 7, 21, 42, and 56 days for thickness and maturation, and the OB was examined for OMP, SYN, and GFAP. Results were graphed and analyzed in conjunction with WT normal mice for comparison purposes. The graphs in this experiment contained data previously gathered for WT placebo and WT estradiol treated mice. Images contained only KO placebo treated and KO estradiol treatment tissue comparisons.

This experiment addressed whether or not estradiol or placebo treatment in KO mice affected the olfactory system. Thickness assessment examined any atrophic or thickening effects due to either estradiol or placebo treatment. OMP quantification in the OE provided answers to whether thickness outcomes were due to mature ORN turnover. OMP, SYN, and GFAP studies in the OB revealed possible correlations with outcomes in the OE. These results determined whether or not estrogen replacement was beneficial for maturation in KO mice as it was in WT normal mice.

Based on short term estrogen replacement findings in KO mice, I expected that estradiol replaced KO mice would maintain a thicker OE over the time course with more mature ORNs. KO placebo treated mice would lose mature ORNs through the time course until there were few left. Therefore, I predicted lower OMP and SYN immunoreactivity in KO placebo treated mice. Astrocytes will be in short supply in the OB of both treatments after spiking initially in estradiol treated mice due to the OVX effect. It's anticipated that the effects of estradiol on maturation will not be as lucrative as those observed over long term treatment in WT mice.

Results:

Estradiol treated KO mice had a thicker OE compared to that of KO placebo treated mice (Figure 32). An analysis of variance determined significant differences between treatments in KO mice ($F_{1,10}$=11.07, p=0.008). Additionally, the OE of placebo treated KO mice had been reduced to 35 μm at 56 days. This was the thinnest observed. At 3 days, estradiol treated KO mice followed the same decline in thickness as did WT estradiol mice before rebounding at 42 days (Figure 31). Furthermore, WT mice treated with estradiol had the thickest OE overall, but not significantly greater than KO estradiol treated mice ($F_{1,10}$=3.675, p=0.084). It was also determined that WT placebo treated mice did not have a thicker OE than KO placebo treated mice ($F_{1,8}$= 3.609, p=0.094).

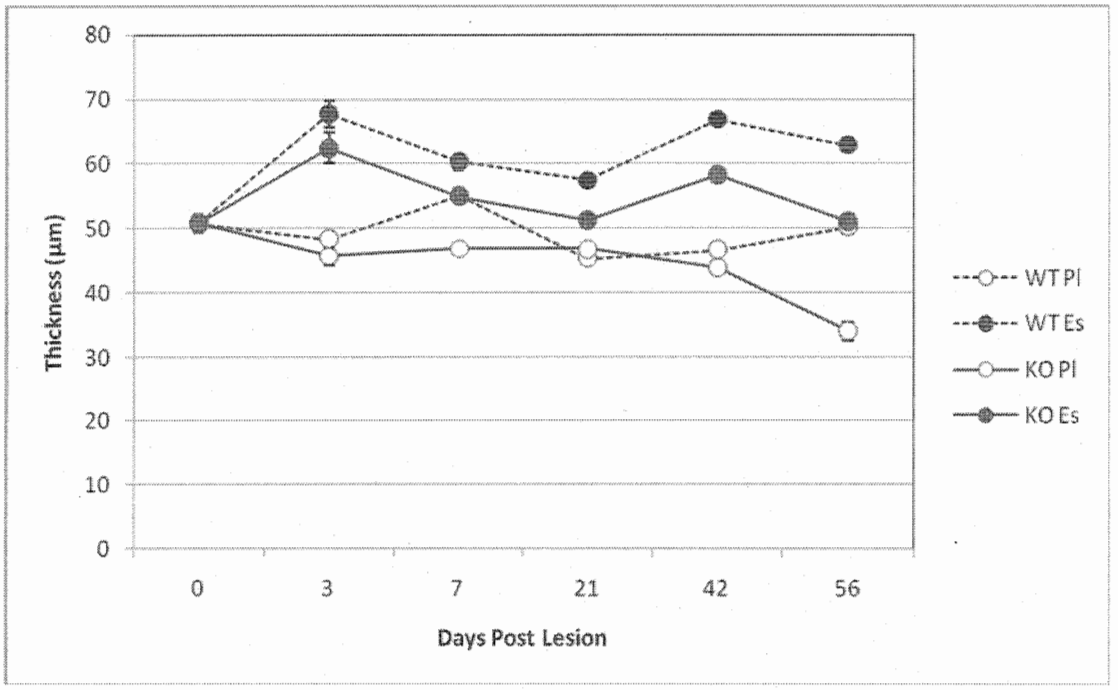


Fig. 31- Graph of OE thickness in KO and WT normal mice over a time course of 56 days. Estradiol treated mice had a thicker OE compared to the OE of placebo treated mice. The OE of placebo treated KO and WT mice was consistently thinner throughout the time course.

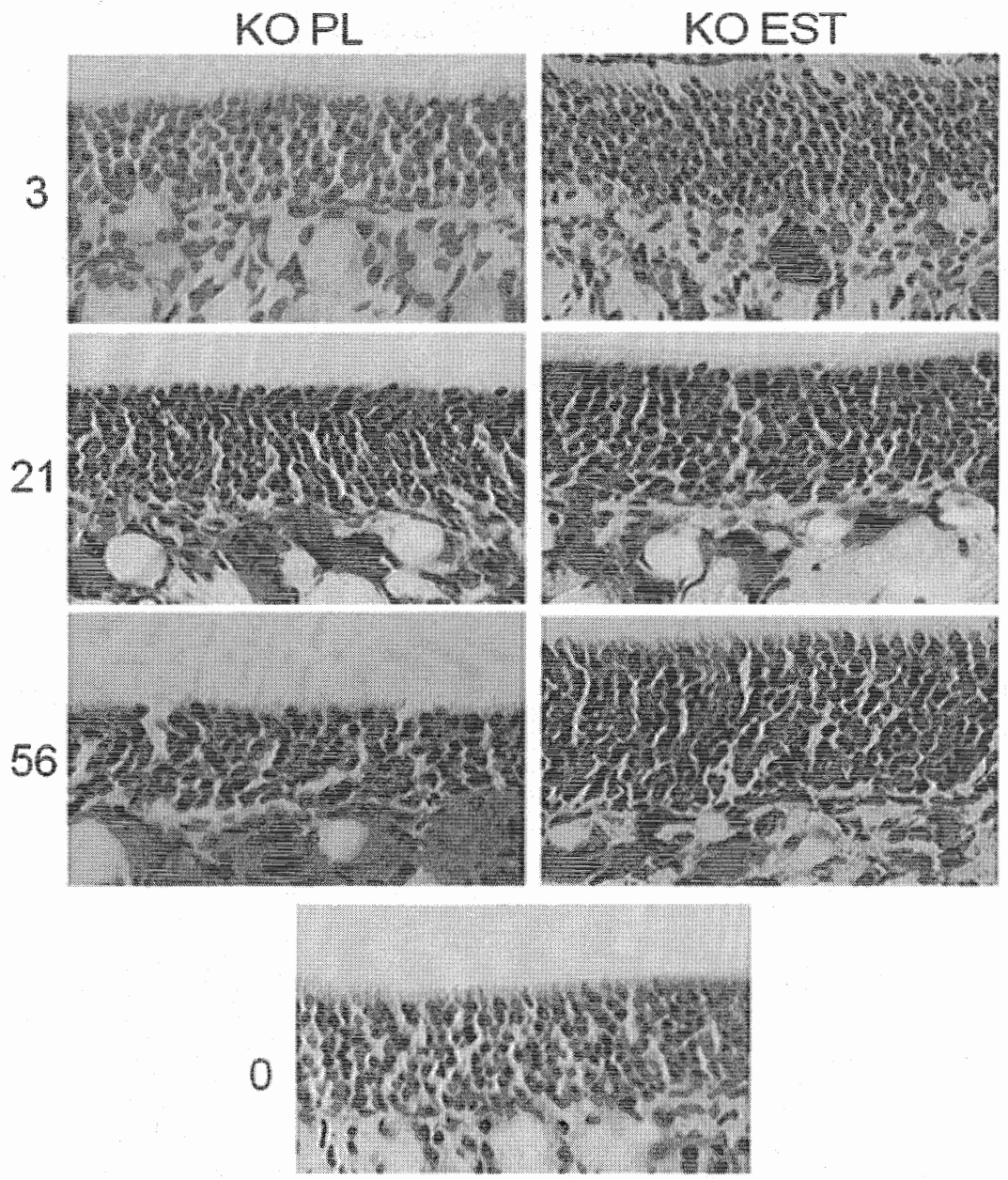


Fig. 32- Cresyl violet staining of the OE in KO normal mice. Thicker OE was observed in estradiol treated mice throughout the 56 day period, whereas a thinner OE was observed in placebo treated. The thinnest OE was observed at 56 days in placebo treated. Scale bar=15 μm.

Maturation in the OE of KO mice was different between treatments over the time course (Figure 34). KO estradiol treated mice had significantly more OMP^+ cells

compared to KO placebo treated ($F_{1,10}$= 22.03, $p<0.001$). KO mice treated with estradiol maintained around 100 OMP^+ cells across all time points with the exception of 119 OMP^+ cells at 3 days. Placebo treated KO mice maintained about 70 OMP^+ cells with the exception of 84 OMP^+ cells at 21 days (Figure 33). Furthermore, WT estradiol treated mice maintained higher maturation compared to KO estradiol treated mice over the 56 days ($F_{1,5}$=9.959, p=0.025). Maturation in placebo treated WT and KO mice did not differ significantly ($F_{1,10}$=1.206, p=0.298).

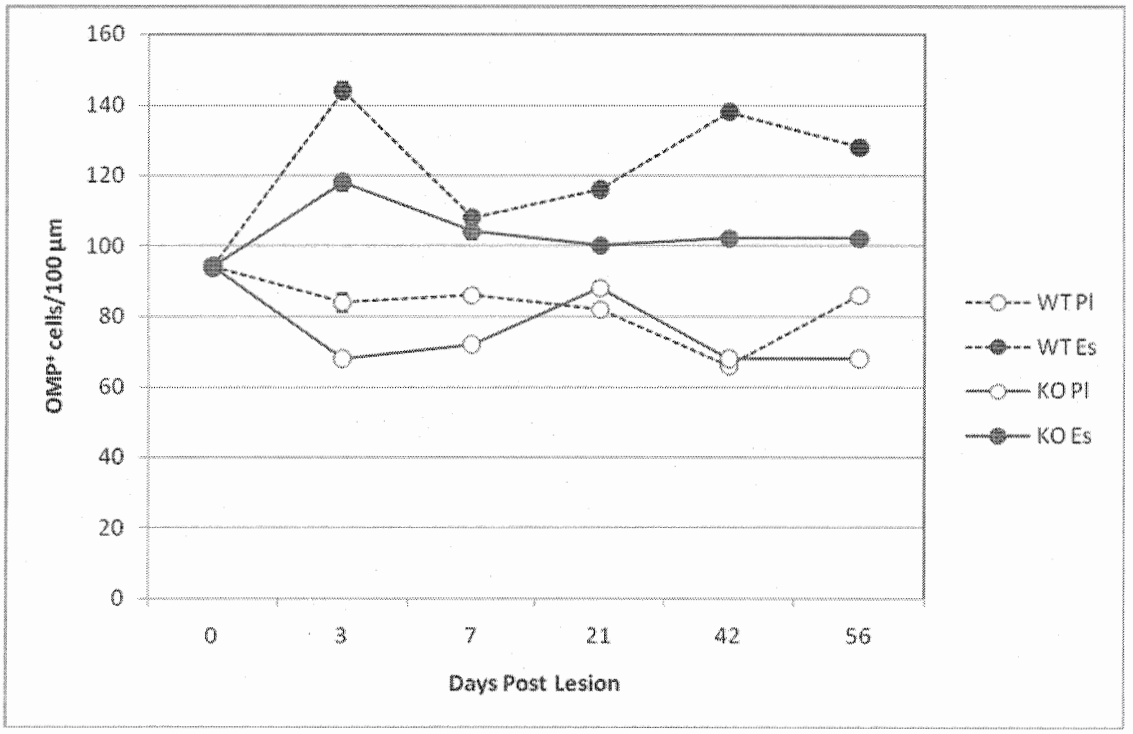


Fig. 33- Graph of OMP in the OE of KO and WT normal mice. Maturation trends graphed over a period of 56 days. KO estradiol treated maintained higher amounts of OMP over the 56 days compared to KO placebo treated. When comparing genotypes, placebo treated WT and KO mice had less OMP cells than WT and KO estradiol treated at all time points. WT estradiol treated mice had the most OMP.

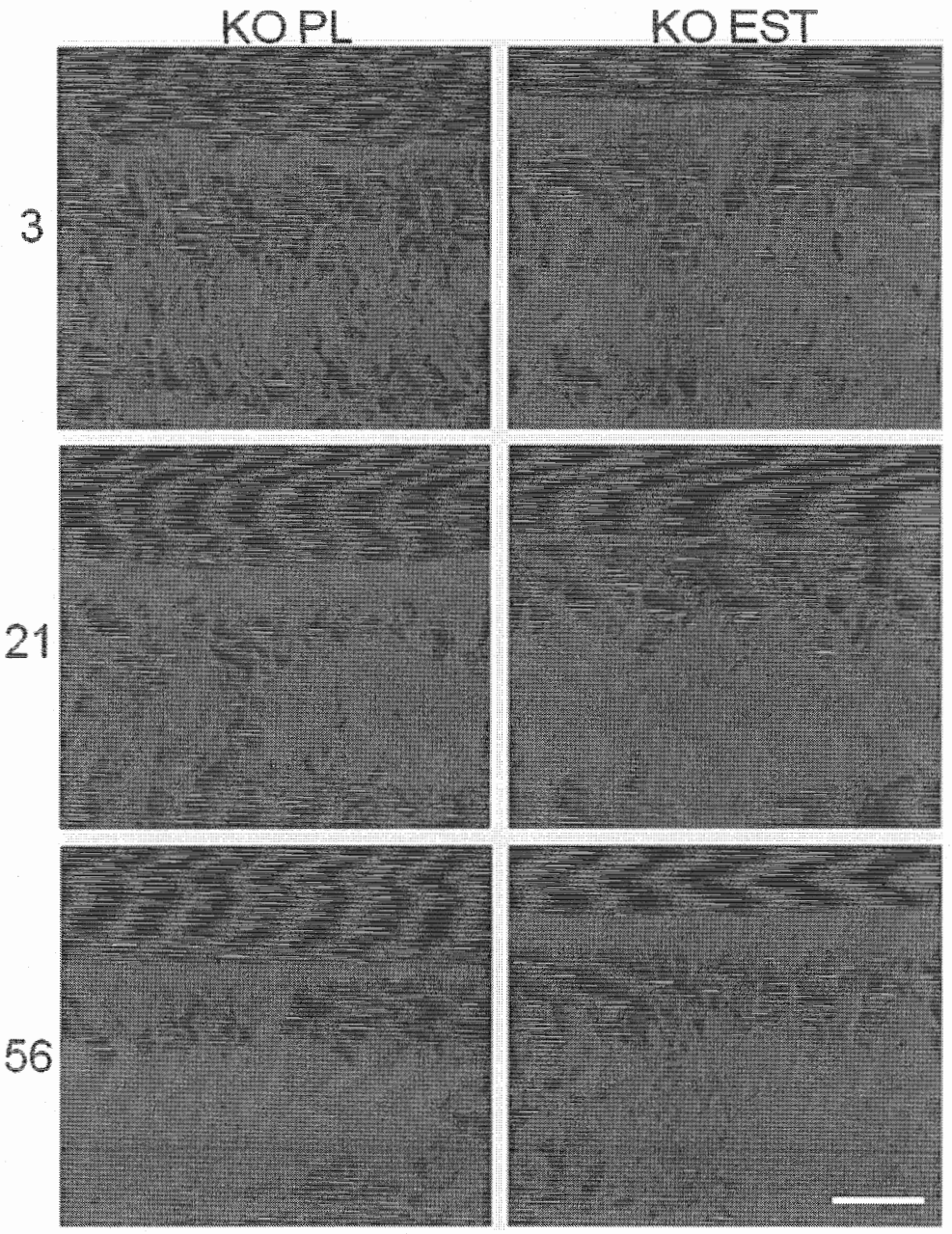


Fig. 34- OMP staining of the OE in KO normal mice. More OMP^{+} cells were observed in estradiol treated mice throughout the 56 day period compared to placebo treated. Scale bar=15 μm.

OMP in the OB differed slightly between treatments in KO mice. The most notable difference was at 3 days. Estradiol treated KO mice had more OMP immunoreactivity compared to KO placebo treated mice at 3 days. At 21 days, the

placebo and estradiol treated mice had similar OMP levels, and at 56 days, estradiol treated mice had more than placebo treated mice (Figure 35). Furthermore, KO mice were unable to achieve and maintain immunoreactive levels present in WT estradiol treated mice (refer back to Figure 13).

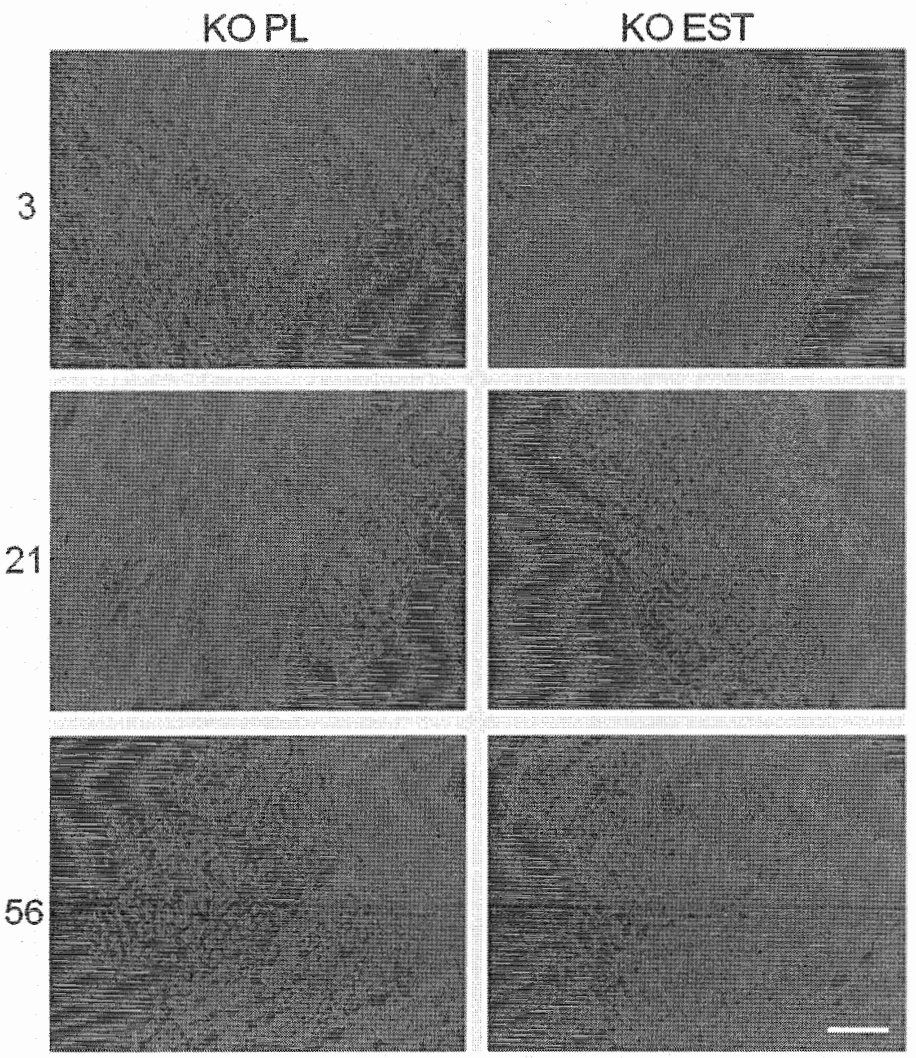


Fig. 35- OMP immunoreactivity in the glomerular layer of the OB of KO normal mice. More OMP was observed in estradiol treated at 3 and 56 days. No significant difference was observed at 21 days. Scale bar=15 μm.

SYN immunoreactivity was similar to OMP in the OB. Estradiol treated mice had more SYN at 3 and 56 days. At 21 days, there was no observable difference between

treatments. KO placebo treated mice had a decline in SYN from 3 to 7 and from 7 to 56 days (Figure 36). Upon comparison of genotypes, KO estradiol treated mice were unable to maintain the same amount of synapses as that of WT estradiol treated mice (refer back to Figure 14).

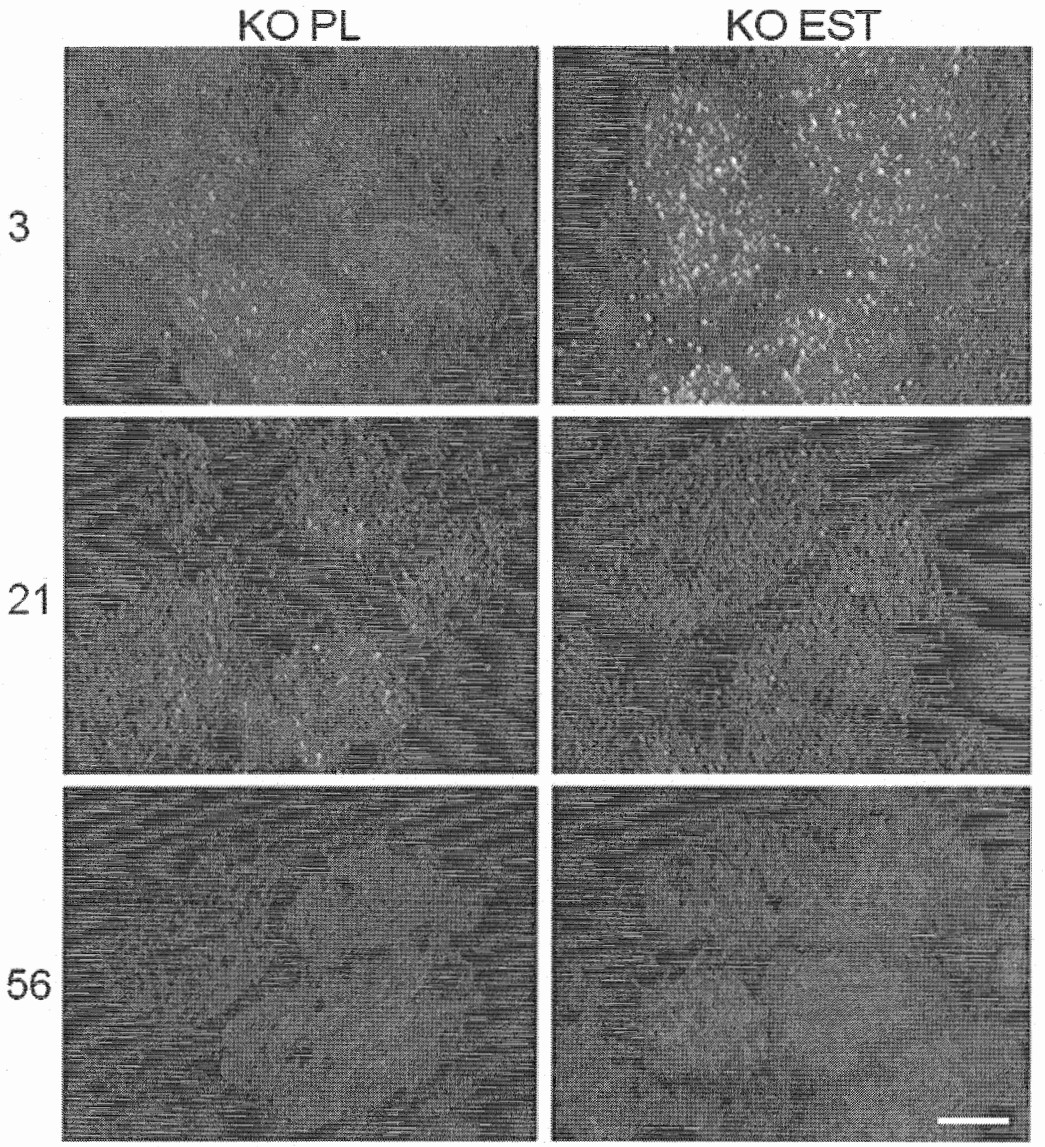


Fig. 36- SYN immunoreactivity in the glomerular layer of the OB of KO normal mice. SYN levels were higher in estradiol treated mice at 3 and 56 days. SYN levels were similar at 21 days in placebo and estradiol treated mice. Scale bar=15 μm.

Astrocytes in the OB varied between estradiol and placebo treatment in KO mice (Figure 38). Estradiol treated mice had more astrocytes at 3, 7, and 21 days when compared to placebo treated mice. At 42 and 56 days, the number of astrocytes dropped lower than that of placebo treated mice (Figure 37). An analysis of variance demonstrated significant difference between treatments over the time course ($F_{1,10}$=30.69, p=0.032). Furthermore, there was no significant difference between estradiol treatment in KO mice and estradiol treatment in WT mice ($F_{1,10}$=4.551, p=0.086) and no significant difference between placebo treatment in KO mice and placebo treatment in WT mice ($F_{1,10}$=2.514, p=0.144).

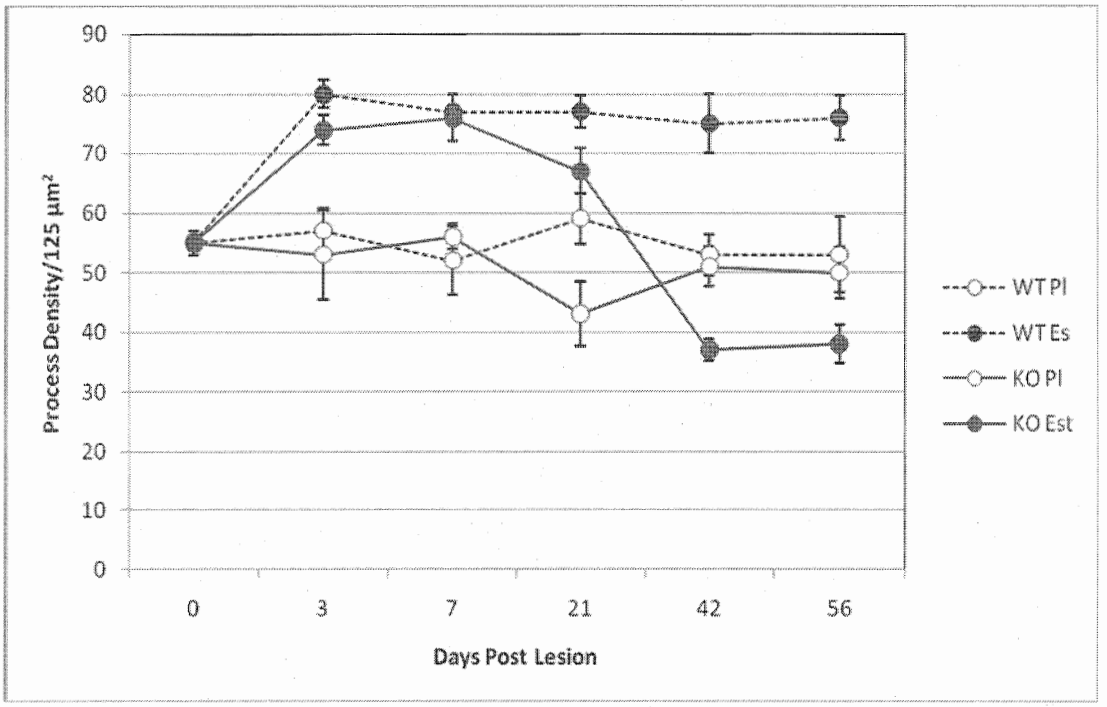


Fig. 37- Graph of astrocyte process density in the glomerular layer of the OB in WT and KO normal mice. Estradiol treated KO mice had more astrocytes present at 3 and 7 days before experiencing a drop from 21-56 days. Placebo treated KO mice densities remained fairly consistent over the time course with a small drop at 21 days. WT mice treated with estradiol had the most astrocytes in the OB and numbers did not fluctuate over time.

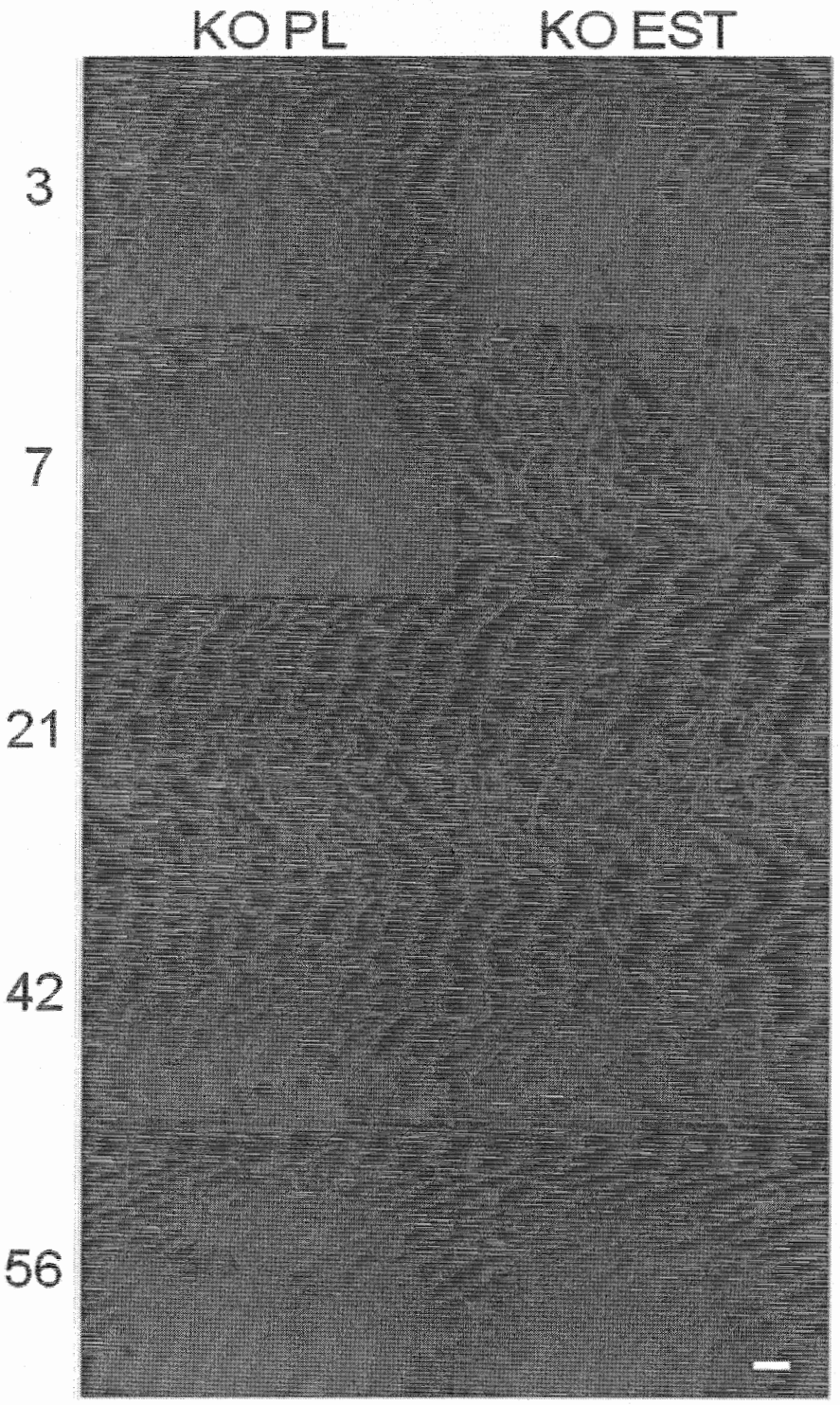


Fig. 38- GFAP assessment in the OB of KO normal mice. Immunohistochemical analysis showed astrocyte processes throughout the glomerular layer. Estradiol treated mice had more astrocytes than placebo treated at 3, 7, and 21 days. Scale bar=15 µm.

Discussion:

Estradiol had the same general effect in KO mice as it did in WT mice over the time course. KO mice treated with estradiol had a thicker OE with more mature ORN compared to placebo treated mice. Estradiol's effect was not as robust in KO mice since WT mice with estradiol had the thickest OE and the most mature ORN throughout the time course. Not having estradiol effected maturation sustainability in KO mice the most. Overall, the number of mature ORNs in the OE was reduced but did not approach an expected low.

OMP and SYN immunoreactivity in the OB corresponded with the amount of mature ORNs present in the OE. Immunoreactivity was greater in KO estradiol treated mice throughout the time course, but it was still less than that observed in WT estradiol treated mice. This result suggests that estradiol augmented maturation and synapse sustainability via apoE.

It was expected that astrocytes would spike in estradiol treated due to OVX, and placebo treated mice would be in short supply from 0 days onward. KO placebo treated mice maintained low occurrence of astrocytes. KO estradiol treated mice had a large increase at early time points followed by a dramatic decrease at later time points. The only result correlated with this observation was maturation. Early on maturation increased in the OE and the OB in estradiol treated mice. Astrocytes were present at this time to promote synaptogenesis. However, without apoE the new mature ORNs died and astrocytes were no longer customary. In comparison, WT estradiol treated mice maintained high astrocyte densities throughout the time course to maintain elevated maturation.

Chapter 5: Estrogen in KO Injured Mice: A Comparative Analysis With WT Injured Mice

An evaluation of repair in KO mice was performed to determine if estradiol's effect on olfactory nerve recovery required the presence of apoE. This study defined the importance of estrogen replacement and it's facilitation of nerve repair via an apoE mechanism. KO mice received either an estradiol or placebo pellet and were evaluated at the same time points as previous studies. The OE was assessed on thickness, cell division, immature ORNs, and mature ORNs. The OB was assessed on OMP and SYN immunoreactivity and GFAP quantification. Results were graphed and compared with WT estradiol treated and WT placebo treated mice. Images contained only KO placebo and KO estradiol treatment tissue comparisons.

Thickness data initially revealed when differences in recovery patterns occurred between placebo and estradiol treated mice. Examination of BrdU, Gap43, and OMP pinpointed which cells in the OE were affected and at what time during recovery. A comparison to the OE of WT injured mice determined if apoE was needed for efficient repair. OMP and SYN immunoreactivity in the OB disclosed any differences in mature ORN re-establishment and synaptogenesis between estradiol and placebo treatments. A comparison with WT injured mice observations discerned the importance of apoE in the OB during recovery.

Based on previous findings, I expected that estradiol would not be as effective during repair in KO mice as was in WT mice. In KO mice, cell division would be lower in placebo treated mice compared to estradiol treated. Hence recovery would be delayed in placebo treated mice.

Previously, apoE was important in promoting maturation in the absence of injury and during repair. I anticipated that immature ORNs would be unable to reach maturity in both treatments. Immature ORNs would continually degenerate over the time course.

Furthermore, there would be minimal OMP and SYN immunoreactivity in the OB among treatments throughout the time course since ORNs would not reach the OB. There would be no need for astrocyte incidence in the OB, since there would be no apoE promoting growth and synaptogenesis.

Results:

KO injured mice were unable to sustain recovery at later time points. The significant difference between WT and KO genotypes was evident at 42 and 56 days (Figure 39). At these time points, the KO did not increase thickness with either placebo or estradiol treatment ($F_{1,4}$=10.85, $p<0.001$). It was noticed that estradiol treated thickness in KO was highest at 21 days followed by an atrophy at 42 and 56 days, whereas placebo treated KO maintained that same thickness from 21 to 56 days (Figure 40).

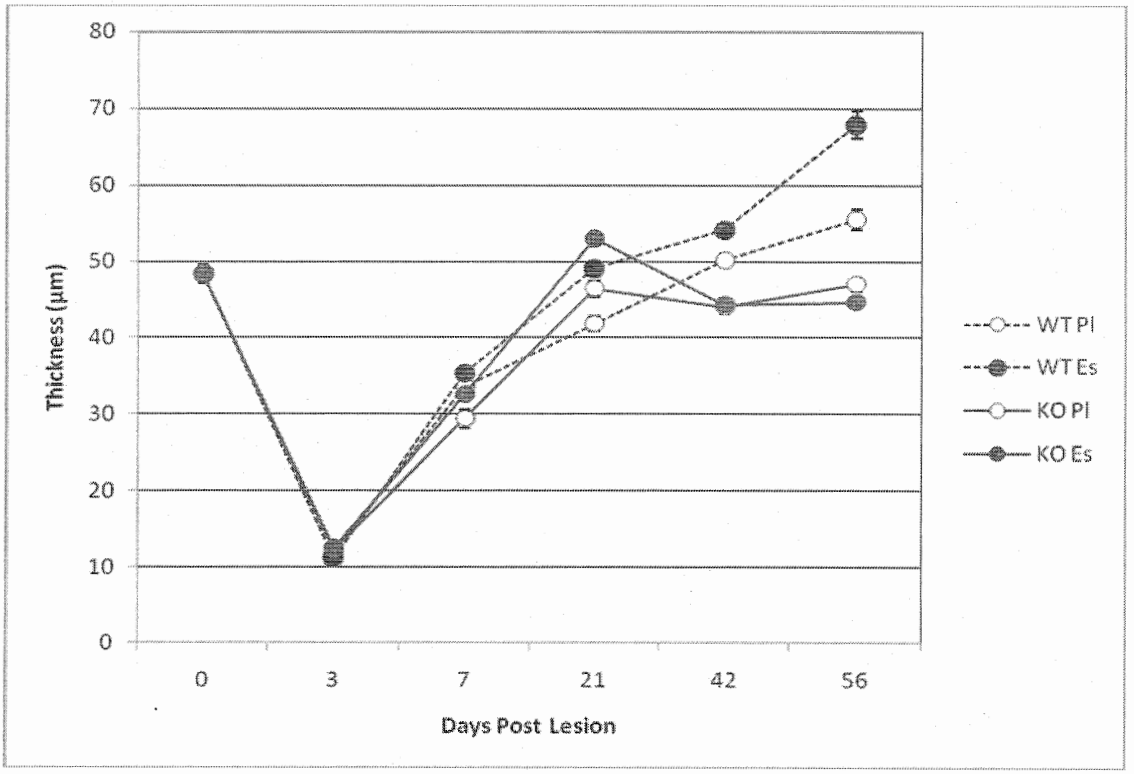


Fig. 39- Graph of OE thickness in KO and WT injured mice. At 3 days there was no difference in OE thickness between treatments. All mice recovered at 7 and 21 days, but then resurgence of the OE was halted at 42 and 56 days in both treatments of KO. This was significantly different than estradiol and placebo treated WT mice where recovery was continuous throughout all time points.

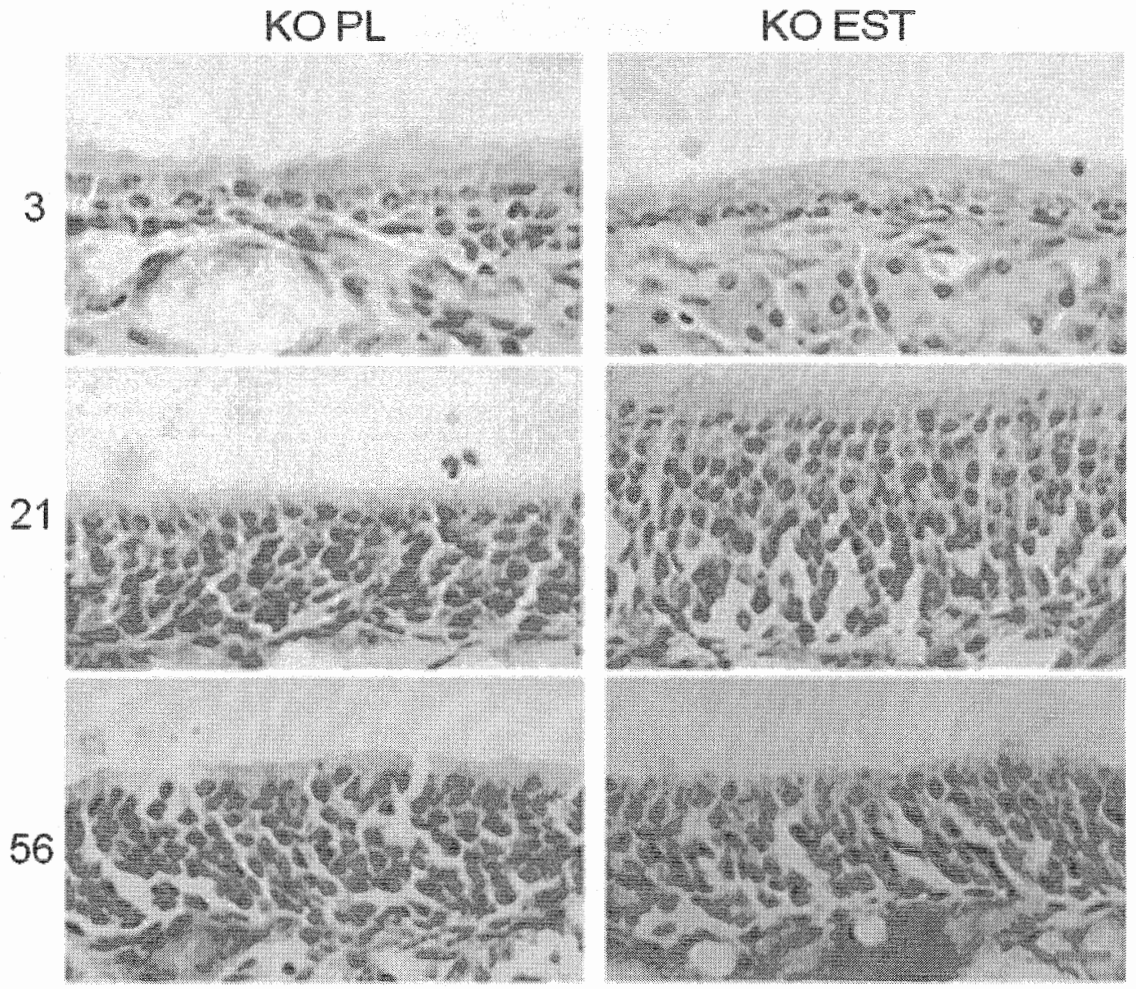


Fig. 40- Cresyl violet staining in the OE of KO injured mice. No observable difference was seen in the OE at 3 days. The OE was thicker in estradiol treated at 21 days and both treatments exhibited thin OEs with incomplete recovery at 56 days. Scale bar=15 μm.

Cell division in KO injured mice was variable throughout the time course of repair. Both estradiol and placebo treated mice had minimal $BrdU^+$ cells at 3 days (Figure 42). At 7 days, there was an increase in both treatments to about 20 dividing cells per millimeter. Then at 21 days, cell division decreased to about 10 dividing cells per millimeter in both treatments. At 42 days, estradiol treated mice experienced a spike in cell division to about 55 cells per millimeter followed by a decrease to about 35 cells

per millimeter at 56 days. Placebo treated continually increased to 32 cells per millimeter at 42 days and 56 cells per millimeter at 56 days (Figure 41). In the course of repair there was no significant difference overall between treatments in KO mice ($F_{1,4}$=2.301, p=0.075). There was a significant difference when compared to neurogenesis in WT mice. $BrdU^+$ cells were substantially greater in the WT than the KO, particularly within the first 7 days ($F_{1,4}$=50.82, p<0.001). Estradiol amplified the amount of cell division in WT and had no augmented effect in KO ($F_{1,4}$=4.96, p=0.002).

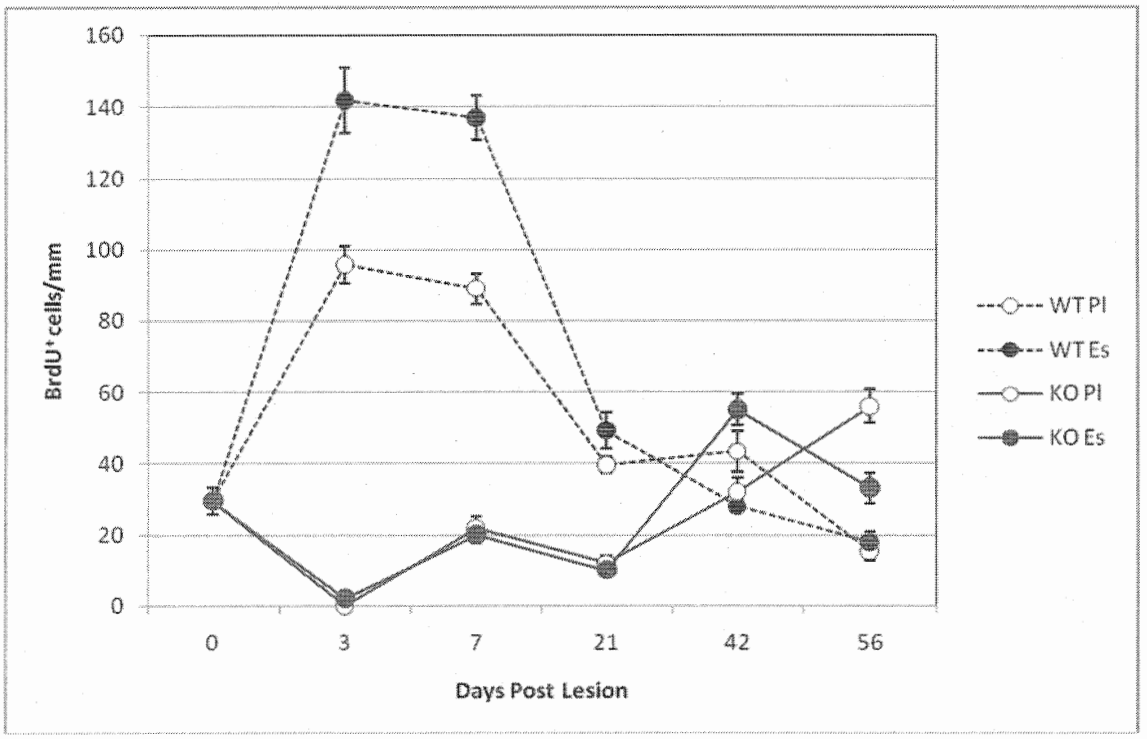


Fig. 41- Graph of cell division in the basal cell layer of the OE in KO and WT injured mice. A variable pattern of increases and decreases in cell division was observed throughout the time course in KO. Unlike KO, WT mice followed a pattern of increased cell division at earlier time points, particularly at 3 and 7 days.

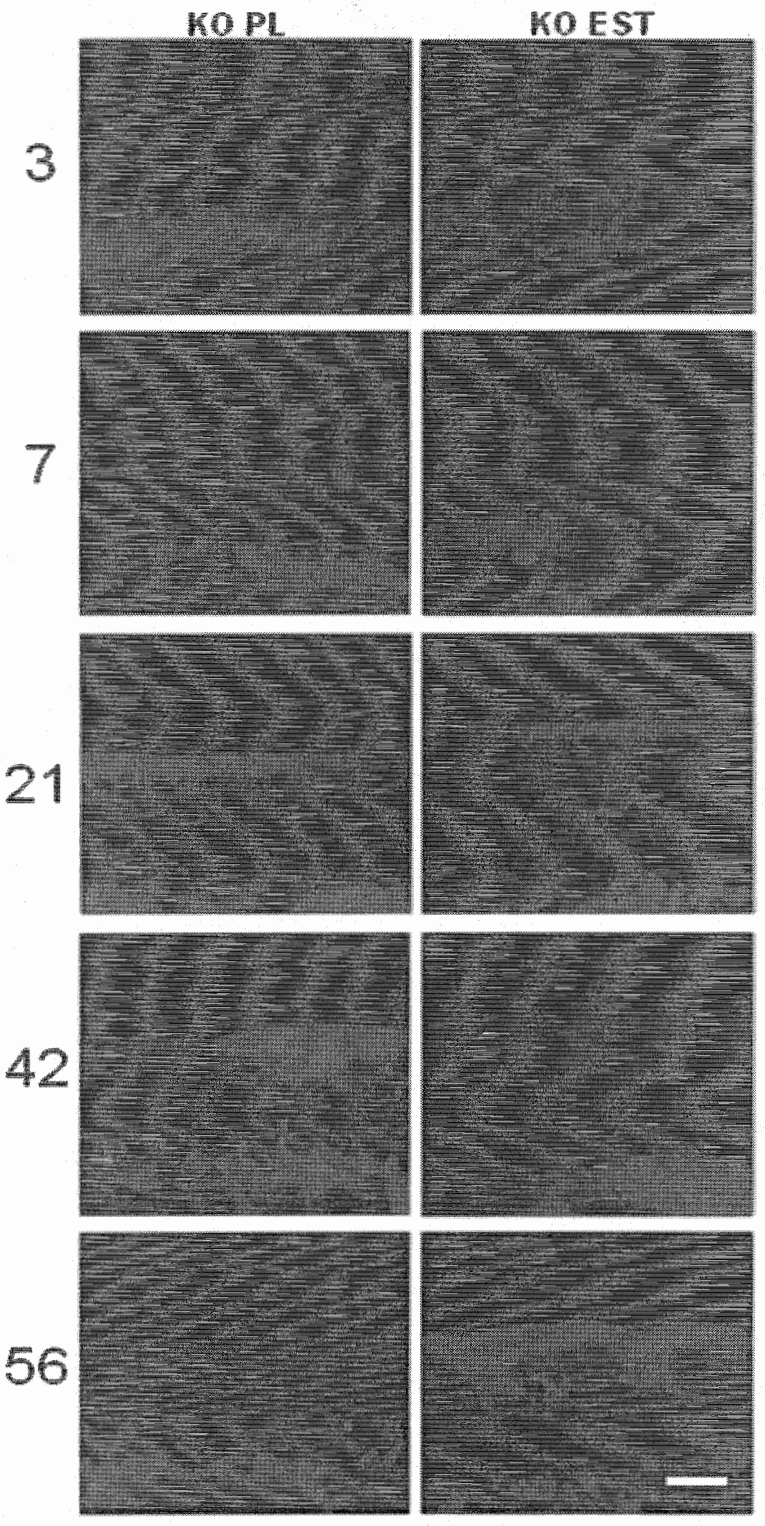


Fig. 42- BrdU labeled cells in the basal cell layer of the OE of KO injured mice. Minimal cell division was observed 3, 7, and 21 days. Cell division was higher at 42 and 56 days in both treatments. The most cell division observed in estradiol treated was at 42 days and the most in placebo was at 56 days. Scale bar=15 μm

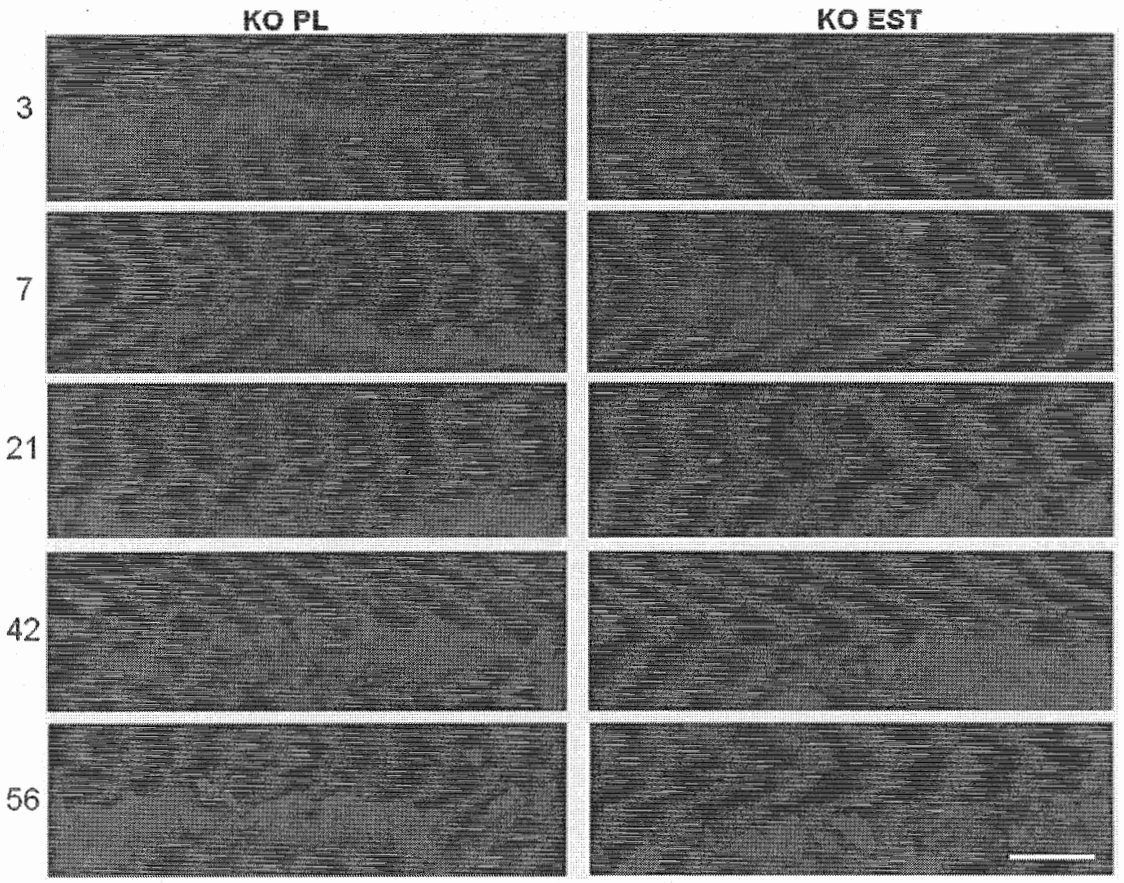


Fig. 42 MAGNIFIED- Scale bar=15 μm

Estradiol and placebo treated KO mice demonstrated similar patterns of immature ORN reconstitution with the highest amount observed at 21 days (Figure 44). Immature ORNs emerged at 7 days in both treatments with estradiol having around 8 $Gap43^+$ cells and placebo having a few per 100 μm. Both treatments experienced a spike at 21 days. At this time point, placebo treated mice peaked at about 22 $Gap43^+$ cells per 100 μm, and estradiol peaked at 26 $Gap43^+$ cells per 100 μm. Then both treatments dropped at 42 and a rose again at 56 days (Figure 43). An analysis of variance determined no significant difference between treatments in the KO ($F_{1,10}$=0.053, p=0.823). In comparison to WT, Gap43 was much lower across all time points in KO mice. WT mice treated with

estradiol experienced an increase in Gap43 at 7 days and KO mice did not ($F_{1,10}$=5.670, p=0.022).

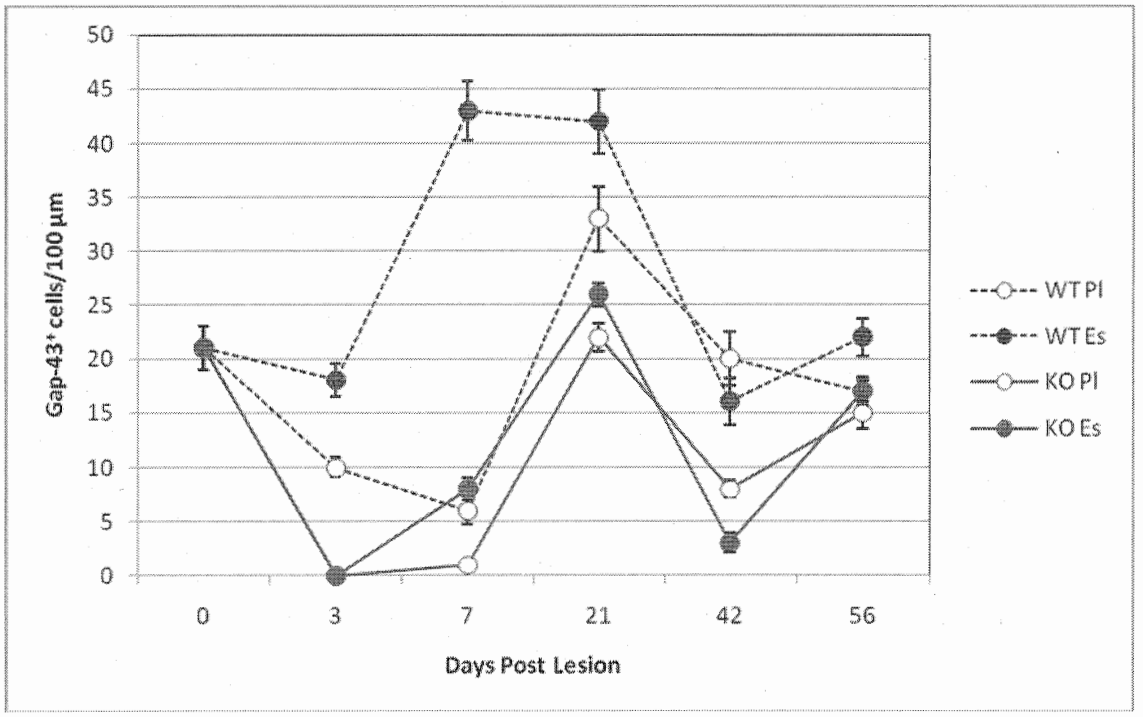


Fig. 43- Graph of immature ORN reconstitution in the OE of KO and WT injured mice. In KO mice there was virtually no Gap43$^+$ cells at 3 days and a small number at 7 days with more cells present in estradiol treated. Gap43$^+$ cells peaked at 21 days in both treatments and decreased at 42 where estradiol had slightly less immature cells than placebo treated. Both KO treatments experienced a similar increase at 56 days. WT placebo treated followed a similar pattern to the KO with the exception of more Gap43 at 3 days. WT estradiol treated had much higher Gap43 across the time course.

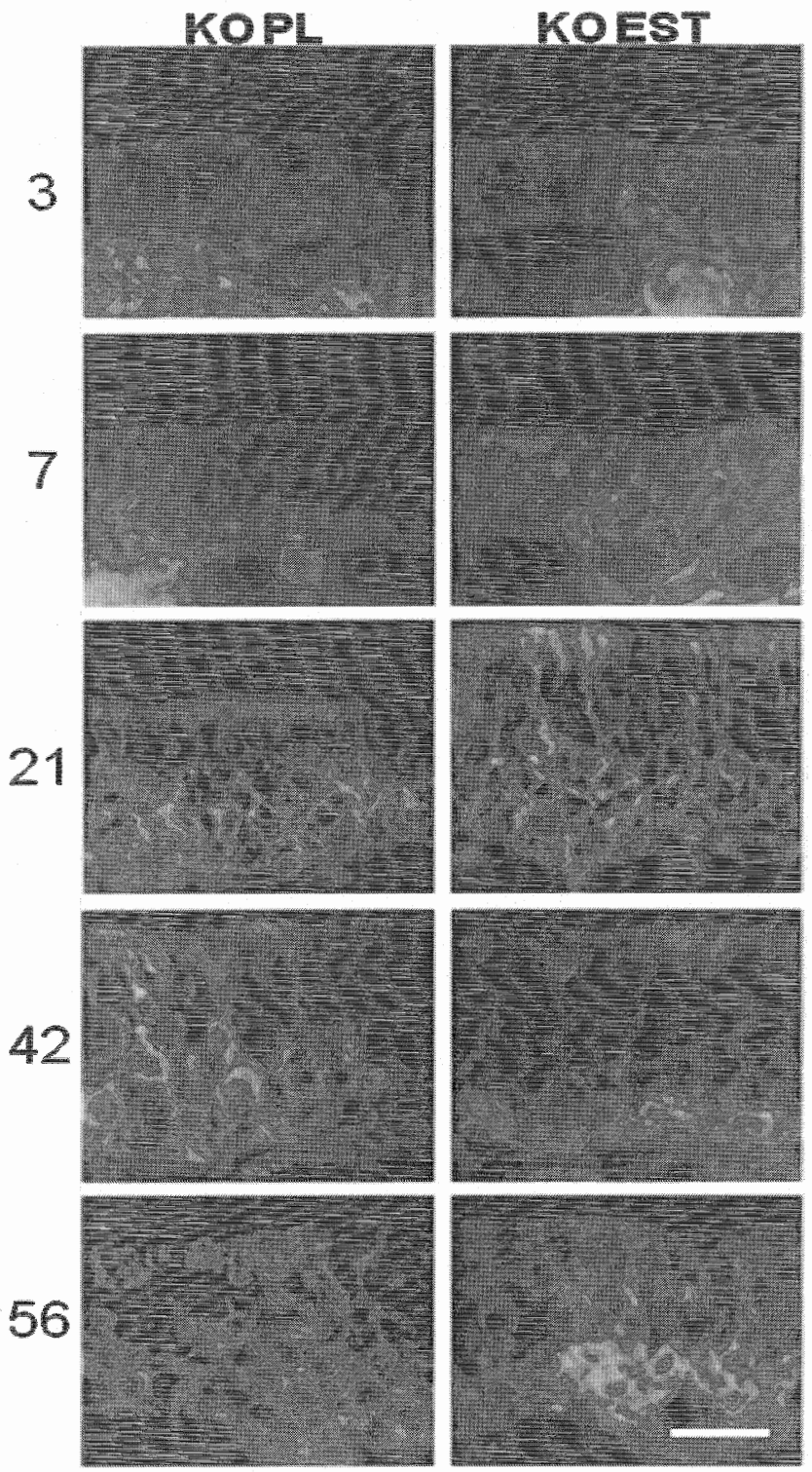


Fig. 44-Gap43 labeled cells in the OE of KO injured mice. Gap43 ORNs were not observed until 7 days with more immunoreactive cells in estradiol treated. The most Gap43^{+} cells were at observed 21 days and significant amount of immature ORN were still developing at 56 days in both treatments. Scale bar=15 μm

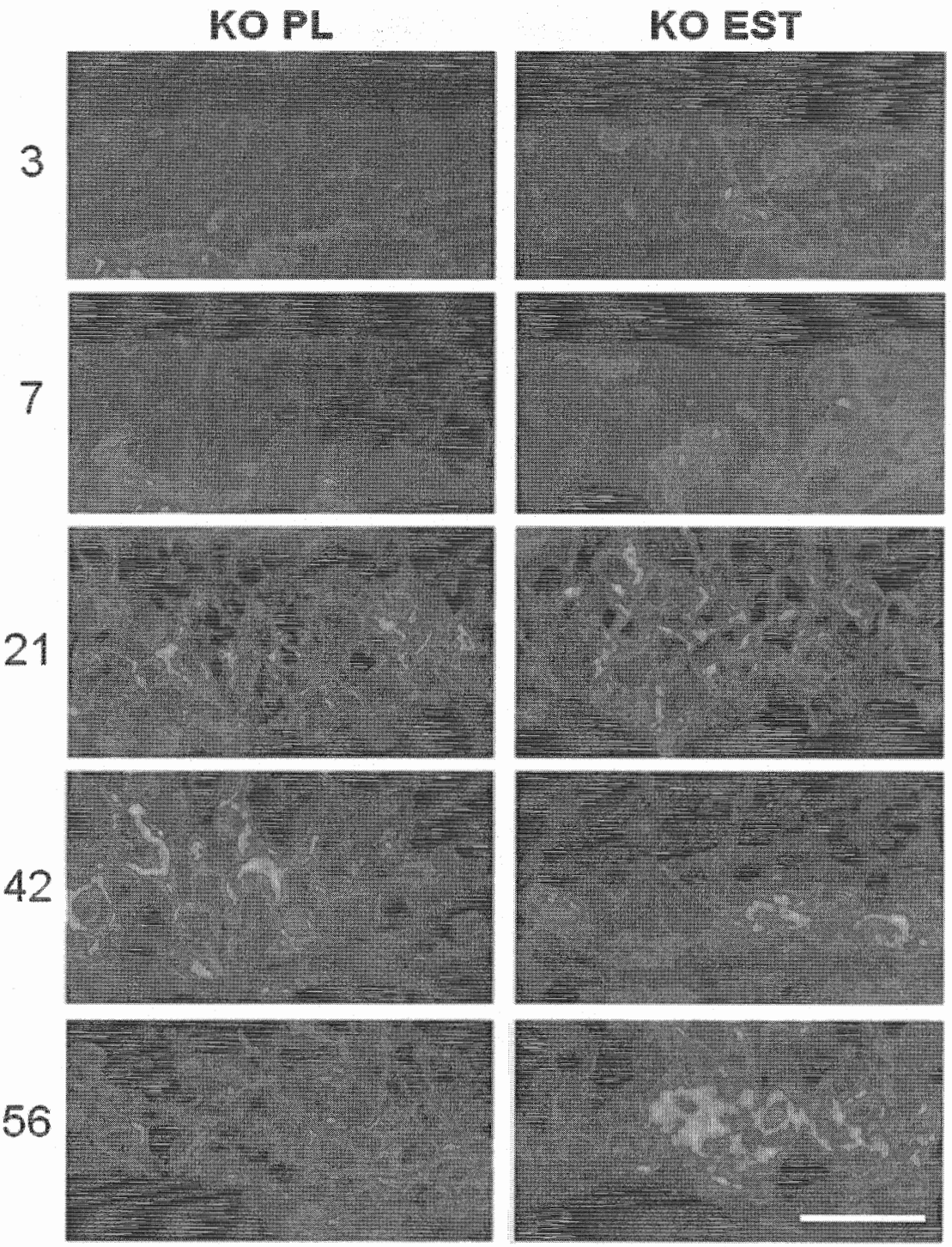


Fig. 44 MAGNIFIED - Scale bar=15 µm

Maturation patterns in KO were fairly uniform across time points (Figure 45 & 46). The number of OMP^+ cells steadily increased with no distinguished difference between treatments ($F_{1,10}$=0.0004, p=0.985). Maturation was not as prominent as in WT which suggested that apoE was required for facilitation of estradiol's effect on repair ($F_{1,4}$=17.91, p<0.001).

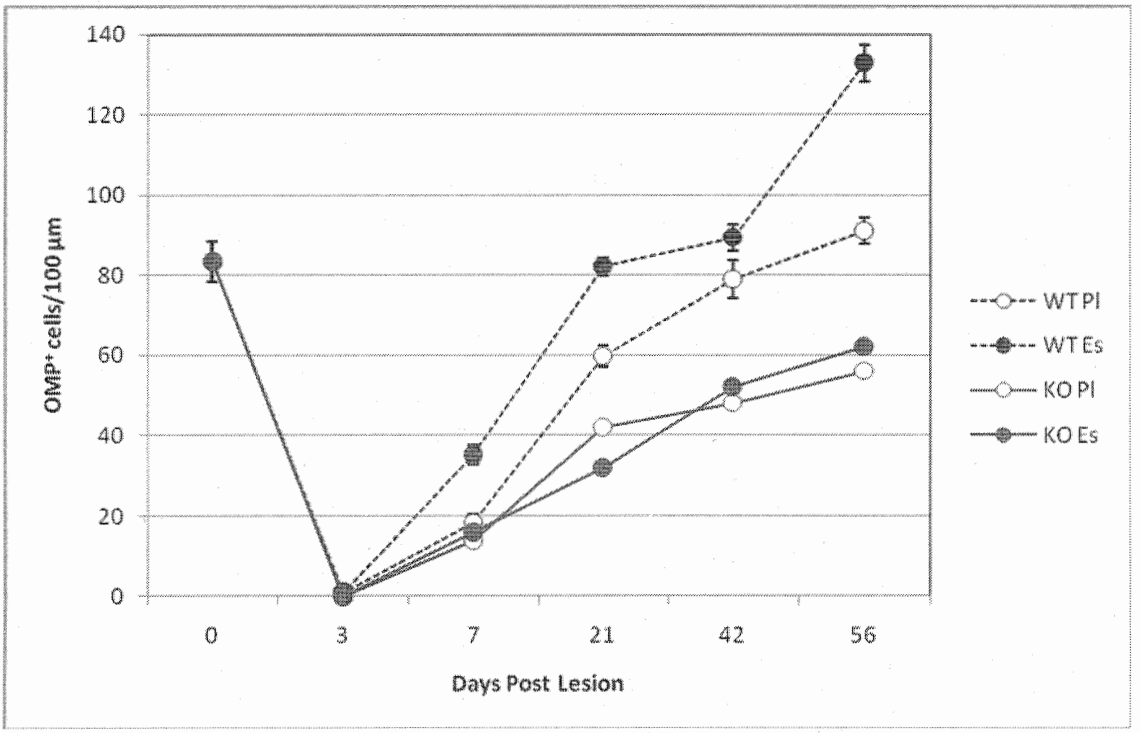


Fig. 45- Graph of OMP in the OE of WT and KO injured mice. KO mice followed similar maturation patterns throughout repair indicating that estradiol was only effective in WT mice. WT mice with estradiol had an OE that matured faster and in greater numbers than KO.

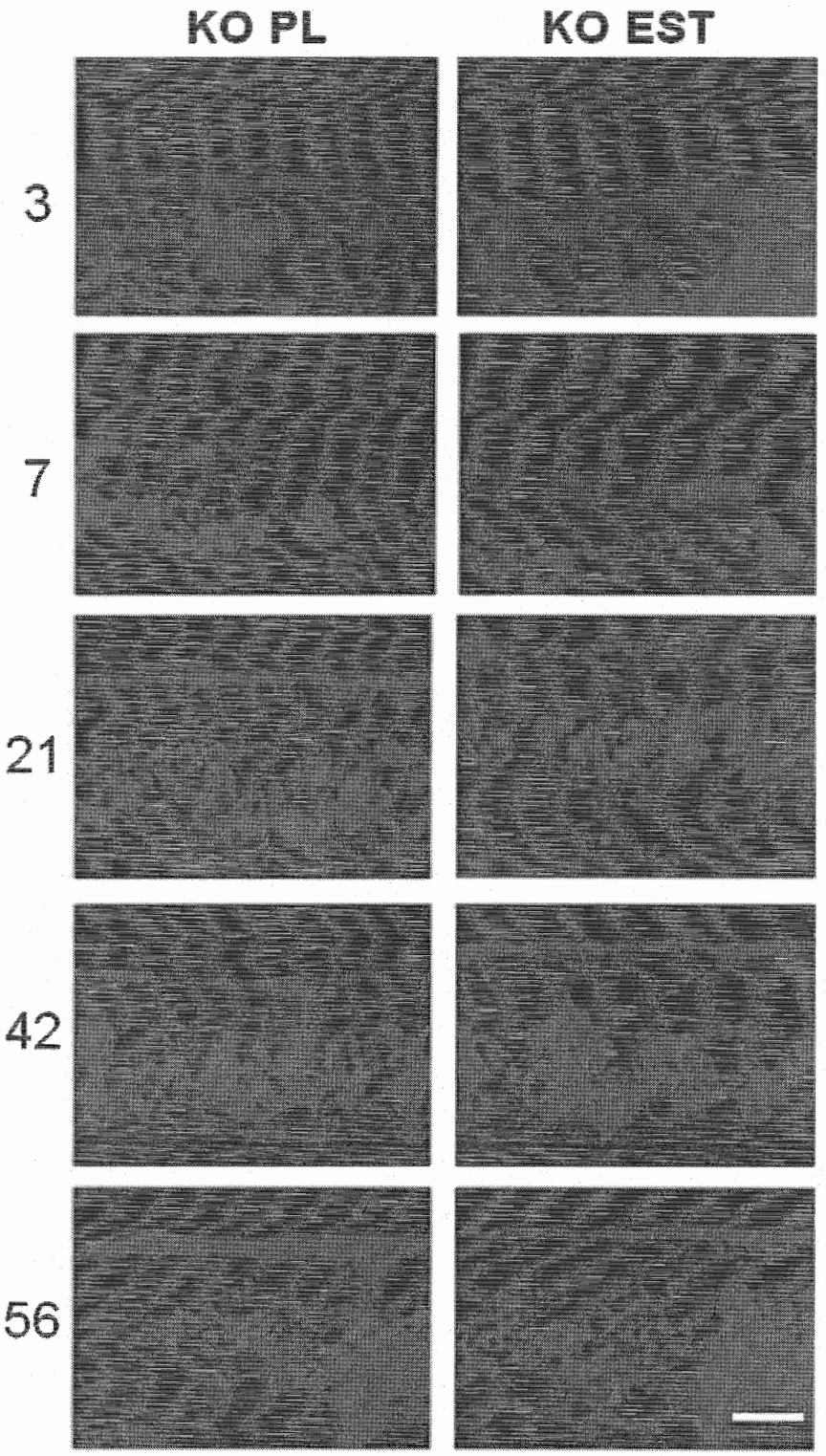


Fig. 46- OMP labeled cells in the OE of KO injured mice. OMP^{+} cells were first labeled at 7 days. The number of mature ORNs increased slightly over the time course, however there was no difference between placebo and estradiol treated. Scale bar=15 μm

OMP immunoreactivity in the glomerular layer of the OB corresponded with results from the OE. The only notable difference between treatments was at 21 days where OMP immunoreactivity was slightly less in estradiol treated (Figure 47). Overall, KO mice did not achieve the same level of OMP in the OB as did the WT mice. WT mice had higher levels of OMP in the glomeruli regardless of treatment (refer back to Figure 27).

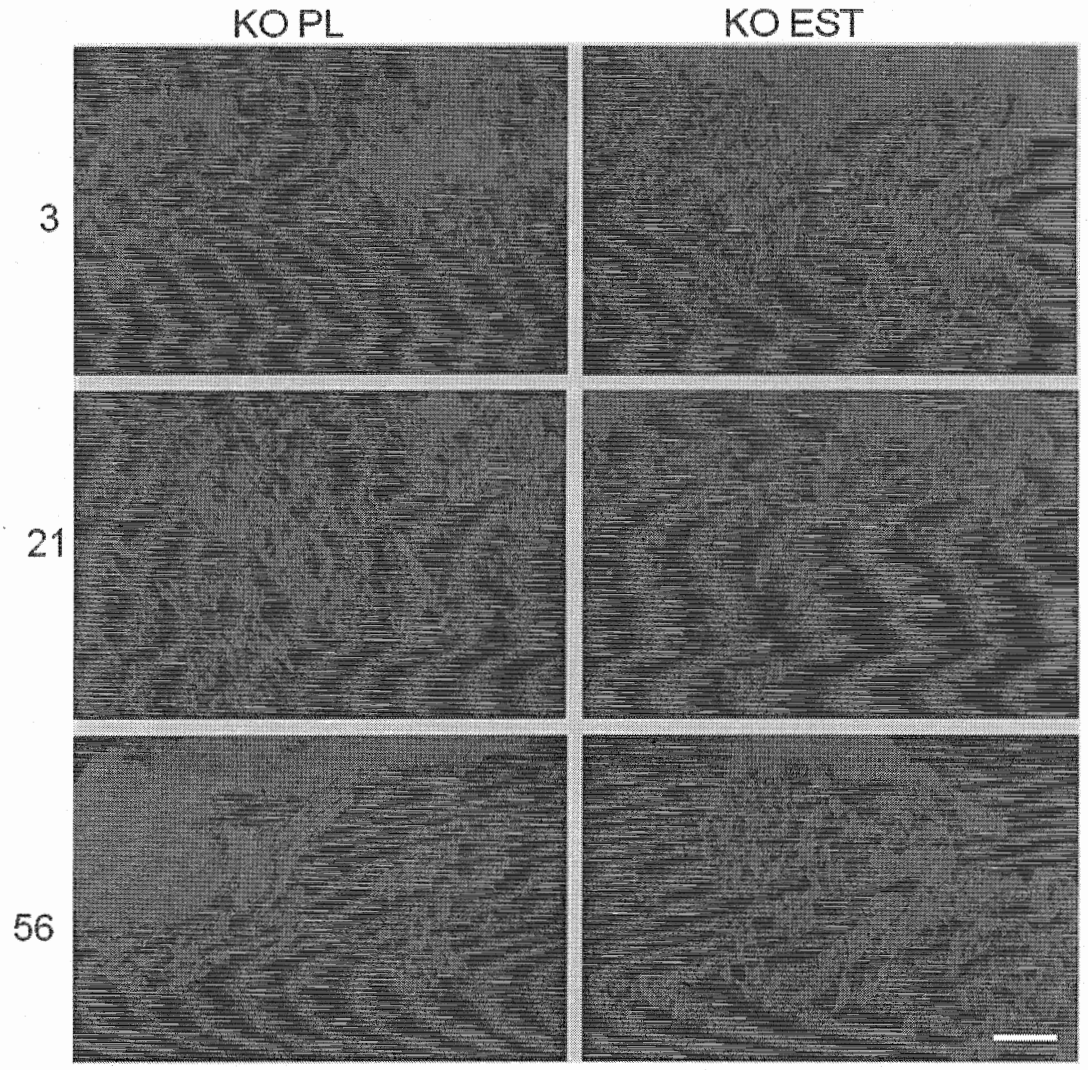


Fig. 47- OMP immunoreactivity in the glomerular layer of the OB in KO injured mice. OMP was minimal in the glomeruli of both treatments at 3 days. There was a progressive increase in placebo at 21 and 56 days. There was no increase in estradiol from 3 to 21 days however there was from 21 to 56 days. Scale bar=15 μm.

Synaptophysin was highest at 21 days in the OB in both treatments. SYN increased considerably from 3 to 21 days, and decreased slightly from 21 to 56 days (Figure 48). This pattern was different than that of WT where synapse formation increased over the time course of repair. SYN was highest at 56 days in WT estradiol treated mice (refer back to Figure 28).

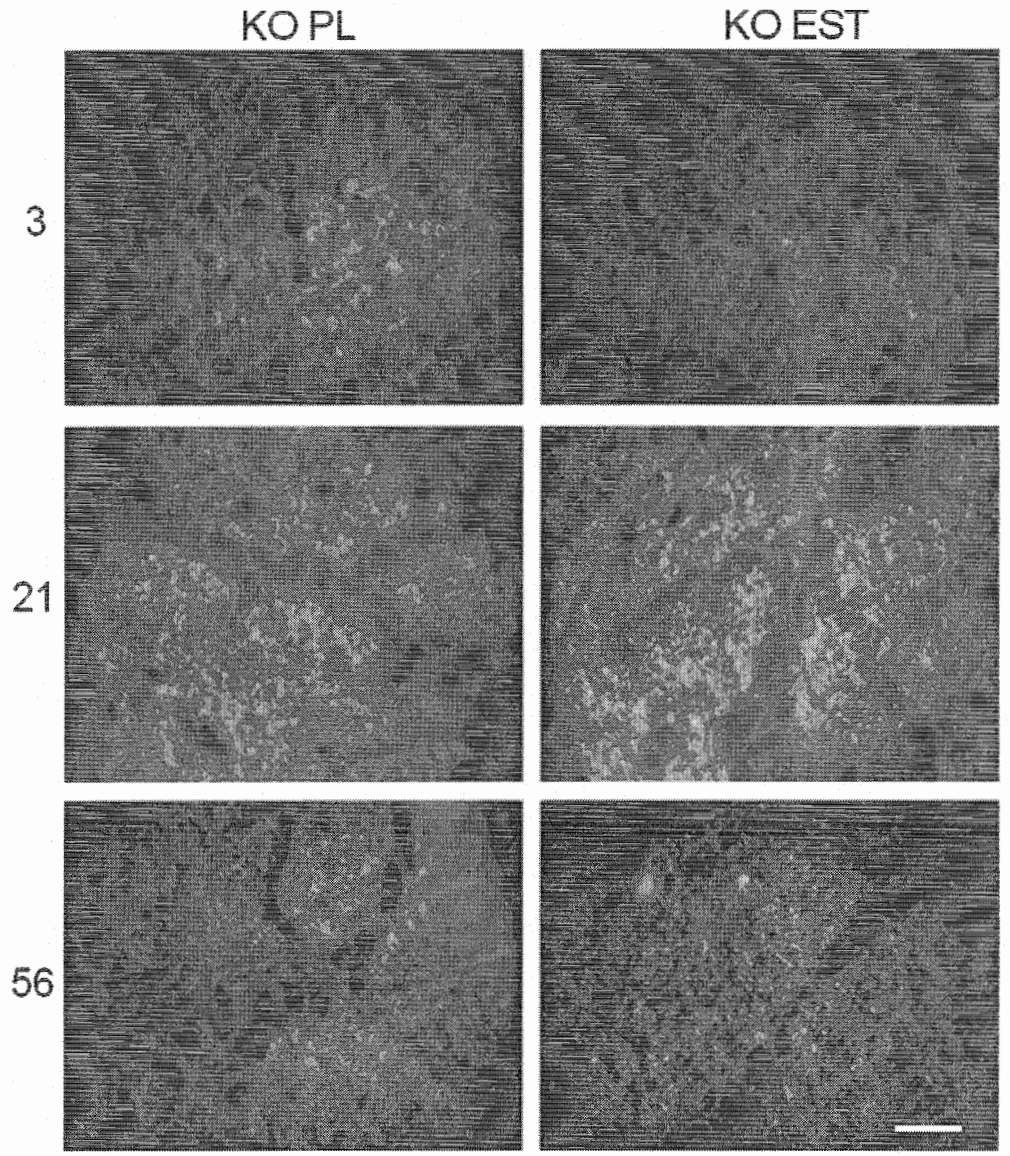


Fig. 48- SYN immunoreactivity in the glomerular layer of the OB in KO injured mice. SYN was at low levels 3 days after lesion in both treatments and increased to higher levels at 21 days. SYN then decreased in both treatments in comparable amounts. Scale bar=15 μm.

Lesion slightly increased GFAP immunostaining in KO mice across the time course (Figure 50). Astrocyte densities stayed around 80 processes per 125 μm^2 through 3, 7, and 21 days in both treatments. At 42 days, both placebo and estradiol treated mice densities increased to around 140 processes per 125 μm^2. Then at 56 days, placebo treated mice dropped back down to 80 processes per 125 μm^2, and estradiol treated remained up around 130 processes per 125 μm^2 ($F_{1,4}$=4.295, p=0.006). Astrocytes in WT experienced dissimilar increases and decreases. WT increased at 3 and 7 days before dramatically decreasing at 21 days and rising at further time points (Figure 49).

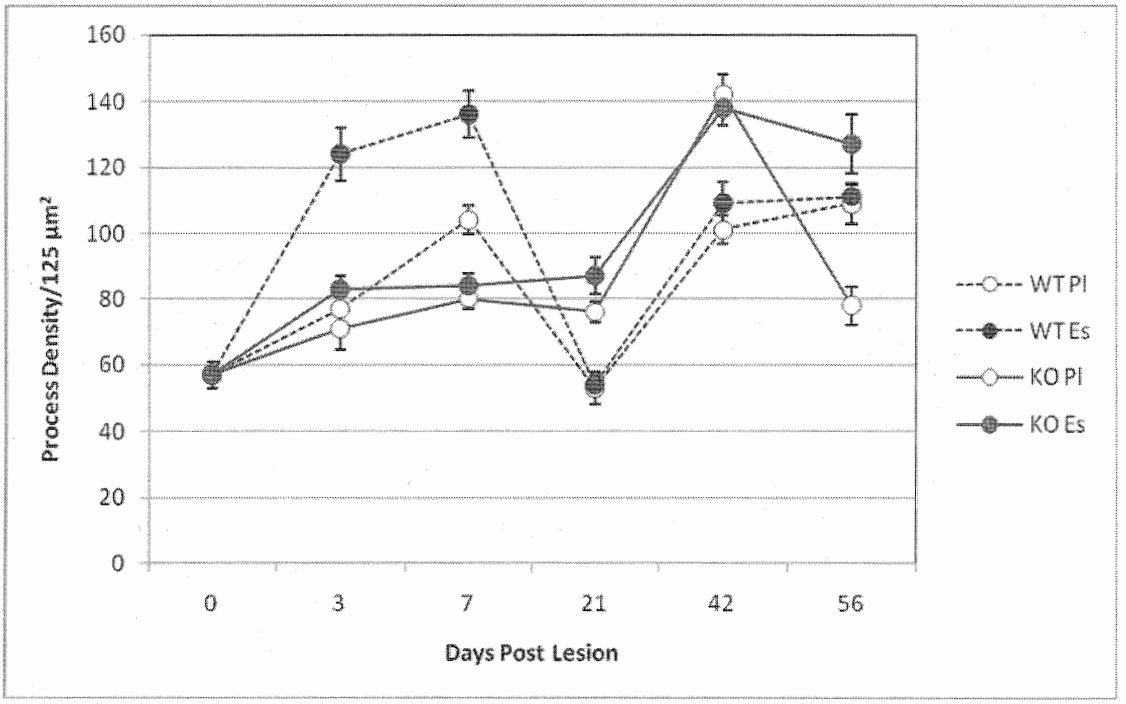


Fig. 49- Graph of astrocyte process density in the glomerular layer of the OB in WT and KO mice. Both treatments in KO remained at a stable density until spiking at 42 days. KO estradiol treated then decreased slightly and KO placebo treated decreased significantly at 56 days. WT mice had an increase in astrocytes early on followed by a decrease at 21 days. WT density then increased at 42 and 56 days.

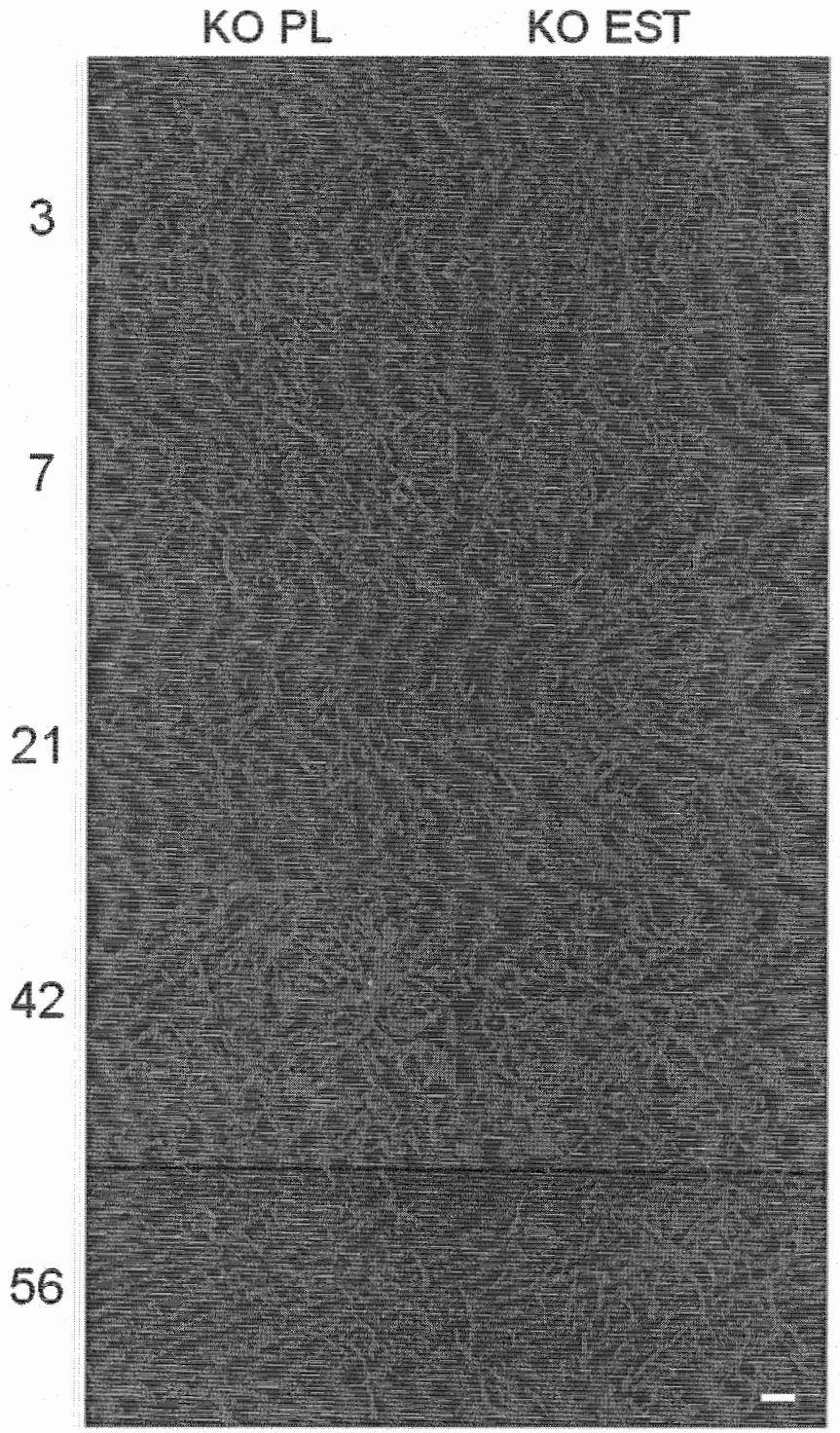


Fig. 50- GFAP assessment in the OB of KO injured mice. Immunohistochemical analysis showed equal astrocyte densities between treatments at 3, 7, and 21 days. Placebo treated then increased at 42 days and decreased at 56 days. Estradiol increased at 42 and 56 days. Scale bar=15 μm.

Discussion:

Neither treatment was effective in sufficiently restoring the ORN population because of limited neurogenesis in the OE. At early time points, cell division in KO mice was substantially less than that of WT mice. Consequently, the number of ORNs reaching maturity by 21 days was less. This result confirmed that apoE was needed in the OE to promote rapid cell division at 3 and 7 days. Without apoE, basal cells do not divide at 3 days and have inadequate division at 7 days.

After 21 days, development was slow-moving relative to WT mice. There was a peak in the amount of immature neurons at 21 days, however this amount was much less than that observed in WT injured mice. Therefore, the number of neurons which matured by 42 and 56 days was less, which resulted in a thinner OE with less OMP^+ cells in both treatments.

Later in the time course, the OE of KO mice responded to less than expected mature ORNs by increasing cell division. At 42 days, the number of immature ORNs was greatly reduced in both treatments. At this time cell division was amplified in both treatments and the number of immature ORNs increased by 56 days. Additionally, cell division was still greater at 56 days.

An increase in cell division at 42 and 56 days was most likely elicited by feedback from the OB. At 42 days the axons of newly mature neurons, observed as immature neurons at 21 days, innervated the OB. Astrocytes increased to promote synaptic branching. Without apoE no new synapses formed. This was apparent by less observable SYN immunoreactivity at 56 days. Consequently, cell division in the OE

increased because mature ORNs were few and synaptic activity of the olfactory nerve was not completely restored.

This study determined that activity in the OE was dependent on feedback from the OB. Estradiol and apoE were vital in restoring olfactory nerve function. Without apoE, estradiol did not facilitate nerve repair and restore normal olfactory function.

Discussion

Estrogen and ApoE in Normal Olfaction

Estradiol was essential in promoting maturation during normal olfactory nerve maintenance. More maturation improved olfactory nerve function. Olfaction is faced with the loss or dying of ORNs due to noxious chemicals in the environment (61, 69, 70). Having more mature ORNs stabilized olfactory nerve signaling.

Estradiol facilitated this effect by increasing the occurrence of astrocytes in the OB. Studies have shown that astrocytes respond to estradiol treatment by increasing apoE mRNA in the brain (71). Primarily, apoE is synthesized and secreted by the astrocytes (72).

ApoE most likely functioned in distribution of lipids and regulation of cholesterol homeostasis by interacting with its target cells in the OB. Target cells in the OB are likely to be the terminal ends of growing ORNs. ApoE provides lipids to growth cones on axons of neurons developing toward maturation (73-75). In order for developing ORNs to mature, they must synapse with the mitral and tufted cells in the glomeruli of the OB. ApoE has been associated with the terminal processes of the olfactory nerve in the glomerular layer. Recent findings have shown that apoE containing lipoproteins stimulated neurite outgrowth (73, 77, 78). These findings are evidenced in higher synaptophysin immunoreactivity.

ApoE utilizes receptors on ORNs. Neurons have LDL receptors present on their axons, cell bodies, and dendrites. ApoE binds to receptors on neurons to promote

cholesterol and lipid uptake for efficient neuronal growth (5, 11). A previous study has shown that apoE3 enhances axonal and dendritic growth compared to apoE4 (16). Others have shown the presence of apoE receptors in active growth cones of lengthy axons. Lengthy axons need extra-cellular cholesterol at the site of axonal lengthening (1, 11, 18). This evidence further supports the fact that apoE in the OB provided the cholesterol needed for enhanced maturation via estradiol.

A lack of apoE and estradiol was slightly detrimental to maturation as evidenced in the placebo treated KO mice. This signified that a loss of both components can decrease maturation and limit neurite outgrowth and synaptic signaling in the OB. As neurons were lost over time, they were inefficiently replaced leading to a deficient olfactory system.

Estrogen and ApoE in Olfactory Nerve repair

ApoE was a critical intermediary for the beneficial effects of estradiol on nerve repair. Estradiol's effects were seen in both the OE and OB of WT mice. Over the time course, apoE mice treated with estradiol repaired faster and more effectively.

Studies have shown that the majority of dividing cells in the first week following lesioning are non-neuronal cells such as supporting (SUS) cells (69, 79-81). Previous studies have determined at 7 days cell division of ORNs spike giving rise to the immature ORN population (82, 83). ApoE mice without estradiol followed this repair pattern. Cell division increased at 3 and 7 days, and the immature ORN population peaked at time points subsequent to 7 days. On the other hand, ApoE mice with estradiol had much more cell division at 3 and 7 days. Immature ORNs emerged as early as 3 days and

experienced a peak at 7 days. It was clear that estradiol facilitated rapid cell division of not only supporting cells but ORNs earlier than expected under normal recovery patterns.

Consequently, repopulation of mature ORNs was achieved sooner with estradiol and apoE. Studies have shown from the 2nd week post-lesion, immature ORN begin to differentiate into mature ORNs (62, 82, 83). WT mice without estradiol were at the appropriate stage of reconstitution, whereas WT mice with estradiol had already reached control levels of mature ORN by 21 days. By the end of the time course, WT mice with estradiol had significantly more mature ORNs present than at control levels. WT mice without estradiol repopulated mature ORNs to the control levels by 56 days.

Enhanced maturation in the OE of estradiol treated WT mice was due to high apoE levels in the OB. Verhaagen, et al, (1990) determined two stages in reconstitution of ORN population in the neuroepithelium with the second stage being dependent on the OB. The first stage was described as formation of the immature neuron population in the OE, explained previously. The second stage was development of mature ORNs. Development of mature ORNs was dependent of the OB being present. When the OB was removed, immature neurons did not have their synapse target and differentiation into mature ORNs was compromised (68).

ApoE levels in the OB were critical during the second stage of development. Just as in normal mice, more apoE promoted enhanced ORN maturation by binding to lengthy axons developing toward their synaptic target. It's likely that apoE advanced synaptic branching during synapse formation by increasing the amount of neurite outgrowth. Astrocytes were responsible for keeping apoE levels high in estradiol treated mice while axonal growth was taking place.

Mice which did not have apoE were unable to repopulate their neuroepithelium regardless of treatment. Consistently low maturation and increased degeneration of immature ORN yielded inefficient nerve repair. The most evident deficiencies were in cell division and mature ORN development in the OE. Less cell division gave rise to less immature ORNs, and the immature ORNs present at 21 days struggled to reach maturity because apoE was not present in the OB to advance growth and synaptogenesis.

The data showed that OE responded from feedback in the OB due to inadequate maturation in KO mice. Both treatments increased cell division and the number of immature ORNs at later time points. It's likely that the majority of immature ORNs present at 56 days would not differentiate to mature ORNs and the feedback cycle would continue.

Estrogen and ApoE in Neurological Diseases

The protective effects of estrogen in the nervous system have been demonstrated by numerous studies. Estradiol has been found to enhance neurite growth, axonal elongation, cell survival, and synaptogenesis (84-86). In this study, estrogen facilitated neuroprotectiveness by the means of apoE secreted by astroglial cells in the CNS through the following mechanisms:

1. Estrogen increased olfactory nerve sustainability by promoting rapid maturation of ORNs in WT mice.
2. Estrogen manifested higher maturation throughout the time course in WT mice.

3. Estrogen supported synaptic plasticity between mature ORNs and bulbar neurons in WT mice.

Published studies have clearly documented that estrogen facilitates neuronal repair following injury in animal models (26, 27). Only one study has examined the effects of estradiol on the olfactory system during repair. Results from that study determined that estradiol replacement significantly improved recovery of olfactory discrimination performance post OE lesion in rats (41). This thesis study made clear the physiologic basis of this phenomenon. Experiments performed revealed that estrogen facilitated nerve recovery by the means of apoE through the following mechanisms:

1. Estrogen increased basal cell proliferation in the OE and promoted faster recovery of the ORN population post injury in WT mice.
2. Estrogen increased maturation by promoting axonal growth of ORNs and re-innervation in the OB of WT mice.
3. Estrogen enhanced synapse development of newly formed ORNs with bulbar neurons via neurite outgrowth in WT mice.

Overall findings revealed apoE3 genotype as a link to ET effectiveness in treating neurological diseases. Olfactory dysfunction is one of the first signs of AD. Dysfunction occurs in individuals with mild cognitive impairment to more severe cognitive deterioration, as well as around 90% of early onset AD and PD individuals (87, 88). Estrogen protects against olfactory dysfunction by increasing the amount of maturation in the olfactory system via apoE.

It is not known whether estrogen provides its neuroprotective effects via ER binding in the OB. It is also unclear which cells in the olfactory neuroepithlieum interact

with estrogens. Estrogen receptors have been found in the OE in conjunction with the Bowman's gland where enzymatic activity is high (89). Estrogen receptors ERα and ERβ have been characterized in the OB. ERβ expressing cells were present in the glomerular, external plexiform, and granule cell layer of the OB (42, 44). Results from this study suggest that astroglial cells express ERβ. Binding of estrogens in the nuclei of target astroglial cells controls apoE fluctuations. ApoE can then be utilized in nerve maintenance and repair.

Conclusion

The data revealed that estradiol treatment results in improved neuronal function. ApoE is a vital mediator for the beneficial effects of estrogens on nerve maintenance and nerve repair within the intact and injured nervous system.

Estradiol protects from neuronal injury by maintaining a strong nerve bundle full of mature neurons. ApoE works to grow these mature neurons and preserve their synapses. When apoE is not present, estradiol does not effectively enhance maturation. A lack of apoE and estrogen may be detrimental to nerve sustainability.

Estradiol facilitates nerve repair by increasing stem cell division, promoting faster recovery, escalating maturation, and enhancing synaptogenesis. ApoE has a generalized effect in the OE to increase cell division and promote recovery with estradiol treatment. ApoE in the OB grows mature neurons and encourages neurite outgrowth at the site of synapse with bulbar neurons in estradiol treated mice.

The data suggests that astrocytes are responsible for apoE fluctuations in the OB. Estrogen is a hormone that increases the occurrence of astroglial cells in both the intact and injured nerve to augment maturation. The interaction of apoE and estradiol to generate increased cell division in the OE is unknown.

Moreover, it is evident that estradiol is beneficial but not needed for sufficient repair in apoE gene expressing mice. However, a lack of apoE is a disadvantage whether estradiol is present or not. Nerve repair is poor due to insufficient cell division and incapable maturation in apoE deficient mice.

Future studies should focus on characterizing ERα and ERβ in the olfactory system. Localization of receptors will determine which cells estradiol interacts with to produce the results observed in this study.

ApoE3 genotype is necessary for estrogen therapy's success in treating neurological diseases. ApoE3 individuals benefit through treatment by increasing and sustaining the amount of mature neurons transmitting information throughout the central and peripheral nervous system. Moreover, estrogen may compensate for a loss of cognition by directing apoE3 to the site of damage. ApoE3 functions in recycling lipids from degenerating neurons, encouraging growth of newly formed neurons, and preserving and enhancing synaptic plasticity.

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