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The effects of ApoE on olfactory neuron plasticity in mice

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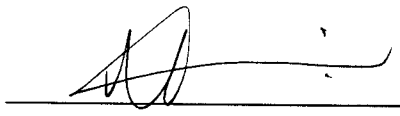

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The Effects of ApoE on Olfactory Neuron Plasticity in Mice.

BY

Ikemefuna T. Nwosu

THESIS

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I HEREBY RECOMMEND THAT THIS THESIS BE ACCEPTED AS FULFILLING
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7 December 2006
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ABSTRACT

Apolipoprotein E (apoE), a lipid transporting protein, is a major risk factor for developing Alzheimer's disease (AD). Previous studies have shown that apoE is expressed at high concentrations in the olfactory nerve (ON) and around the glomeruli of the olfactory bulb (OB) and that the apoE levels in these regions increase substantially following lesioning of the source of the olfactory nerves, the olfactory epithelium (OE). However, the mechanism(s) underlying the contribution of apoE to neuronal functioning and developing AD are not clearly understood. To investigate and better clarify the role of apoE in nerve regeneration, I examined the presence and concentration of apoE in the olfactory epithelium (OE), the rate of cell proliferation in the OE, the rate of regeneration of the OE, the rate of maturation of neurons in the OE and the rate of synaptogenesis of neurons of the OE all in response to induced injury to the olfactory epithelium.

I evaluated the immunoreactivity of apoE in apoE wild type (WT) mice and apoE deficient mice (apoE knock-out (KO)). ApoE immunoreactivity was found only in the OE of WT mice. ApoE was localized to several cell types including ON, olfactory ensheathing glial (OEG), endothelial cells and Sustentecular (Sus) cells.

I predicted that apoE levels in the OE of WT mice, in response to injury of the OE would increase as it does in the OB. To test this hypothesis, I induced injury to the OE of apoE WT mice and allowed them time to heal. Data revealed that apoE levels initially declined followed by an upregulation to above control levels in the second week post-lesioning. ApoE levels dropped slightly after the seventh week back towards control levels.

It has been found that the CNS is capable of neurogenesis. Thus I examined the influence of apoE on the cell proliferation rate of the OE in response to induced injury to the OE. Cell proliferation in the OE of WT and KO mice was detected by BrdU, a DNA marker that detects cells undergoing mitosis. My results indicated no significant difference in cell proliferation rates for both strains of mice as the rate increased after the third day and peaked at the seventh followed by an abrupt decline to normal during the second week.

My next study involved determining whether apoE influenced the rate of maturation of ON in the regenerating OE. Using OMP, a marker for mature ON, I examined the OE of apoE WT and KO mice. The data suggests that there is no observed delay in the rate of ON maturation in the apoE KO mice.

Previous studies indicated that ON maturation is partly dependant on the ability of immature ON to form synapses in the target organs. ApoE has also been implicated in the development and functioning synapses. I postulated that ON in apoE WT mice would undergo synaptogenesis more efficiently than apoE KO mice. To test this hypothesis I used synaptophysin (SYN), a synaptic marker, to determine the rate of synaptogenesis between WT and KO mice. Synaptogenesis occurred in apoE KO mice at about 2 weeks slower than it did in WT mice.

These results demonstrate that the OE is able to undergo neuronal regeneration and maturation. However, the differential pattern of this phenomenon between apoE WT and KO mice over eight weeks after peripheral lesioning of the OE illustrates the profound effect apoE has on not only neuronal regeneration but plasticity as well.

DEDICATION

I would like to thank God for allowing me the opportunity to chase my dreams this far. When ever I was ready to throw in the towel, there always seemed to be a situation or person at the right time with the right words. Thank you!

To my wife Uzo and son Immanuel, you have put up with my extended absences for these past couple of years. I am done now! Thank you for understanding.

To my parents H.R.H. Dr. Gibson and Mrs. Nwosu, thank you for supporting me and giving me the stability of a loving family. My long and extended journey towards my "American Dream" is now one major step closer thanks to your love and financial support.

I am grateful to Dr. Britto Nathan who has molded me into a scientist with an overwhelming ability to persevere till I get an answer.

My committee members; Dr Gary Bulla and Dr. Charles Costa who have always had an open door to me, have helped me straighten out my thoughts and always encouraged me to press on. Thank you!

During these years I have come to met wonderful members of faculty who have each deposited a seed of greatness in me. As I forge on to be an instructor, I know at certain times I will fall back on the priceless jewels that have been deposited in me.

To my colleagues in the laboratory, I couldn't have done it without your help. I am full of gratitude for all the days we spent working out my problems.

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My utmost gratitude is extended to Dr. and Mrs. Mukuti Upadhyay (Economics and Biological sciences Departments respectively) for helping me with statistical analysis and tissue preparation respectively.

I would also like to thank Dr. David Turnbull and Tara Hubschmitt for all the long hours they put in the proofreading.

I would like to thank Dr. Nathan for this very intense period of my life. I have been stretched beyond what I could ever have imagined possible.

To my most loyal fans Egon Frech and Roxanne Summers, You have provided me with parental love, care and support that I needed even before I figured I needed assistance. Thanks for being there every step of the way.

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1. INTRODUCTION

ApoE is a 34 kDa protein consisting of 299 amino acid residues. It is a component of a class of lipoproteins that serves in the redistribution of cholesterol and other lipids among tissues and cells by interacting with lipoprotein receptors on target cells [1-4]. Receptor-lipoprotein binding initiates cellular uptake and degradation of lipoproteins, making lipid available for use in the regulation of intracellular cholesterol metabolism. The primary structures of apoE from 10 different species have been determined [5]. It has been found that apoE exists in three major isoforms namely; apoE2, apoE3 and apoE4. They are produced by three alleles (e2, e3 and e4, respectively) at a single gene locus on chromosome 19 in humans [1]. These common isoforms of apoE in humans differ by amino acids at positions 112 and 158 [6-9]. The most common isoform, apoE3, contains cysteine and arginine at positions 112 and 158, respectively. Both positions contain cysteine in apoE2 and arginine in apoE4. These amino acid changes in positions 112 and 158 confer different lipid binding properties and are responsible for the association of each isoform to different diseases. Mice have one form of apoE, which is similar to human apoE3 in its structural and functional properties, including receptor binding and lipoprotein preferences [10,5].

1.1. ApoE in the Nervous System

ApoE is the major apolipoprotein in the brain and cerebrospinal fluid (CSF) [10-12]. Astrocytes and microglia are the major apoE producing cells in the brain. Previous studies have shown that apoE is the only apolipoprotein in the CSF that can interact with

lipoprotein receptors [10,12]. Cells within the brain express five major receptors for apoE-containing lipoproteins: a) the low density lipoprotein (LDL) receptor, b) the LDL receptor-related protein (LRP), c) the very low density lipoprotein (VLDL) receptor, d) the glycoprotein (gp) 330, and e) the apoE receptor 2 (APOER2) [13]. It has demonstrated that human apoE-containing CSF lipoproteins bind to fibroblast LDL receptors and that the LDL receptor and LRP mediate the binding and internalization of apoE-containing lipoproteins in cultured neurons [14]. These studies demonstrate that apoE and apoE-containing lipoproteins are present within the brain where they can interact with neurons. Moreover, the presence of lipoprotein receptors on neurons suggests that lipoprotein transport by apoE is important for normal functioning of adult neurons.

1.2. Neurological Diseases Related to apoE

Genetic studies have revealed that inheritance of apoE4 alleles increases the risk and rate of progression of late-onset Alzheimer's disease [15-17]. Immunocytochemical studies indicate an increase of neurofibrillary tangles and amyloid plaques, the two hallmarks of AD, in autopsied brain samples from apoE4 carriers as compared to non-apoE4 carriers [18,19]. Both plaques and tangles appear earlier in the apoE4 individuals. Furthermore, a failure of plasticity is also indicated in AD patients with apoE4. Arendt et al demonstrated that AD brains of apoE4 patients show greater degeneration of neurons, and decreased dendritic growth in several areas of the brain, as compared to non-apoE4 patients [20].

In addition to AD, apoE modifies the risks and progression of several other chronic neurological diseases. Individuals with apoE4 alleles appear to have a higher risk and an earlier age of onset of Parkinson's disease [21,22]. ApoE4 inheritance increases the risk of developing dementia following head trauma [23-25]. Recovery following stroke or head trauma is prolonged and significantly decreased in apoE4 carriers as compared to apoE3 individuals [26-28]. This phenomenon is also observed in football players and boxers who are prone to head injury [29]. These findings strongly suggest a close link between apoE polymorphism and the development of neurological disorders, but the mechanism behind the pathological effects of apoE remains unknown.

1.3. ApoE and Peripheral Nerve Regeneration

Peripheral nerve maintenance and repair are suggested as possible roles of apoE in the PNS [2,23,30-32]. In rats, the synthesis of apoE increases several-fold within 3-weeks post-injury of peripheral nerve [33,34]. Morphological studies demonstrated that resident macrophages and monocyte-derived macrophages produce large quantities of apoE, which is then secreted in the form of lipoprotein-like particles [33]. These lipoprotein-like particles are proposed to scavenge cholesterol released from the degenerating myelin, and provide them to the growth cones of sprouting axons by a LDL receptor-mediated endocytosis for membrane biosynthesis [2,35,36]. Deficiency of apoE in apoE KO mice leads to impaired blood-sciatic nerve barrier and to loss of unmyelinated fibers in the sciatic nerve [30,37]. However, regenerating sciatic nerves in both apoE KO and WT mice were morphologically indistinguishable at 2 and 4 weeks post nerve injury [38,39].

1.4 ApoE and CNS plasticity

ApoE may be important in plasticity and repair in the CNS although data are equivocal. Newly synthesized apoE increases following optic nerve injury in rats, but absolute levels do not [32]. Axonal degeneration and myelin sheath alterations were observed in the optic nerve of apoE KO mice [41]. ApoE mRNA is upregulated in the entorhinal cortex following perforant pathway lesioning in rats. ApoE KO mice have fewer synapses, vacuolized and swollen dendrites in the hippocampus, and a reduced recovery following perforant pathway lesioning [41-43]. In contrast, two other studies have not observed any obvious neuropathology in apoE KO animals [44,45]. Thus the importance of apoE in the CNS is controversial, and warrants further studies.

1.5. The Olfactory System as a Model for Regeneration

The adult olfactory system presents an ideal model for studying proteins involved in neuronal growth-related processes (Fig. 1). First, the ORN regenerate from stem cells throughout the life of the organism [46,47]. The exceptional ability of the ORN to regenerate makes it an important neural system for the study of mechanisms functioning in neuronal degeneration and post-lesion plasticity. Previous studies have developed a reasonably good timeline for the regeneration of the olfactory nerve following acute lesions of the receptor mucosa in mice [48-52].

Second, the cytoarchitecture and connectivity of the bulb are relatively simple, permitting reasonably simple interpretations of experimental manipulations [53,54]. Furthermore, the bulb is a closed structure permitting accurate volumetric analyses for use in subsequent studies. Third, spatial and temporal separation exists between

degeneration and regeneration. Spatially, the olfactory nerve is limited to the periphery of the olfactory bulb. Temporally, the olfactory bulb response to nerve lesions occurs over several months. These unique properties of the olfactory system, combined with the use of apoE KO mice, provide a powerful model system for the study of apoE effects on neuronal plasticity.

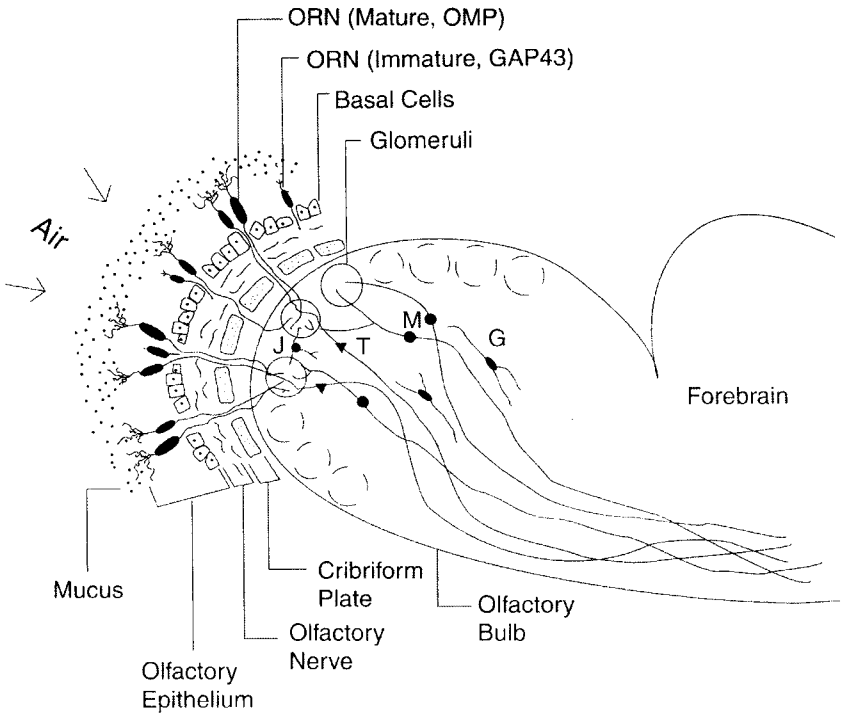

Figure 1. Organization of the olfactory system. The cell bodies of the olfactory receptor neurons (ORN) are located in the olfactory epithelium. ORN continuously regenerate from the basal cells. Their axons pass through the cribriform plate and terminate in the glomeruli of the olfactory bulb. In the glomeruli, they synapse with juxtaglomerular (J), tufted (T) and mitral (M) cells. Mitral cells and tufted cells project their axons in between the granule cells, and terminate in a number of targets in the forebrain.

1.6. Aim of Study

Recent findings demonstrate that apoE containing lipoproteins stimulate neurite outgrowth in a variety of neuronal cultures [55,56]. ApoE mRNA and protein have been found to increase at the site of neural regeneration [57]. Previous studies also show that apoE is associated with the terminal processes of olfactory nerves around the glomeruli of the olfactory bulb. Moreover, apoE increases significantly in this region following a reversible lesion of the OE. Subsequent studies found that olfactory nerve regeneration and morphological recovery of the OB was significantly delayed in apoE KO mice compared to WT mice following OE lesioning [57,58]. These observations may confer on apoE, a critical role in olfactory receptor neuron (ORN) plasticity; however the mechanism whereby apoE mediates these processes is unknown. This study examined nerve regeneration and subsequent maturation of ORN in the olfactory system of apoE KO and wild-type (WT) littermate mice to better understand the potential mechanisms through which apoE may support ORN plasticity.

1.6.1. ApoE in the OE

To investigate the possible mechanisms through which apoE might influence regeneration in the olfactory system, it was important to first determine if apoE was expressed in the OE and furthermore, if the apoE levels in the OE changed during the time course of regeneration. It was hypothesized that apoE would be present in the OE of WT mice. From previous studies of apoE expression in the OB in response to injury [57,58]. It was also hypothesized that, apoE levels would change over the time course of regeneration. These hypotheses were tested by first chemically inducing injury to the OE

of adult WT and KO mice and then used immunohistochemical methods to determine the localization and observe the dynamics of apoE in the OE.

1.6.2. ApoE and Cell Proliferation in the Regenerating OE

These results possibly implicate apoE in the regeneration of the OE. This led to the proposal that apoE is important for cell proliferation in the regenerating OE after injury and that KO mice would have delayed cell proliferation. Using BrdU immunoreactivity to label cells undergoing mitosis, cell proliferation was observed over the time course between apoE KO and wild-type (WT) littermate mice.

1.6.3. ApoE and ORN Maturation in the Regenerating OE

To further understand the effects of apoE on the plasticity of Olfactory Receptor Neurons, the possible role of apoE in the maturation of ORN was investigated. It was proposed that the KO mice may have lower number of mature ORN than WT as the OE regenerates.

1.6.4. ApoE and Synaptogenesis of ORN in the OB

Synaptogenesis is believed to occur only in mature ORN that make contact with the OB [36]. Recent studies place apoE ladened with cholesterol as the glial-derived factor that strongly promotes synaptogenesis in neuronal cultures [59,60]. Therefore the possibility that apoE may support synaptogenesis of mature ORN was investigated. A further prediction was made that KO mice would have a delayed synaptogenesis in the absence of apoE. SYN immunoreactivity in the OB of mice over the time course revealed that KO mice have a delayed rate synaptogenesis of approximately 3 weeks.

Based on the results from this study, apoE is seen to be a critical element of ORN regeneration. It is proposed that the mechanism through which it mediates its effect on ORN plasticity is through facilitating ORN differentiation and supporting synaptogenesis. Together, these data suggest that the lack apoE may delay the regenerating ORN growth and maturity as well as forming synapses efficiently. It is known that synapse loss can lead to neuronal degeneration due to the absence of trophic factors reaching the cell body via the synapse [46,61,62]. The lack of apoE in KO mice and its effects may be analogous to that of apoE4 in humans and thus present a possible etiological mechanism for the onset of neurological diseases such as AD.

2. METHODOLOGY

2.1. Materials

2.1.1. Nasal Irrigation

WT (C57BL/6J strain) and homozygous apoE KO mice, which have been backcrossed at least 10 times on to the C57BL/6J parental strain, were obtained from Jackson Laboratory (Bar Harbor, ME). Pentobarbital 40 mg/kg, (Cat. # 57-33-0) was purchased from Sigma-Aldrich (St. Louis, MO). Triton-X100 (TX) (Cat # BP 151-500) was purchased from, Fisher Scientific (St. Louis, MO).

2.1.2. BrdU Labeling

Bromodeoxyurindine (BrdU) 50 mg/kg, (Cat. # B5002-1g) was purchased from Sigma (St. Louis, MO).

2.1.3. Sacrifice and Tissue Preparation

Paraformaldehyde (Cat. # 441244-1KG) was purchased from Sigma-Aldrich (St. Louis, MO). Sucrose (Cat. # S-9378), Gelatin (Cat. # G-1890-500) and Potassium chromium(III) sulfate dodecahydrate - "Chrome Alum" (Cat. # 54493-0) were purchased from Sigma (St. Louis, MO). Cal-EX Decalcifying Solution (Cat. # CS510-D), Tissue freezing medium (Cat. # 15-183-13) was purchased from Fisher Scientific (St. Louis, MO).

2.1.4. Nissl Staining

Cresyl Violet acetate (Cat. # C5042-10G) was obtained from Sigma (St. Louis, MO). Permount (Cat. # SP15-500) was purchased from Fisher Scientific (St. Louis, MO).

2.1.5. Immunohistochemistry for apoE

Goat anti-human apolipoproteinE (apoE) (Cat. # 178479) was purchased from Calbiochem (San Diego, CA). FITC-conjugated Donkey anti-goat IgG secondary antibody (Cat. # 705-095-147) and normal donkey serum (Cat. # 017-000-121) were obtained from Jackson ImmunoResearch (West Grove, PA). Anti-photobleach mounting media (Cat. # H-1000) was purchased from Vector Laboratories (Burlingame, CA)

2.1.6. Immunohistochemistry for BrdU

Rat anti-Bromodeoxyuridine (BrdU) (Cat. # OBT0030CX) was purchased from Accurate Corporation (Westbury, NY). Cy3-conjugated goat anti-rat IgG secondary antibody (Cat. # 112-165-068) was purchased from Jackson ImmunoResearch (West Grove, PA). Hanks' balanced salt solution (HBBS) (Cat. # 14175-095) and Fetal bovine serum (dialyzed) (Cat. # 26400-044) was purchased from Invitrogen Corporation (Grand Island, NY). HCl (Cat. # A144-500) was obtained from Fisher Scientific (St. Louis, MO). Anti-photobleach mounting media (Cat. # H-1000) was purchased from Vector Laboratories (Burlingame, CA).

2.1.7. Immunohistochemistry for OMP

Goat anti-rat olfactory marker protein (OMP) was obtained as a generous gift from Dr. Margolis, (University of Maryland, MD). FITC-conjugated Donkey anti-goat

IgG secondary antibody (Cat. # 705-095-146) and normal donkey serum (Cat. # 017-000-121) were obtained from Jackson ImmunoResearch (West Grove, PA). Anti-photobleach mounting media (Cat. # H-1000) was purchased from Vector Laboratories (Burlingame, CA).

2.1.8. Immunohistochemistry for Synaptophysin

Rabbit anti-Synaptophysin (SYN) (Cat. # CMC 109) was purchased from Cell Marque Corporation (Hot Springs, AR). FITC-conjugated goat anti-rabbit IgG secondary antibody was obtained from Jackson ImmunoResearch (West Grove, PA). normal goat serum (Cat. # S-1000) was obtained from Vector Laboratories (Burlingame, CA). Anti-photobleach mounting media (Cat. # H-1000) was purchased from Vector Laboratories (Burlingame, CA).

2.2. Methods

2.2.1 Nasal Irrigation

Fifty six wild type C57BL6J and apoE KO mice, two to four months old, were obtained from the departmental mouse colony and were lesioned as previously described [61,62,82]. For each time point, an equal number of wild type (n=4) and apoE KO mice (n=4) were anesthetized with pentobarbital (40 mg/kg, intraperitoneal) and placed in an immobilizing apparatus. Aliquots of 50 µl of 0.7% (v/v) Triton-X100 (TX) in saline, or 50 µl of saline (SA) alone (control) were injected into each nostril using a 25-gauge needle with a rounded tip. The excess solution was drained from the nasal passages by gently shaking the mice. This technique results in extensive bilateral damage to the OE with approximately 70-80% of the adult OE lesioned [77]. This procedure, however, spares the basal cells, which subsequently divide and differentiate into new ORN [48,49,52]. Animals were placed under a heat lamp while recovering from anesthesia and then were housed in the animal care facility until sacrifice.

2.2.2. BrdU Labeling

Following nasal irrigation, animals were maintained for 0, 1, 3, 7, 14, 21, 42, and 56 days post-irrigation. This time course was selected to cover the range of degeneration and recovery as previously reported [71]. The 0-day post-irrigated animals received an intraperitoneal injection of (BrdU, 50 mg/kg) 12 hours before lesioning and sacrifice; the data from these animals provided basal levels of marker proteins used in this study. The

other post-irrigated animals received an intraperitoneal injection of (BrdU, 50 mg/kg) 12 hours before sacrifice.

2.2.3. Sacrifice and Tissue Preparation

Mice were deeply anesthetized with pentobarbital (80 mg/kg, i.p.) and perfused transcardially with 4°C PBS followed by freshly prepared 4% paraformaldehyde in PBS at 4°C. Turbinates were removed and post fixed in the same solution for one hour. Then they were placed in decalcifying solution for one hour before their canine teeth were excised. Following this, the turbinates were cryoprotected in 30% sucrose in PBS at 4°C for 24 hours. Fixed turbinates were embedded in Tissue Freezing Medium, frozen on dry ice and stored at -80°C.

The olfactory bulbs (OB) were harvested from animals (above) and were post-fixed in 4% paraformaldehyde in PBS at 4°C for 1 hour. Following fixation, OB were cryoprotected overnight in 30% sucrose in PBS at 4°C for 24 hours, frozen in dry ice and stored at -80°C.

Tissue samples from the TX- and SA-treated WT and KO mice were available at days 0, 1, 3, 7, 14, 21, 42 and 56. Turbinates and the OB from the two groups of four mice each per time point were cut at 12 μm thick on a Reichert-Jung 2800 Frigocut E cryostat. Serial sections were thaw mounted onto slides pre-subbed with 2% gelatin and 0.5% Chrome Alum. After air drying the slides, they were rinsed in 0.1M PBS and dried at 37°C in an oven. Sections were stored at 4°C until needed.

2.2.4. Nissl Staining

Sections were washed in 0.1M PBS for 5 minutes then defatted with xylene for 30 minutes. This was followed by hydration through a series of ethanol (100, 95 and 70%) for 10 minutes each. Sections were washed in tap water for 5 minutes, then stained with cresyl violet acetate for 5 minutes. Sections were rinsed in tap water for 10 minutes and differentiated in acidified ethanol for approximately 10 seconds. This was followed by standard dehydration in ethanol series and further delipidation in xylene for 30 minutes. The slides were coverslipped using permount.

2.2.5. BrdU Immunohistochemistry

Sections were washed in 0.1M PBS for 5 minutes and then permeabilized in 0.1% Triton X-100 for 30 minutes at room temperature. Denaturation was carried out using 2N HCl at room temperature for 60 minutes and then neutralized with Hanks' balanced salt solution (HBBS) for 10 minutes. Nonspecific blocking of the sections was carried out with 10% fetal bovine serum (in PBS) within a humid chamber followed by a 10 minute rinse in 0.1 M PBS. An anti-BrdU primary antibody (1:1000 in blocking solution) was applied to the sections and incubated overnight at 4°C. After a 10-minute PBS rinse, sections were incubated for 1 hour in Cy3-conjugated goat anti-rat IgG secondary antibody (1:50 in blocking solution) at room temperature. Slides were rinsed in 0.1M PBS for 10 minutes and then coverslipped using Vectorshield mounting media. Immunoreactive cells were counted and digitally photographed on an Olympus BX50 microscope with appropriate fluorescence excitation filters.

2.2.6. ApoE/OMP/SYN Immunohistochemistry

Sections were washed in 0.1M PBS for 5 minutes and then permeabilized in 0.2% Triton X-100 for 30 minutes at room temperature. Next they were rinsed with PBS and then subjected to a dehydration/rehydration series in ethanol (70, 95, 100, 95, and 70%) for 2 minutes each. Following a 5-minute rinse in PBS, nonspecific immunoreactivity was attenuated by incubation sections in 2.25% gelatin (in 0.1 M PBS) for 1 hour and rinsed again with PBS. The sections were incubated overnight at room temperature in a primary antibody diluted in its appropriate corresponding 4% serum (in 0.1M PBS). The primary antibodies included: anti-ApoE (1:4000), anti-OMP (1:2000), anti-SYN (1:2000). Following primary antibody incubation, sections were rinsed for 10 minutes in 0.1M PBS and incubated for 1 hour at room temperature with the appropriate antibody: FITC-donkey anti-goat IgG (1:1000) and FITC-goat anti-rabbit IgG (1: 1000). Dilutions were made in 4% corresponding serum. Finally, slides were rinsed in 0.1M PBS for 10 minutes and then coverslipped using Vectorshield mounting media. Specificity was determined by incubation with normal serum in place of the primary antisera, which resulted in no staining. Immunoreactive cells were digitally photographed on an Olympus BX50 microscope with appropriate fluorescence excitation filters. Saline and TX-irrigated mice from the two genotypes were processed on the same day with the same reagents to permit comparison of staining density.

2.2.7. Microscopy and imaging

Immunocytochemistry was visualized with appropriate fluorescence excitation filters for fluorescent microscopy (Olympus BX-50). Tiff format images of the nasal

septum of the turbinates or the medial edge of the OB were taken with a Pixera digital camera for microscopy at identical manual exposure settings for all experiments within each study. Saline and TX-irrigated mice from the two genotypes were examined under the microscope on the same day to ensure comparison of staining density. Synaptophysin fluorescence was measured by quantifying mean optical fluorescence of each image using Scion Image software with a 256-bit deep camera. The upper and lower 20 bits were cut off to eliminate nonspecific fluorescence and a mean within this range was obtained.

2.2.8. Morphometric Analysis of OE During Degeneration and Regeneration

To examine if apoE has an impact on the normal remodeling that occurs in the OE after lesioning, I performed morphometric analysis. Essentially, I measured thickness in the OE of cresyl violet stained sections from WT and KO littermates.

2.2.9. Quantification of BrdU

Stained BrdU–positive nuclei were quantified using computer-assisted morphometry and densitometry (Scion Image, Bethseda, MD) as described [73,58,83]. Analysis was performed at two middle rostrocaudal levels of the turbinates controlled in all animals. I selected three anatomically matching areas for cell counting throughout the extent of the OE, and all immunopositive cells were counted within the defined region. The data were expressed as labeling indices; that is, the ratio of the total number of BrdU-positive cells to the linear length of OE analyzed. Published studies have used this

kind of quantification, since stereological analysis cannot be used in OE, due to its variation induced by lesioning [70,73,84].

2.2.10. Quantification of ApoE

Immunofluorescence was evaluated by fluorescence microscopy using 600X magnification. Pictures were taken from similar representative portions of the nasal septum of the OE of each animal from each time point. A complete set of pictures representing the entire time course was assembled into one panel using Adobe Photoshop 7.0 (San Jose, CA) and collectively adjusted for brightness and contrast. Following this adjustment, the panel of pictures was analyzed for differences in localization and levels of apoE present throughout the OE.

2.2.11. Quantification of OMP

Images of OMP immunoreactivity in the OE *were captured using a digital camera (Pixera, Los Gatos, CA) mounted to an Olympus microscope.* Stained OMP–positive neurons were quantified using computer-assisted morphometry (Scion Image, Frederick, MD) as described [57]. Analysis was performed in anatomically similar areas along the nasal septum of the OE by counting all the positively stained nuclei within the defined region. The data was expressed as labeling indices; that is, the ratio of the total number of OMP-positive cells to the linear length of OE analyzed.

2.2.12. Quantification of SYN

Quantification of SYN immunoreactivity in the OB was performed according to published methods [85]. Briefly, microscopic lighting was adjusted such that the

difference between pure black and 100% transmission was 256 gray levels. Images from the glomerular layer in SYN stained sections, obtained from the approximate mid-point of the medial edge of the OB, were captured using a digital camera (Pixera, Los Gatos, CA) mounted to an Olympus microscope. Images were also taken from the subependymal zone, which showed diffuse staining using this protocol, and therefore were used as a measure of nonspecific immunoreactivity. Optical density analysis of the images was performed with Scion Image (Frederick, MD). Measurements within a square selection (40 square pixels) were made from the core of the glomeruli and the subependymal zone. The corrected OD was obtained by subtracting the OD measurements of the subependymal layer from the OD of the glomeruli. The data were generated by using the 40 square pixel area to obtain 3 OD measurements each from 10 contiguous glomeruli.

2.2.13. Statistical analysis

All quantification procedures were repeated 3 times. The data in individual experiments were presented as mean±standard error and statistical analysis (ANOVA, Repeated Measures ANOVA) was performed using SYSTAT.

3. RESULTS

3.1. ApoE in the OE

In this study, the nasal passages of WT mice were lesioned by intranasal irrigation of Triton X-100 in saline. Following lesioning, the OE of WT mice were examined using immunohistochemical techniques. ApoE was observed in the WT mice (Figure 2). ApoE was localized in the cilia of SUS cells and lightly around the perikarya of ORN. In the lamina propria (LP) underlying the neuronal layer, the endothelial cells of blood vessels were intensely stained for apoE. The apoE staining pattern in the olfactory nerve bundles (ONB) was less intense, but was uniformly distributed throughout the core of the nerve bundles. Heavily stained cells, probably olfactory ensheathing glia (OEG), surrounded the nerve fascicles. The distribution and morphology of these intensely stained cells suggested they were OEG. These results revealed that apoE is expressed in the adult OE and lamina propria at strategic locations where it could facilitate the differentiation, maturation and axonal growth of the ORN, perhaps by recycling lipids from degenerating ORN for use by growing axons.

The time course of apoE expression following triton (TX) lesioning was also studied using immunohistochemical techniques. Changes in apoE levels were observed both in the ORN layer and the LP. However, apoE levels in the LP had considerably more dramatic changes in expression during the time course in comparison to the changes observed in the ORN layer. ApoE levels through out the OE and LP peaked at one day after lesioning and then continued to decline until fourteen days post lesioning where levels appeared to be much lower than the control

mice. After fourteen days, apoE expression was gradually upregulated until it peaked at forty two days post lesioning, thereafter gradually returning to control levels by fifty six days (Figure 3). ApoE immunoreactivity was faint or absent in the respiratory epithelium (RE) which was located usually in the ventral extremities of the nasal septum (Figure 4).

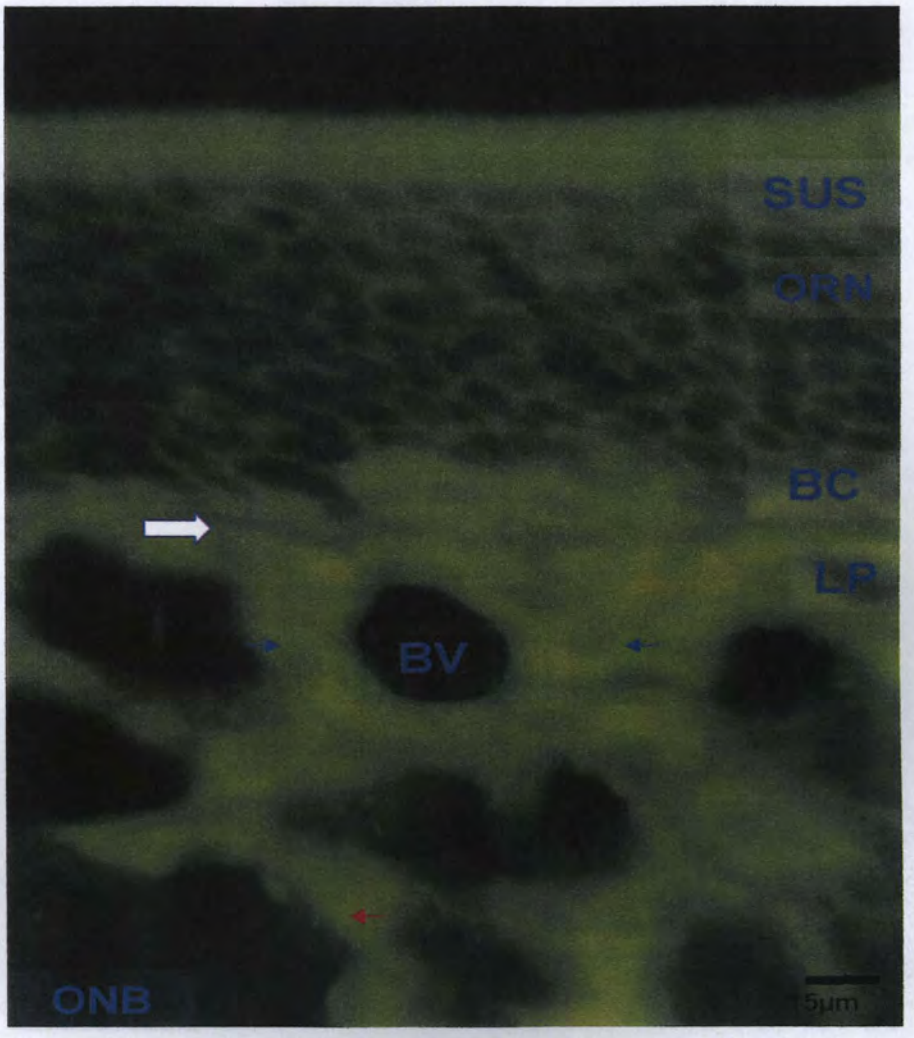

Figure 2: Anti-apoE staining of WT mice OE.
Expression of ApoE Immunoreactivity in the SUS Cells and Glial Cells can be seen predominantly. SUS= Sustentacular Cells, ORN= Olfactory Receptor Neurons, BC= Basal Cells, LP= Lamina Propria, BV= Blood Vessels, ONB= Olfactory Nerve Bundle, White arrow = Basal Lamina, Blue thin arrows =Endothelial cells, Red arrow = Olfactory Ensheathing Glial Cells (OEG). Scale = 15 µm.

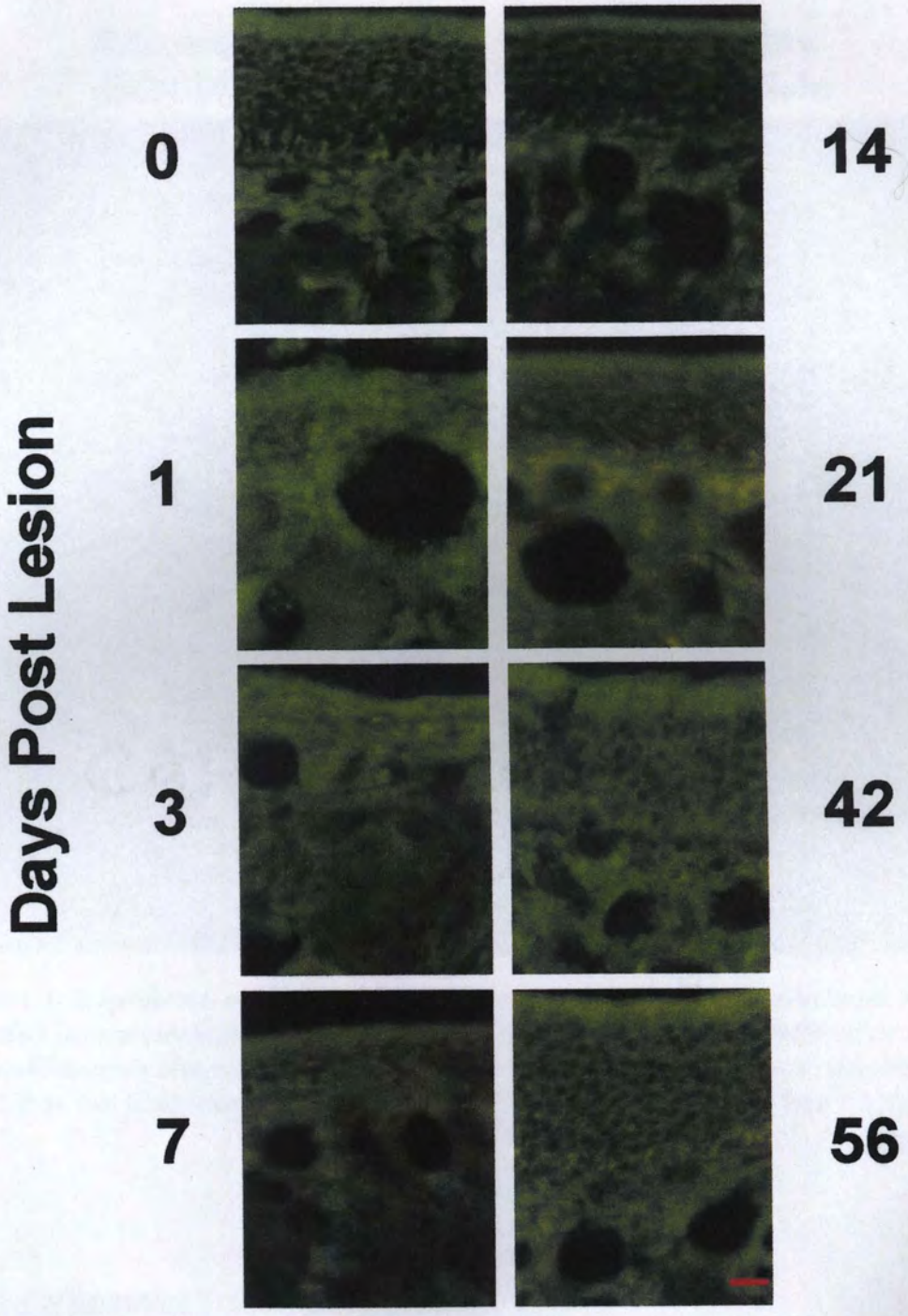

Figure 3: ApoE expression during the reconstitution of the OE of WT mice following TX-100 lesioning. In control OE (A) apoE is expressed prominently in the OEG of the LP and SUS cells with faint staining in the ORN layer. At 1 DPL apoE is upregulated in the BL and OEG of the LP followed by a period of decline (3, 7 & 14 DPL) to levels below that of the control. ApoE expression is observed to be upregulated significantly from 21 DPL. ApoE is observed to return to levels comparable to control non-lesioned animals from between 7 to 8 weeks.
Scale Bar = 15μm

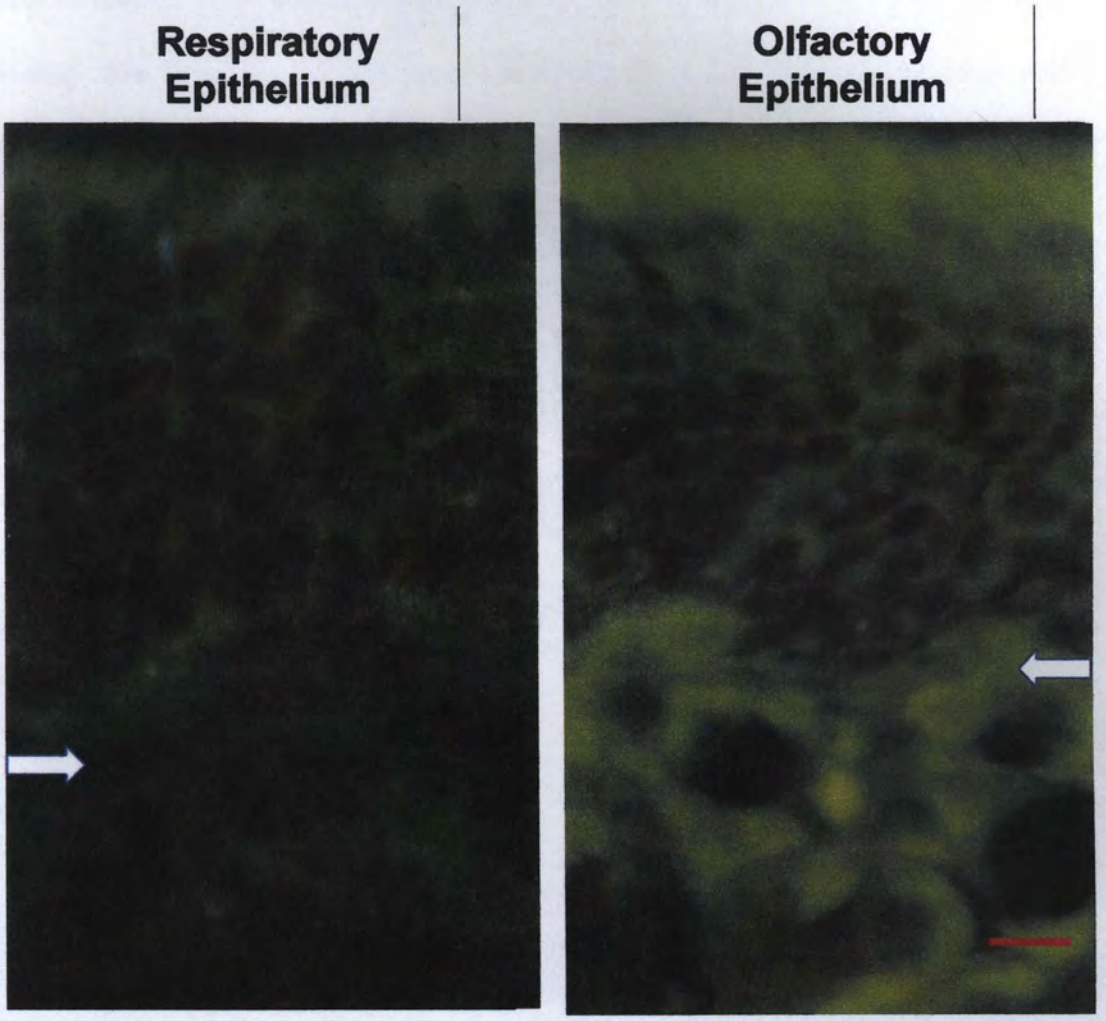

Figure 4: Expression of apoE in the Respiratory & Olfactory Epithelium. Very faint/no immunoreactivity was observed in the Respiratory Epithelium compared to the Olfactory Neuroepithelium. Note the absence of apoE Immunoreactivity in the LP of the Respiratory Epithelium. White arrows = LP. Scale bar = 15 µm.

3.2. Effect of Lesioning to the OE

As mentioned earlier in the text, the olfactory epithelium serves as a unique and useful model to study neural regeneration (Figure 5). As a part of this study, the OE was lesioned and assessed by cresyl-violet staining, to determine the extent of the damage to the OE. Morphological studies of the lesioned mice showed that there was

extensive loss of ORN and SUS cells from the OE by the end of the first day after lesioning. The basal lamina had been visibly denuded, only sparing the basal and stem cells. There was a corresponding accumulation of cellular debris in the nasal passages following lesion The time course study of the reconstituting OE observed no difference in the rate of recovery of the OE between WT and KO mice ($F_{7,28}=355.2$; $P>0.001$) (Figures 6 &7).

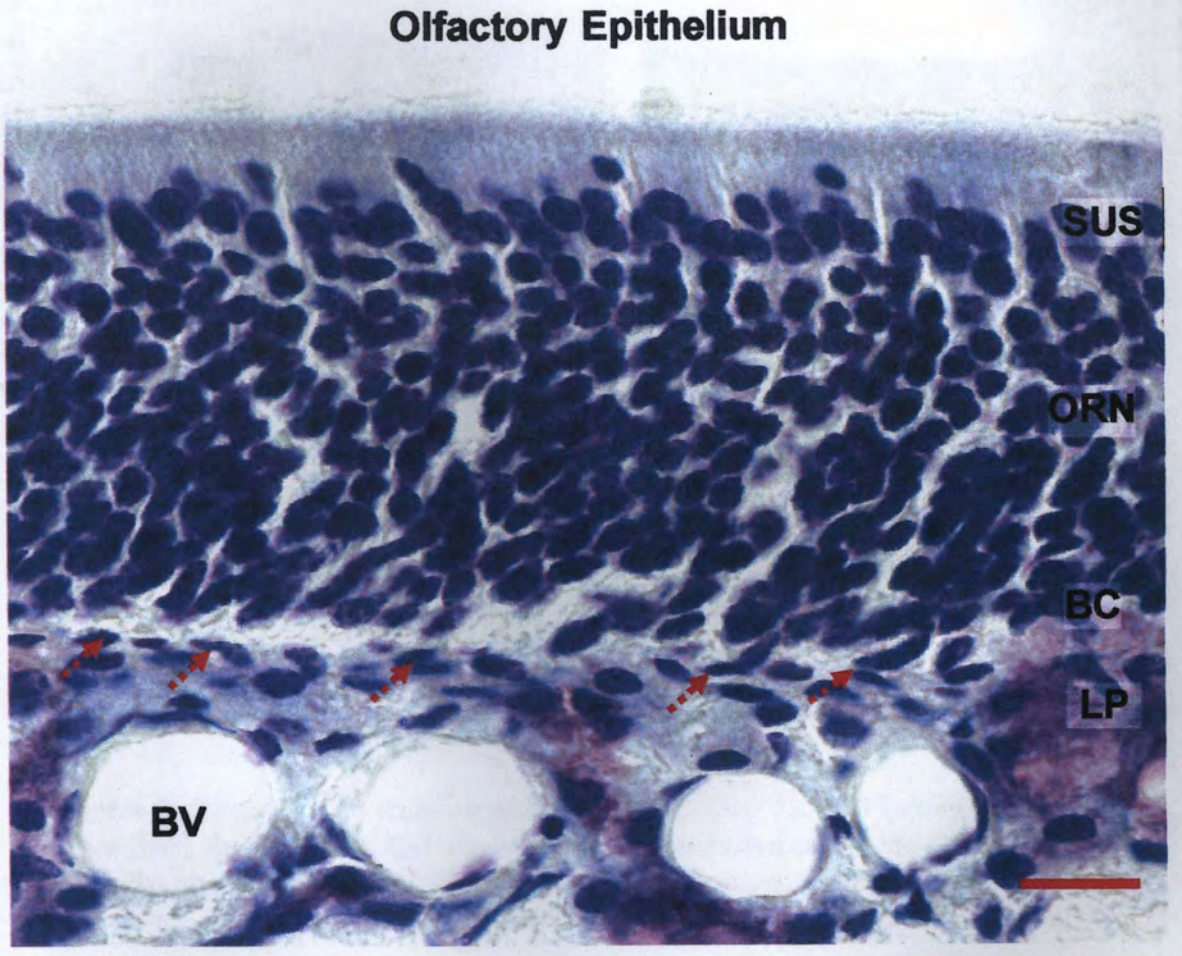

Figure 5: Cresyl-violet staining of normal Olfactory Epithelium. SUS= Sustentacular Cells, ORN= Olfactory Receptor Neurons, BC= Basal Cells, LP= Lamina Propria, BV= Blood Vessels. Red arrows indicate Horizontal basal cells. Scale = 20 µm.

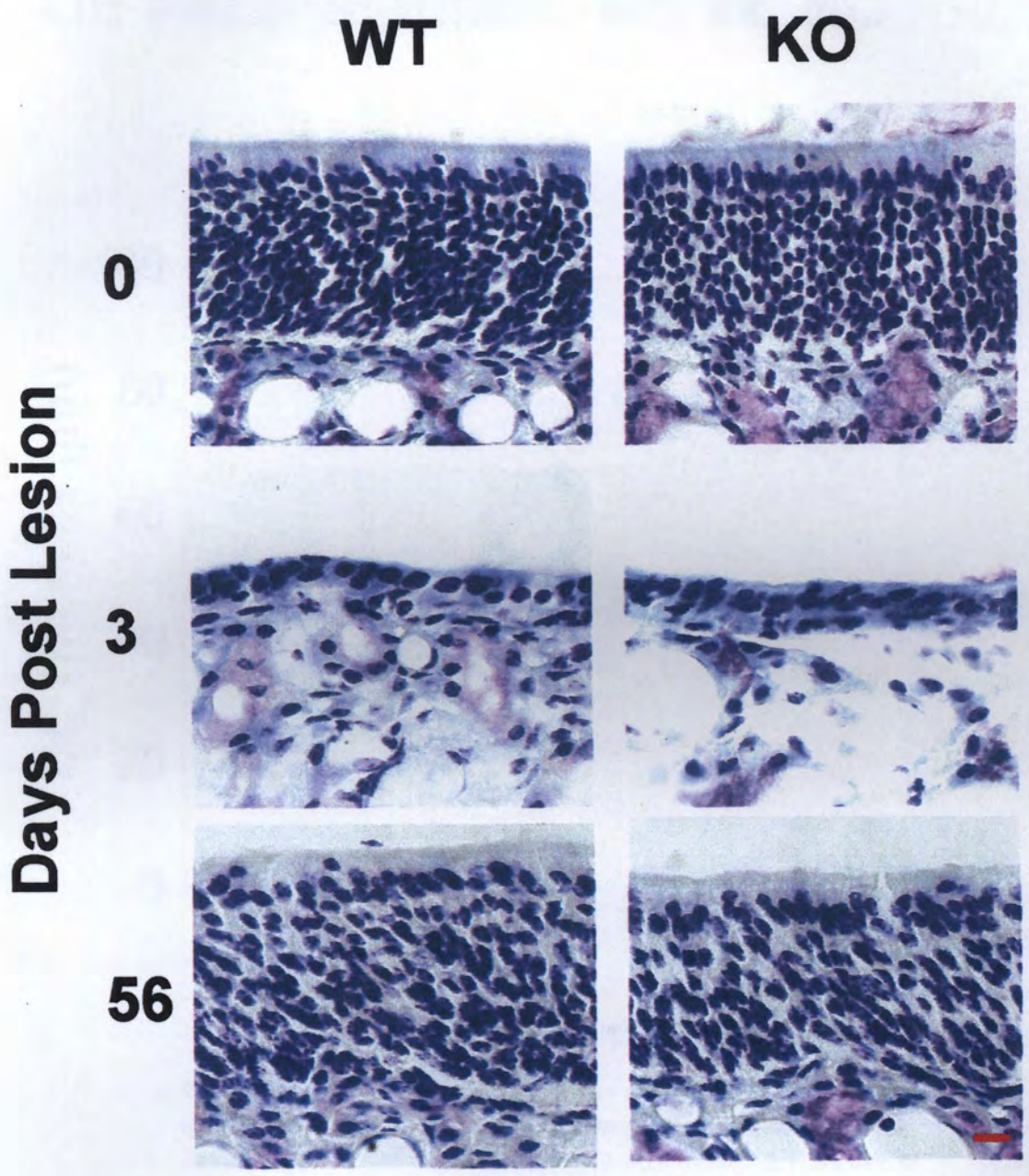

Figure 6: Cresyl-violet staining of the OE following TX-100 Lesioning. WT mice (left) compared to KO mice (right) in controlled unlesioned epithelium (0 DPL) and at 3 days and 56 days after the lesioning. There is no significant difference OE reconstitution between WT and KO mice. Scale bar= 15μm

OE Reconstitution: WT vs. KO mice

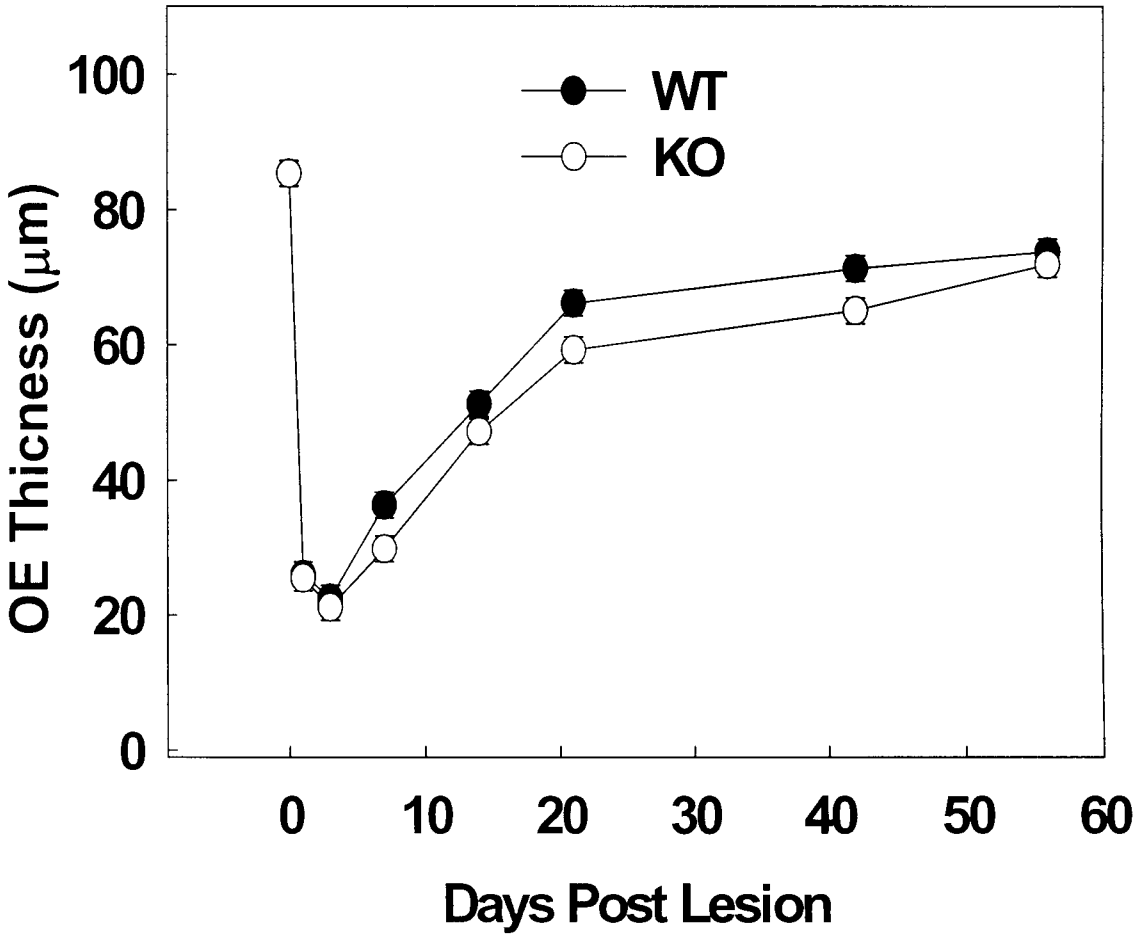

Figure 7: Time course study of the rate of regeneration of the OE thickness following Triton lesioning. At each time point after lesioning WT and KO mice have a significant difference in thickness, yet the rate of recovery of OE is very similar. Lesioning treatment resulted in a sharp decrease in the thickness of the OE after 3 days. This trend was reversed from the seventh day. The highest rate of recovery was observed from the seventh day till the third week where the recovery rate slowed down till the eight week. The OE by the eight week had only recovered about 80% of its pre-lesioning thickness.

3.3. ApoE and Cell Proliferation in the Regenerating OE

Since the previous data from this study indicated that the lack of apoE did not limit the repopulation of the OE of KO mice, it was important to determine whether apoE had any effect on the rate of neurogenesis. It hypothesized that apoE would be a limiting factor on the rate of neurogenesis as the stem cells would require lipids and cholesterol for mitotic division. This postulate was tested by comparing ORN regeneration in both WT and KO mice and was assessed by BrdU immunoreactivity.

BrdU localization was observed to be patchy randomly sited along the length of the nasal septum. BrdU(+) cells where existing, were localized to just above the basal lamina through out the time course. This coincided with the known location of horizontal and globose basal cells that serve as progenitor cells for ORN and supporting cells. The only exception was 1 week after lesioning when BrdU(+) cells were observed across the width of the OE (Figure 8).

The time course data of BrdU immunoreactivity in both the WT and KO mice, was characterized by an immediate increase in number of cells proliferating, followed by a dramatic peak at three to seven days post lesioning after which its levels dropped back to normal by fourteen days post lesioning The results also revealed that there was not a significant difference ($F_{1,4}=0.037$; $P>0.856$) between WT and KO in the time course of BrdU immunoreactivity (Figure 9).

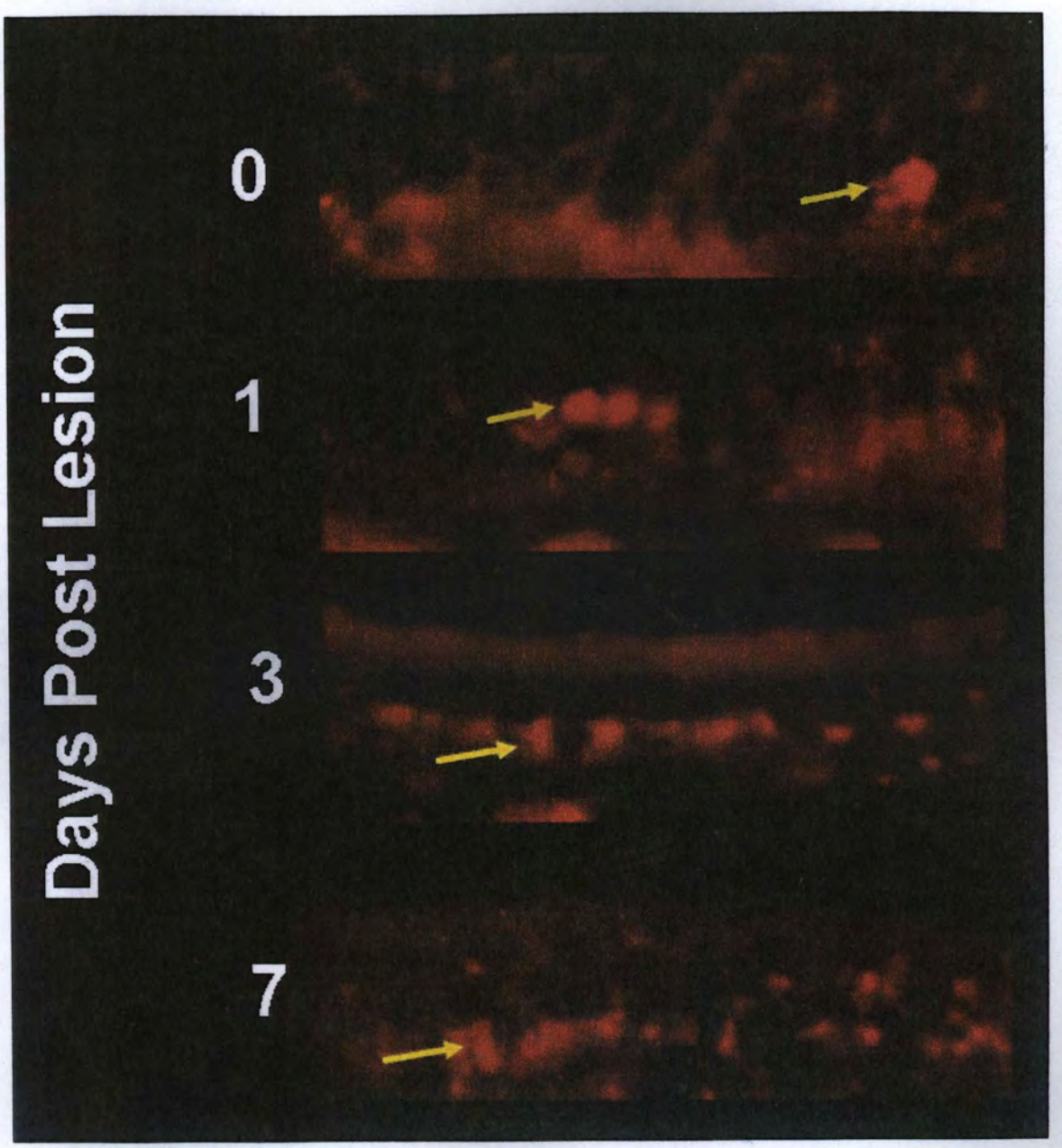

Figure 8: Immunohistochemical localization of BrdU Immunoreactivity in the OE of WT mice following TX-100 lesioning. Brdu localization in an unlesioned animal day, followed by 1, 3 and 7 days post triton lesioning. There is a dramatic increase in BrdU localization along the basal cell layer from day 1 to day 7. BrdU Positive Cells (Cy3 Conjugated) are indicated by thin yellow arrows. Scale Bar = 15μm

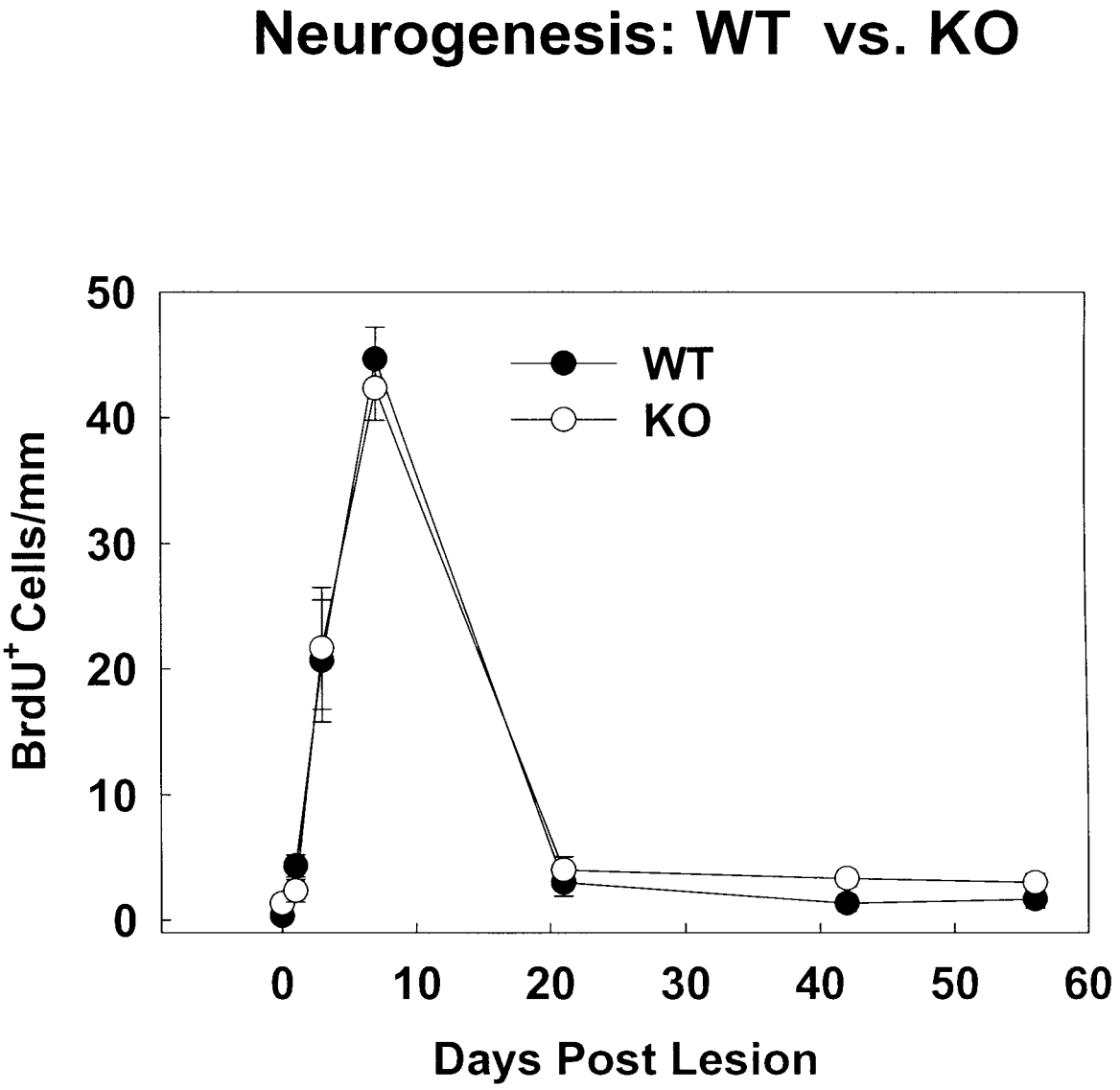

Figure 9: Time course of the changes in the numbers of BrdU(+) cells following Triton lesioning as measured by immunohistochemistry. At each time point WT and KO mice have no significant difference. The lesioning treatment resulted in a sharp increase in the number of Brdu(+) cells after 3 days. This trend continued till the seventh day where it peaked at more than 20-fold the control levels. Brdu levels began a sharp decline reaching the control levels the following week and remained there for the duration of the time course.

3.4 ApoE and ORN Maturation in the Regenerating OE

From previous studies, it was observed that there is delayed olfactory nerve regeneration in KO mice [57]. It was therefore expected that the regenerating ORN of KO mice might also experience delays in maturation. After staining an unlesioned control OE with OMP, mature ORN were localized to olfactory nerve bundles (Figure 8) and to about the middle two thirds of the OE (Figure 10).

The WT mice had higher OMP immunoreactivity than KO mice for each time point including the controls. During the time course, it was found that OMP levels dropped immediately at one day post lesion. Between the third and seventh days after lesioning, OMP (+) cells were barely detectible as the OE is devoid of mature neurons (Figures 11, 12). After seven to fourteen days, the OE of WT mice was characterized by a significant increase in the number of mature ORN as determined by the thickening of the OMP(+) band of cells in the middle layer of the reconstituting OE. The OE of KO mice followed the same trend albeit at lower levels. It was also found that there was no statistical difference between WT and KO mice ($F_{1,4} = 34.08, P<0.004$)

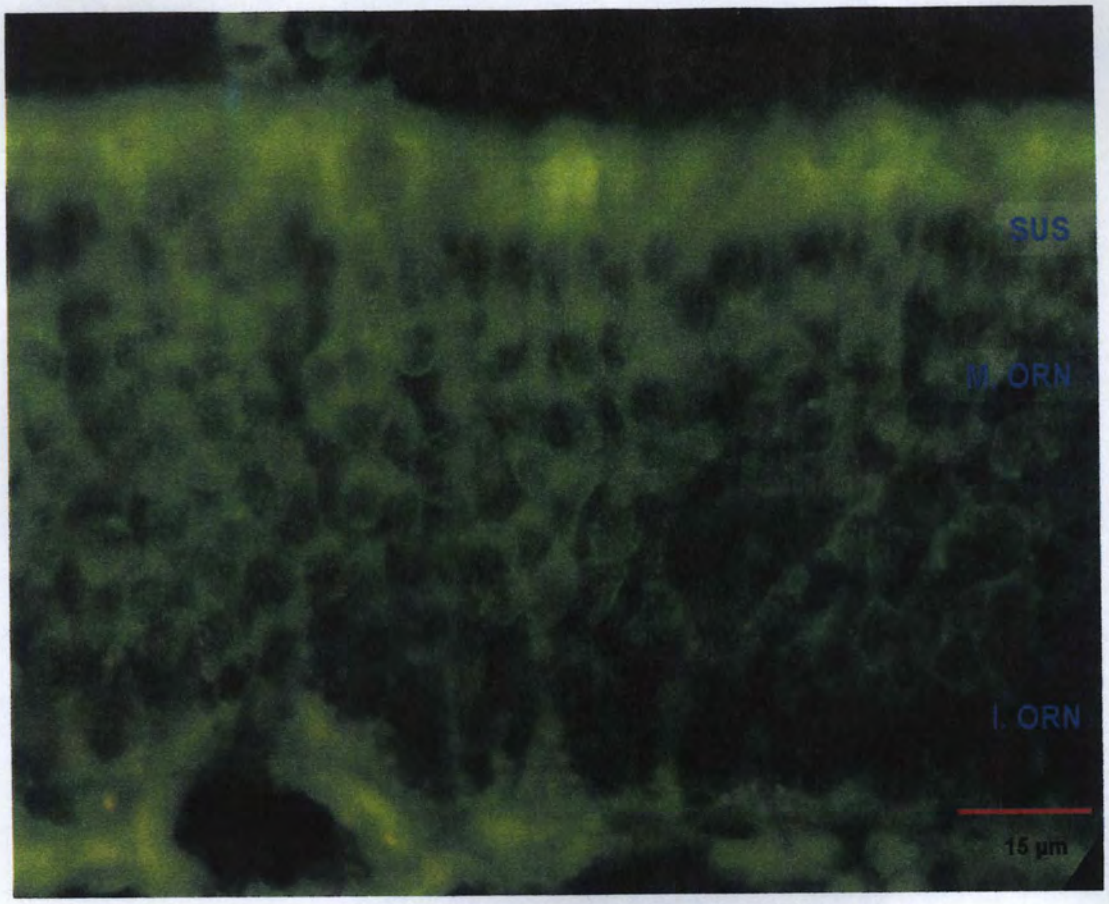

Figure 10. Immunolocalization of OMP in the OE of an unlesioned mouse. The punctiform labeling of OMP(+) cells is seen to localized juust below the SUS cells and above the immature ORN depicted as OMP(-) cells. SUS= Sustentacular Cells, M.ORN= Mature Olfactory Receptor Neurons, IORN= Immature Olfactory Receptor Neurons. Arrow Indicates the Basal Lamina. Scale= 15 µm.

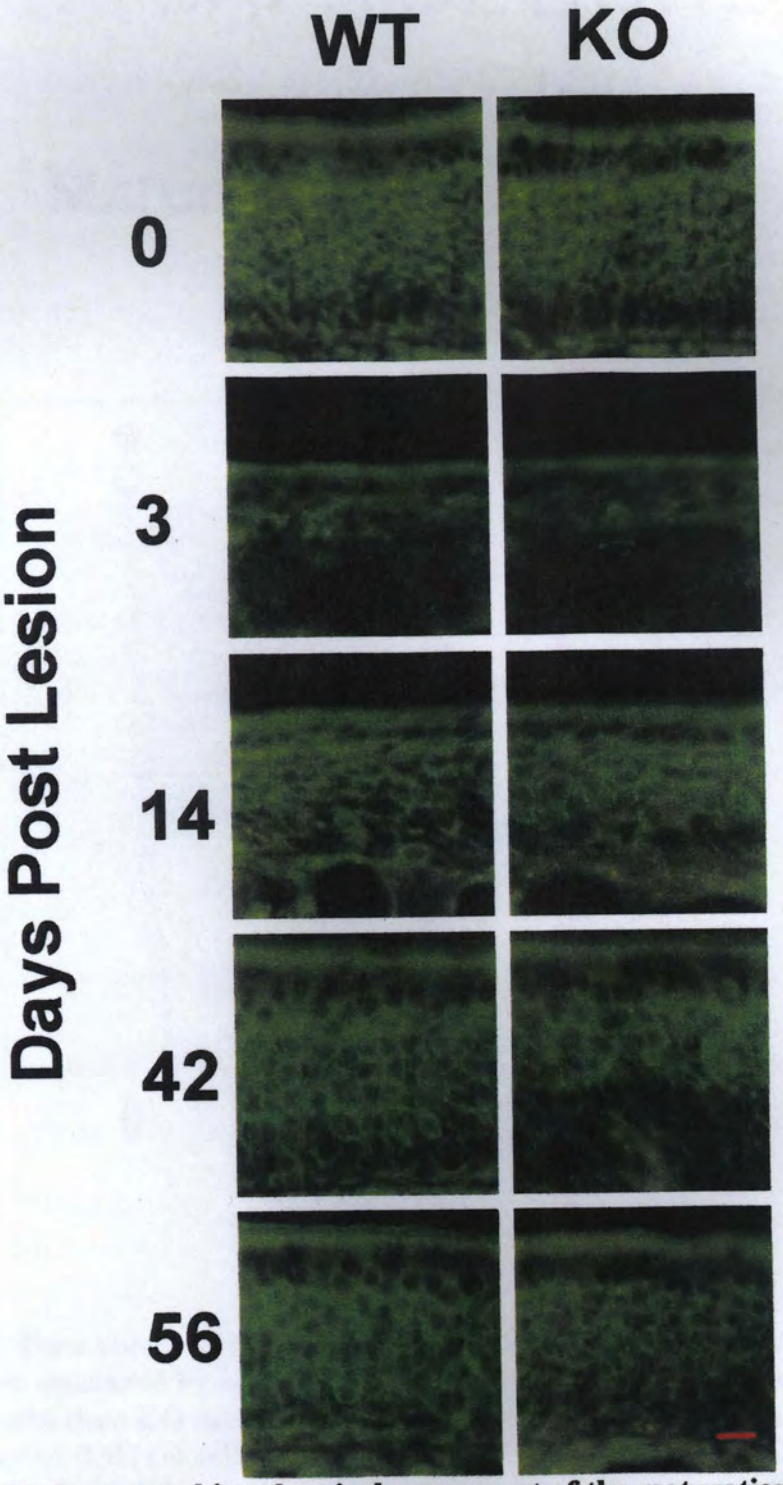

Figure 11. Immmunohistochemical assessment of the maturation of the OE following TX-100 lesioning. WT mice (left) compared to KO mice (right) in controlled unlesioned epithelium (0) and at 3 days, 21 days, 42 days and 56 days after the lesioning. The OE of WT is observed to express more OMP(+) cells than KO at each time point. The full complement of OMP(+) cells is restored at between 42 and 56 DPL. Scale bar = 15μm

Maturation: WT vs. KO mice

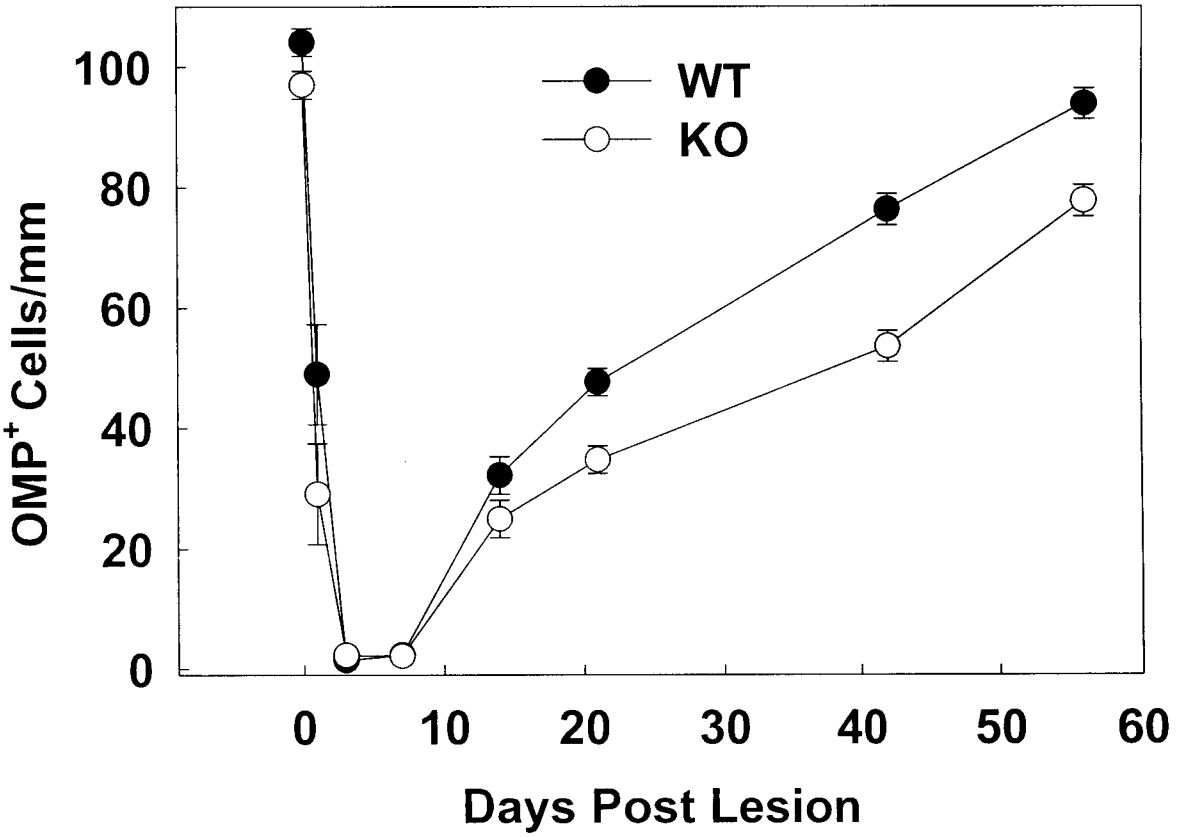

Figure 12: Time course of the changes in the numbers of OMP(+) cells following Triton lesioning as measured by immunohistochemistry. At each time point WT mice has more OMP (+) cells than KO mice. The lesioning treatment resulted in a sharp decrease in the number of OMP(+) cells for the first week. The number of OMP(+) cells began a steady increase reaching just below the control levels by the eighth week.

3.5 ApoE and Synaptogenesis of ORN in the OB

Maturing ORN have to form synapses with the OB to be able to acquire trophic substances that aid the ORN in survival. It was postulated that apoE would be crucial for the rate of synaptogenesis in the OB and that KO mice would face a delayed rate in synaptogenesis. This hypothesis was tested by using anti-synaptophysin SYN to stain the OB.

It was found that the SYN stained the neuropil located in individual glomeruli of the OB. The SYN (synaptogenesis) time course revealed an almost parallel trend to the time course of OMP (cell maturation). After initial declines in SYN levels, the OB did begin to recover significantly between twenty-one and forty-two days after lesioning in the WT mice. In contrast, the recovery in SYN levels in the KO mice was slower and it occurred between day forty-two and fifty-six days and was not complete by the latter date (Figures 13 & 14). The data found this difference in WT and KO recovery of SYN in the OB to be significant ($F_{7,32}=3.476$; $p<0.007$). ApoE was also found to promote synaptic density in the brain of WT mice ($F_{1,32}=26.58$; $p<0.001$). Therefore apoE has a profound effect not only on the rate of synaptogenesis during ORN development but on the maintenance of synaptic density.

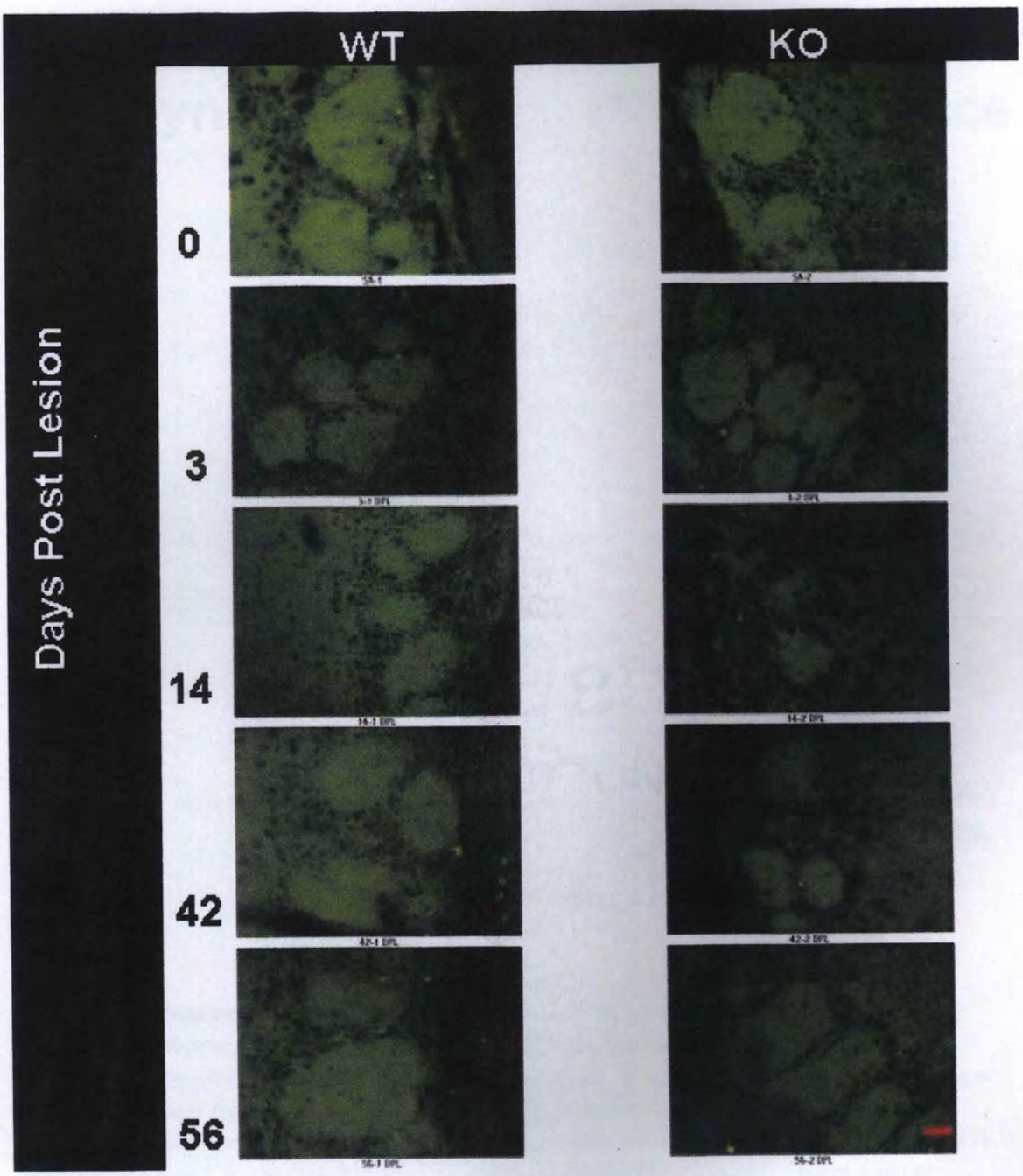

Figure 13: Immunohistochemical assessment of synaptogenesis in the OB following TX-100 lesioning. SYN immunoreactivity seen in WT mice compared to KO mice at various time points. SYN levels are high in both WT and KO control mice at 0 days post lesion. After initial declines in SYN levels at 3 days post lesion, synaptogenesis seems to reoccur earlier in WT mice, as evidenced by the recovering glomeruli form and SYN levels by 14 DPL. The OB of KO is observed to recover about 3 weeks behind WT mice. Scale bar = 15μm

Synaptogenesis: WT vs. KO mice

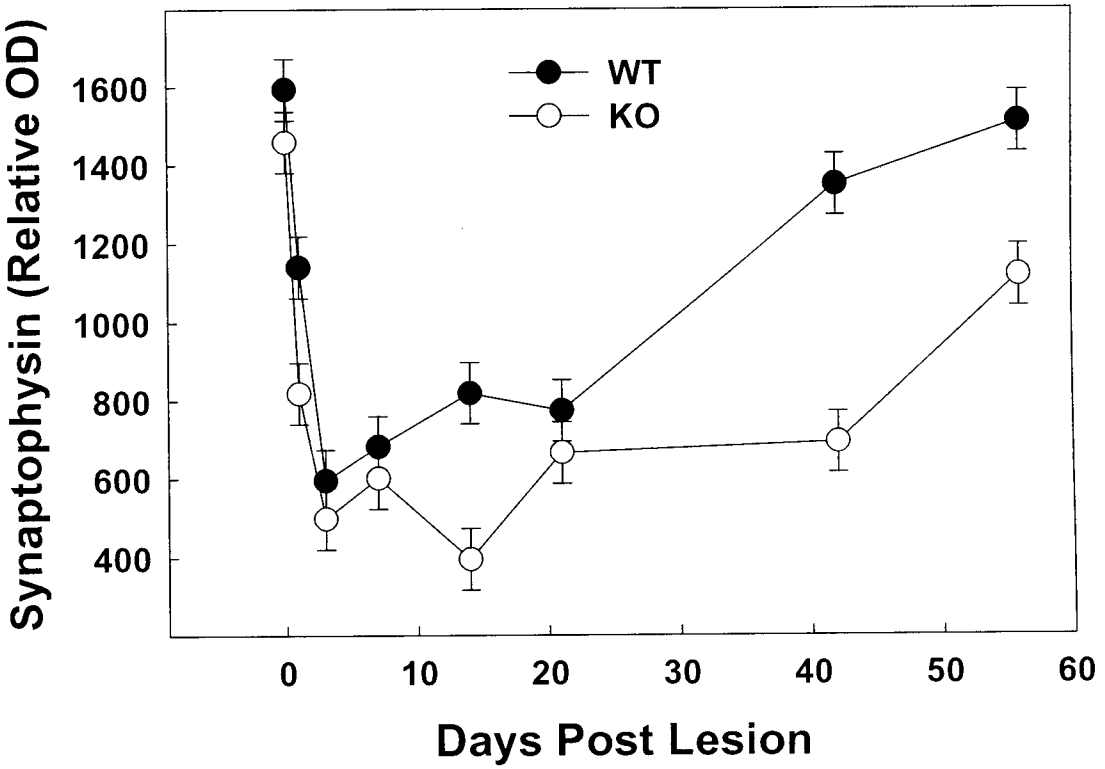

Figure14: Time course of the changes in the levels of anti-synaptophysin(SYN) following lesioning as measured by immunohistochemistry. The lesioning treatment resulted in a drastic decrease in the SYN levels for the first three days. The SYN levels began to increase after 1 week in the WT mice followed by a sharp increase observed between the third and eight weeks. In the KO mice SYN levels continued to decline till the second week and then began a slow rate of increase till the seventh week. The eight week in KO mice was marked by a dramatic increase in the rate of synaptogenesis. It was observed that the SYN levels in KO mice remained lower than WT mice.

4. DISCUSSION

There are numerous etiologies ascribed to AD ranging from plaque formation to oxidative stress in neurons [63]. Important studies have indicated that cholesterol metabolism is a very important aspect of ORN development and plasticity and inevitably implicated as a possible pathway for some neurological disorders [2,20,64,65]. Cholesterol has also been found to be a rate limiting molecule necessary for synapse formation and functioning [86]. Therefore, this investigation explored the possibility of linking the AD etiologies of abnormal lipid metabolism and synaptic formation by examining the effects of apoE on: (a) regeneration in the OE following lesioning, (b) cell proliferation in the regenerating OE following lesioning, (c) on ORN maturation in the regenerating OE following lesioning, and (d) on synaptogenesis of ORN in the OB following lesioning. Also conducted, were preliminary investigations on the localization and expression of apoE in the OE in response to lesioning. These studies compared the apoE WT and KO littermate mice in order to elucidate a mechanism(s) whereby apoE supports ORN plasticity.

4.1. Morphometric Analysis of Regeneration in the OE

The intact adult OE of mice is comprised of ciliated pseudostratified columnar epithelium which sits on a vascular lamina propria. The epithelium consists of bi-polar ORN, sustentacular cells (SUS) and microvillar cells and ranges about 13-18 cells thick from the basal lamina (Figure 5). The uppermost or apical end of the OE is populated by SUS cells. These were identified simply by their unique position in the OE as well as by their characteristically larger nuclei. The majority of the OE is

comprised of mature ORN that are localized in the middle of the epithelium. These cells express OMP and were identified as OMP(+) cells. Just below the mature ORN lie the immature ORN (identified as OMP(-) cells). The globose and horizontal basal cells are located just above the lamiana propria.

After the WT and KO mice received intranasal lesioning with triton TX-100, the OE regenerated over a period several weeks as reported by others [61,62]. The first week was characterized by a loss of most of the neurons and supporting cell populations as depicted by the OE being only 2-3 cells thick (apically) by 3 DPL (Figure 6). This period is also marked by the presence of cellular debris. The massive neuronal death signaled cell proliferation marked by the reconstituting of the OE between 7 and 21 DPL. By the third week, the OE though about 10 cells thick, lacked the anatomical organization of the OE of the control mice. Only after 42 DPL did the reconstituted OE resemble the original OE in both organization and thickness. There was no significant difference in the time- course of the OE regeneration (as characterized by epithelial thickness) between WT and KO mice. Therefore the data indicates that apoE is not a limiting factor in the repopulation of the ORN of mice.

4.2. ApoE in the OE

Previous studies of apoE localization and time course expression in the OB of WT and KO have revealed the presence of apoE in the OE [4,31]. This study has corroborated the previous evidence by showing that apoE is expressed throughout the OE and its underlying lamina propria. In the OE, SUS cells expressed considerable levels of apoE in their perikarya and villar processes, located at the apical surface of

OE, and in their end feet, which are in close association with basal cells lying above the basal lamina (Figure 2). ApoE (as a transporting ligand) in the SUS cells is likely to enhance their normal functioning, particularly in the recycling of membrane components from degenerating cells. This would also be compatible with the previously published role of apoE in recycling membrane of degenerating axons and supplying cholesterol to growing axons [2,5,23,78,79].

It is known that neurons possess LDL receptors in their axons, cell bodies and dendrites to which apoE binds, in the process of the internalization of cholesterol molecules packaged as lipoproteins by Glial cells for neurite growth [11,66,67]. A recent study also identified apoE and the cholesterol it carries as the glial factor that stimulates synaptogenesis within *in vitro* neuron cultures [59]. In this study, apoE was found to be present around the ORN (but only at low levels) of WT mice though not at levels to indicate that apoE secretion occurs within the ORN. Collectively, these corroborative evidences would explain apoE incorporation into neurons as being necessary for the increased lipid metabolism and structural demands associated with regeneration [57]. Aborization, axonal growth and synaptogenesis would probably require more lipids than normally available within the neuron. Thus, apoE secreted by supporting cells would be utilized to supply the deficit in cholesterols and lipids needed for the neuron to efficiently grow. ApoE KO mice are invariably disadvantaged in lacking apoE but it is suggested that the other apolipoproteins would function in the absence of apoE through some redundant pathways [86].

In the lamina propria, apoE was expressed at high levels in the endothelial cells of the blood vessels. Olfactory nerve bundles showed slight staining of apoE, whereas the

surrounding OEG were intensely stained (Figure 2). The finding of apoE in OEG cells would also suggest OEG also play a supporting role for ORN by secreting apoE that is needed to deliver rate limiting levels of cholesterol to the growing axons that form the ONB. In this regard, apoE could facilitate the various functions attributed to SUS and OEG (supporting cells), particularly in recycling of lipid membrane components liberated from the continuously senescing ORN population of a mature OE. This is verified by publications that show OEG as the cells that form a myelin sheath (lipid rich) around the olfactory nerve bundles (ONB) in the lamina propria [68].

4.2.1. ApoE Dynamics in the Regenerating OE

Interestingly, the changes in levels of apoE expression observed in the OE of WT mice were very distinct in the lamina propria. Data revealed a significant initial increase in apoE levels on day 1 in the lamina propria of both mice. This was followed by a decline in levels till the end of the second week where levels fell below normal (levels of control mice). The third week was marked by increase in apoE levels similar to the first day and then a gradual decline to normal from the eight week onward (Figure 3). The time course of the OMP and SYN in later studies is observed to follow a similar pattern to the apoE levels. This in turn would implicate apoE levels in the regenerating OE to maturation and synaptogenesis when lipid metabolism or demands are highest.

The respiratory epithelium (RE) of the mice is usually located towards the extreme ventral and anterior portions of the nasal septum and turbinates. Also, the OE is known sometimes to reconstitute as RE (devoid of ORN) instead of OE (populated

with ORN). The RE regions showed little or no apoE-like immunoreactivity relative to the OE (Figure 4). Infact, even the OEG in the underlying LP had no observable apoE immunoreactivity. This dichotomy suggests that apoE is involved with actively growing processes of the ORN.

4.3. ApoE and Cell Proliferation in the Regenerating OE

During the lifetime of a mammal, olfaction is challenged by the natural death of ORN or the loss of ORN due to noxious chemicals in the environment [46,69,70]. Therefore, there is need for the constant regeneration of ORN to prevent the loss of olfaction that is crucial to the survival of many mammals. To achieve this, the system has a strongly regulated process of balancing the elimination and generation of ORN [47,71]. The understanding of this process is very important in the possible development of methods to reverse cell degeneration or enhance cell proliferation in patients suffering from neurodegenerative diseases.

The time-course of cell proliferation as observed by BrdU immunoreactivity in the regenerating OE of WT and KO mice revealed cell proliferation may have began as early as one to two DPL. There was a marked increase in cell proliferation in the OE at three DPL, which later peaked on the seventh day post lesioning. It has been established in other studies that the first week of mitotic division following lesioning, involves primarily the proliferation of non-neuronal cells; namely, supporting cells [69,71-73]. By seven DPL, significant ORN proliferation peaks thus repopulating the OE with immature neurons [50,51].

BrdU immunoreactivity in both WT and KO mice was initially localized to just below the layer of already newly formed SUS cells along the basal lamina – the location of a mixed population nonneuronal and neuronal stem cells at three DPL (Figure 8). Thus, it is also evident that some of the SUS cells are already well developed by the time ORN mitotic division begins in earnest. The results are in accord with previous studies that describe the complete denudation (by Triton-TX lesioning) of the OE except for the stem cells and the duct cells, followed by the initial regeneration of supporting cells before ORN [49,62,74]. It is likey that the SUS cells provide neurogenic factors and physical scaffolding on which immature ORN migrate apically [21,51]. Therefore the timing of their formation suggests that SUS proliferation is vital for the subsequent ORN proliferation.

When ORN proliferation peaks at seven DPL, BrdU(+), cells are found not only along the basal lamina but throughout the OE (Figure 8). It is known that during the regeneration of the OE, some mitotic cells migrate apically above and away from the basal lamina [25,61,62]. There are studies that indicate the appearance of a significant increase in immature ORN by the seven DPL, which correlates with our data that show ORN proliferation peaked at about the same time [49-52,73]. OMP studies in regenerating OE further clarify this point by the localization of OMP in the upper third of the OE one week later [46,47,62]. The lower two-thirds of the OE at this point is populated by immature ORN as evidenced by a lack of OMP localization.

From the 2nd week post-lesion, the OE is populated by a majority of immature neurons (OMP(-) cells) [47,50,51], which are just beginning to differentiate into mature ORN (OMP(+) cells) as they extend their axons into the OB. The data show

that the numbers of BrdU(+) positive cells (proliferating) began to decrease considerably towards normal levels concurrent with the increase in differentiated mature ORN (from immature neurons) (Figures 8 & 10). This is indicative of a possible negative feedback mechanism that signals the proliferation pathway, directing it to slow down as the ORN population reaches a critical point (≃70% of original number) in development [76]. This mechanism is not fully understood. However, it has been postulated that the differentiated cells may signal the decrease in proliferation of their own progenitor cells [46,47,50,51]. There is indirect evidence from previous studies supporting an inverse relationship between ORN in the OE and the rate of proliferation of neuronal progenitor cells in rodents and amphibians [74,77]. Implicit in this view, is the correlation of maximum proliferation (3-7 DPL) and minimal thickness (3-7 DPL) of the OE as well as minimal presence (3-7 DPL) of mature OMP(+) ORN that was as observed in the data between both WT and KO mice.

The data indicated no significant differences in cell proliferation between apoE KO and WT mice during the entire time course. This may be explained by the fact that neurogenesis is independent of both the presence of the OB as the ORN target and the presence of apoE as a possible mitogenic factor. The data are corroborated by studies of chronically bulbectomized mice that have not lost the ability of OE regeneration (though it does not fully reconstitute) [72]. Other studies of regeneration in apoE KO mice describe reconstitution of the OE, although delayed, relative to WT mice [5,18,30].

However, during the decline in cell proliferation that began after the first week of proliferation, a contrasting observation was made that was not significant. KO mice followed a similar time-course trend as WT but at an elevated rate of cell proliferation after the first week (Figure 9). In other words, the OE of the KO mice with respect to WT mice (from the third to eight weeks) is in a higher state of ORN proliferation. This could concur with other studies that have shown that, when ORN from the OE fail to make synaptic connections, higher rates of apoptosis (cell death) and neurogenesis are observed in the OE [49,70,71,74]. The perceived higher level of proliferation could be explained as being necessary to keep the OE populated with enough ORN as it suffers from a higher rate of senescence. In light of apoE KO mice, the observed delay in regeneration of the OB [30,57] may have a concomitant elevation in apoptosis and possibly ORN proliferation as well. It is imperative to redesign this study to accommodate a larger sample size and to identify if the neuronal progenitor cells are indeed proliferating at higher levels than normal in the OE of KO mice during the second phase of cell proliferation.

4.4. ApoE and ORN Maturation in the Regenerating OE

Since it appears that apoE has no direct effect on initial ORN proliferation, it could be then assumed that during the second week post-lesion, the OE of both strains of mice might have similar numbers of immature neurons – OMP(-) cells. This is based on the evidence [72] showing that, after bulbectomy in a rat, the immature ORN proliferation rate is not disrupted. It is crucial to note the existence of a critical window of 7-21 DPL (2 weeks) for ORN in the regenerating OE to differentiate or

mature [48,61,72]. Maturation is hypothesized by Schwob to be: "the onset of OMP expression, the elaboration of cilia, and the disappearance of perikaryal gap-43" [72]. It is well documented in bulbectomy studies that the OE never regenerates to its original capacity and is instead populated by a very large number of immature ORN and characterized by unusually high levels of apoptosis of OMP(+) mature ORN [72,77]. This may very well be the state of the KO regeneration process.

It is therefore essential to elucidate the factors that mediate this and whether apoE does influence this process in any way. This part of the study answers some of these questions by investigating ORN maturation in apoE WT and KO mice.

The unperturbed adult OE is populated by mostly mature ORN that are identified by the OMP marker. It is a 19-kDa, acidic, soluble protein found abundantly in mature olfactory sensory neurons. The axons of mature ORN therefore exhibit OMP immunoreactivity. Since the function of OMP is not known, one cannot postulate on the possible interaction of apoE and OMP in the regenerating OE. OMP immunoreactivity simply serves as a marker or indicator of a cell type – mature ORN.

Immunohistochemical analysis of the OE of both WT and KO mice showed OMP intensely stains the anatomically identified mature ORN. OMP(+) cells were localized to the upper two-thirds of the OE, stretching from below the more apical layer of SUS cells to just above the lower basal layer of OMP(-) immature ORN, as observed by other studies [46,47] The olfactory nerve bundle (ONB) of the lamina propria, which contains the axons of the ORN, also stained strongly for OMP (Figure 10).

It is known that OMP(+) cells begin to significantly appear in the OE one week after the arrival of immature ORN [49,71,74,77] which is fourteen days post lesioning. During the next week, a tremendous spike in OMP immunoreactivity is observed as a wider layer or band of mature ORN forms in the middle part of the OE. This occurs as a result of the differentiation of immature ORN into mature ORN. This study paralleled the above results with observed significant levels of OMP cells, appearing by fourteen days post lesion. This was preceded by an initial disappearance of OMP cells in the OE during the first week following lesioning. This would indicate that the mature ORN had been mostly destroyed and are making a return as the new immature ORN mature by the second week. This process is gradual, as previous studies have colocalized a large number of ORN with both immature and mature neuron markers [61].

Quantitative assessment of OMP levels in the OE of both WT and KO mice after Triton-TX 100 lesioning concurred with the observed OMP time course of previous studies [49,71,74,77]. OMP levels remained below 25% of control animals by the second week and then doubled by the third week and almost approached normal levels by the eighth week post-lesion in WT mice. OMP levels in KO mice closely mimicked this trend, although at each time point the KO mice had lower numbers of OMP(+) cells in the OE (Figure 12).

The data suggest that apoE does not influence the rate of maturation of ORN ($F_{7,28}=2.943$; $P<0.019$). The OE of apoE KO mice does however produce fewer OMP(+) mature ORN than WT per time point (14-42 DPL) during OE reconstitution,

indicating that the KO ORN are constrained by certain factors (other than the OB target deprivation) in maintaining a full complement of OMP(+) per time point.

An earlier study has characterized apoE3 as enhancing the axonal/dendrite growth over apoE4 [14]. Another has isolated apoE receptors in the active growth cones of lengthy axons, where extra-cellular cholesterol is needed at the site of axonal lengthening, since cholesterol is not synthesized there [2,6,11,23]. There is additional evidence suggesting that neuronal growth is dependent upon cytoskeleton stability, which in turn is limited by cholesterol levels [65]. Collectively, this evidence makes the absence of apoE a prime candidate in the poor maintenance of mature of ORN in KO mice by limiting much needed cholesterol [65] This line of reasoning would effectively explain why the data indicate that OE in WT mice have a higher detectible number of OMP (+) cells than KO yet they may be populated initially by the same number of immature ORN and mature at the same rate.

From the information so far presented, apoE does not have any effect on the rate of maturation of the ORN in the OE. Due to the lack of an ascribed biochemical role of the protein OMP in the process of maturation, it is likely then that the lack of apoE may instead influence ORN cytoskeleton stability, which may be a precursor for increased senescence. This would explain the lower levels of OMP(+) cells in KO mice across the time course. Further studies on the quantification of immature ORN, and the rates of apoptosis, may better explain the mechanisms of adult neuronal differentiation.

4.5. ApoE and Synaptogenesis of ORN in the OB

Previous studies have shown apoE expression in the olfactory nerve and around the glomeruli in the olfactory bulb of adult mice [57]. Following OE lesioning, apoE levels were two fold higher than normal in the OB [58]. Reactive glia have been identified as the primary candidates for this surge in apoE in the OB post lesioning. Other studies have identified the elusive glial-derived synaptogenesis-promoting factor as cholesterol-laden lipoprotein [59]. Collectively, these recent studies suggest a role for apoE in the synaptogenesis of the CNS. In the present study, synaptophysin (SYN) level (a synaptic marker), was studied as expressed in the OB of WT and KO mice, following OE lesioning at various time points. Given the possible role of apoE in synapse formation, it was predicted that synaptogenesis should be slower in mice lacking apoE.

It was found that KO mice had less SYN than WT mice across the entire time course. Thus at what ever day post lesioning examined, KO mice always had lower levels of SYN than WT. This is corroborated by other studies that have found apoE KO mice to be neuropathologically normal, although showing numerous CNS defects, including reduced levels of SYN [80, 81,86]

A study involving injury to the entorhinal cortex of KO mice have demonstrated deficits in the recovery of SYN [81]. This study using the OE as the model shows a similar deficit in recovery by KO mice. Following OE lesioning, data suggest that SYN loss in WT reaches the lowest levels at 3-7 DPL and then starts a slow climb to 21 DPL, followed by an acute increase between 21 and 42 DPL. In contrast, the SYN in KO mice reaches the lowest levels at 14 DPL and slowly recovers up to 42 DPL, followed

by an acute increase between 42 and 56 DPL. In brief, the significant peak in synaptogenesis WT mice occurred between day 21 and 42 DPL while in the KO mice, it occurred between day 42 and 56 DPL and was still not complete at 56 DPL.

The data suggest that 21 DPL is a very crucial time point for normal ORN to begin forming synapses under ideal conditions. From other yet-unpublished studies from our laboratory, immunoblot techniques indicate an upregulation of apoE in the OE beginning at 14 DPL and peaking at between 21 and 42 days. This correlation can be explained by the increased demand by the ORN growth cones for cholesterol to satisfy the lipid demands of synaptogenesis. In other words, the ORN requires apoE-laden cholesterol to deliver the rate limiting cholesterol. It is known that due to the ORN inability to produce cholesterol at the growth cones or nerve terminals, ORN are therefore dependent on exogenous sources (via LDL receptor uptake) and endogenous sources (axonal transportation) [86]. Invariably, the KO mice suffer cholesterol deficits that delay both synaptogenesis and the stabilization of synapses due to the fact that the exogenous supply of cholesterol is stymied by the lack of apoE – the trafficking ligand. These suggestions are supported by the following conclusions: levels of cholesterol in the brain is critical for synaptogenesis [65]; long axons in ORN may lack the ability to supply sufficient cholesterol for rapid axonal growth [55,56]; massive synaptogenesis (as observed in regeneration) requires large amounts of cholesterol [59]; cholesterol cannot be synthesized at neuronal terminals [87]; and cholesterol may be necessary for the structural demands of synaptogenesis including membrane formation, synaptic vesicle formation, formation presynaptitic components and clustering of postsynaptic receptors [86]. I therefore propose that apoE indeed

does limit the rate of synaptogenesis in the OB of KO mice by indirectly limiting the availability of cholesterol that is essential to the process.

5. CONCLUSION

The data revealed that: (1) apoE is expressed differently in the various parts and/or cells of the OE during regeneration of OE; (2) maturation of ORN, as measured by OMP is not delayed in KO mice as compared to WT mice post lesioning though KO mice have lower numbers of mature ORN; (3) synaptogenesis between ORN dendrites and the terminals in the OB as determined by SYN, was delayed in KO mice as compared to WT mice post lesioning; (4) neurogenesis in the OE, as identified by BrdU, progresses with no significant difference between KO and WT mice post lesioning.

In conclusion, apoE has been found to be a mitigating factor in the regeneration of ORN by (1) enhancing the maintenance of differentiated ORN in the OE and (2) enabling the process of synaptogenesis to progress unimpeded by the rate-limiting need for cholesterol. A model can be described in which the ability of an injured or regenerating ORN to complete the process of repair or growth is highly dependent upon exogenous lipid or cholesterol availability. Under the stress of repair or synaptic remodeling, it is likely that the intracellular availability of these lipids may be limiting, therefore apoE being the very efficient transporter of lipoproteins, would serve in this crucial role of rapidly internalizing cholesterol. Failure to perform (isoform defect) or in its absence, the stressed ORN is physically and biochemically hindered in the tasks of plasticity. This would explain the observed delays in the rate of and synaptogenesis of KO mice.

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