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Expression of ApoE in the mouse olfactory system during development and post injury

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Expression Of ApoE In The Mouse Olfactory System During Development And Post Injury

BY

Sreenivas Nannapaneni

THESIS

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF

MASTER OF SCIENCE IN BIOLOGICAL SCIENCES

IN THE GRADUATE SCHOOL, EASTERN ILLINOIS UNIVERSITY
CHARLESTON, ILLINOIS

2006
YEAR

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Fall Semester 2006

Abstract:

Apolipoprotein E (apoE), a prominent player in transporting cholesterol and metabolizing plasma lipoproteins, has emerged as a major risk factor in causing Alzheimer's disease (AD). ApoE, extensively expressed in the primary olfactory pathway, binds to its low-density lipoprotein receptor and related receptors to play a role both in development and in control of the immune system. 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 olfactory epithelium (OE). Studies from our laboratory indicate that apoE plays a vital role in the ON regeneration and maturation. However the mechanism(s) underlying the contribution of apoE to neuronal functioning and developing AD are not clearly understood. In the present study, I examined the expression of apoE in the OE, effects of apoE on the neurogenesis and neuronal maturation in apoE wild-type (WT) and apoE deficient knock-out (KO) mice during embryonic and postnatal stages, and finally, the effects of apoE on neuronal regeneration in the OE following unilateral bulbectomy (OBX).

My results indicate that apoE is expressed in the WT mice. On the contrary, there was no apoE staining in the KO mice. My studies revealed that apoE is expressed by Sustentacular cells (Sus), Basal cells, ensheathing glial cells (EGC), endothelial cells, Bowman's gland, olfactory nerve fascicles (ONF) and the end feet of the Sus cells. This was also confirmed by double-labeling immunohistochemistry with markers to specific cell types. This suggests a role for apoE in neuronal maturation and repair.

My results on the developing mouse olfactory system suggest that apoE is expressed in the mice during their embryonic and postnatal stages. They also indicate that expression of apoE during the early stages of development in the OE proper is high compared to the adult state. Interestingly, the expression of apoE in the lamina propria (LP) was seen to be much organized as the development progressed. The results on olfactory marker protein (OMP) indicate that there are OMP^+ neurons in both WT and KO mice during their developmental stages. From the results it is clear that there is delay in neuronal maturation in the KO mice compared to the WT mice. This indicates that apoE is involved in the maturation of neurons probably through recycling lipids.

Previous studies from our laboratory indicate that apoE synthesis is upregulated in the OB following OE lesioning. In this study, unilateral bulbectomy carried out on WT mice revealed that there was apoE expression in the OE following unilateral bulbectomy. They also indicated that the apoE levels increase following bulbectomy on the ipsilateral side to the OBX and remained normal on the contralateral side. However, the thickness of the OE on the ipsilateral side did not return to its normal thickness. The intensity of apoE immunostaining in the ipsilateral OE to OBX was found to be high following bulbectomy at 7 days post OBX, and appeared to decrease at 21 days post OBX. This suggests that apoE is involved in the regeneration of olfactory receptor neurons (ORN) in the OE following unilateral OBX.

DEDICATION

I would like to thank my mentor Dr. Britto P. Nathan for his teaching, his knowledge, his support and patience, for giving me the opportunity to perform research, for always being there for me with advice and help, for believing in me and my ideas and for encouraging me to explore more with every passing day.

I would like to thank Dr. Gary Bulla and Dr. Charles Costa, my graduate committee members, for their teaching and support, for always being there for me with advice and help.

I would like to thank my fellow students: Aseem Hussain, Salina Gairhe, Ike Nwosu, Jung Sook Park, Stephen Clark, Michael Tonsor, Kristopher Yoon & Mrs. Sarita Upadhyay, for two wonderful years of research and study together, for their support, knowledge and friendship.

I would like to thank the whole Department of Biological Sciences for the two wonderful years of working and learning from all within the Department. I am very privileged to be a part of Biological Sciences Department at EIU.

I would like to thank the Graduate School and International Programs at EIU for giving me this unique opportunity for graduate study, research and for reaching new heights in my academic career in contributing to the field of Neuroscience. Specifically, I would like to thank the International Programs Staff.

I especially would like to thank my parents for their love and support, for believing in me, and my best friend Viswatej Vemulapalli, for his friendship and moral support, for helping me tremendously in these two years of my study at EIU.

ACKNOWLEDGEMENTS

I would like to thank Dr. Britto Nathan, for giving me the opportunity to work in his laboratory for the past 2 years and getting the best out of me during my stay in his laboratory.

I would like to thank Dr. Thomas Getchell and his laboratory technicians, University of Kentucky, Lexington, for helping me out with Bulbectomy procedure.

I would like to thank Dr. James Schwob, Tufts University, Boston, for generously providing antibodies.

TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

Introduction:

Human ApoE, a 34 kDa protein, is a polymorphic protein consisting of 299 amino acid residues. It is a member 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 (Herz et al., 1988; Mahley, 1988; Takahashi, 1992 and Yamagishi, 1998). 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 (Weisgraber, 1994). In humans, apoE exists in three major isoforms: apoE2, apoE3 and apoE4. They are produced by three alleles (ε2, ε3 and ε4, respectively) at a single gene locus on chromosome 19 (Herz et al., 1988). These common isoforms of apoE in humans differ by amino acids at positions 112 and 158 (Menzel et al., 1984; Hallman 1991; Davignon, 1988 and Raffai 2001). 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 single amino acid substitutions lead to dramatic differences in the biological properties of apoE, including its affinity for lipoprotein receptors. Mice have one form of apoE, which is similar to human apoE3 in its structural and functional properties, including receptor binding and lipoprotein preferences (Borghini 1995 and Weisgraber 1994).

ApoE is primarily synthesized in the liver, but is also expressed in significant amounts in the nervous system. Previous studies have shown apoE mRNA and protein increase at the site of neural regeneration (Beffert et al., 1998) and that apoE containing

lipoproteins stimulate neurite outgrowth in a variety of neuronal cultures (Bellosta et al., 1995; Holtzman et al., 1995; Ignatius et al., 1987; LeBlanc and Poduslo, 1990; Nathan et al., 1994; Nathan et al., 1995; Nathan et al., 2001; Nathan et al., 2002; Nathan et al., 2004; Snipes et al., 1986; Teter et al., 1999; White et al., 2001). We have previously shown that apoE surrounds glomeruli of the olfactory bulb. Subsequent studies found that olfactory nerve regeneration and morphological recovery of the OB was significantly delayed in mice lacking apoE/KO compared to WT mice following OE lesioning. The olfactory nerve undergoes constant regeneration and axonal growth throughout the life of the organism. One hypothesized function of apoE in the nervous system is efficient recycling of lipids from degenerating neural tissue to growth cones of regenerating neurons to support neuroplasticity. The current study was carried out to localize apoE in the adult mouse OE to complement previous studies of apoE in the OB.

Role of apoE in the Nervous System:

ApoE is the major apolipoprotein in the brain and cerebrospinal fluid (CSF) with astrocytes being the major apoE producing cells in the brain (Pitas et al., 1987). Microglia also produces apoE in the central nervous system (CNS) (Nathan et al., 2001). Neurons are not known to secrete apoE (Pitas et al., 1987). Among CSF lipoproteins, apoE is the only apolipoprotein that can interact with lipoprotein receptors. Cells within the brain express four receptors for apoE-containing lipoproteins: low density lipoprotein (LDL) receptor, LDL receptor-related protein (LRP), VLDL receptor, and glycoprotein (gp) 330. The LDL receptor and LRP receptor are expressed by neurons (Pitas et al., 1987; Boyles et al., 1985). It has been shown that some neurons express the VLDL receptor, and that ependymal cells

express the gp330 receptor (Willnow et al., 1992; Sakai et al., 1994; Kim et al.; 1996 & Kounnas et al., 1994). It has been reported that the LDL receptor and LRP receptor mediate the binding and internalization of apoE-containing lipoproteins in cultured neurons (Bellosta et al., 1995). These studies provide evidence that the apoE and apoE-containing lipoproteins are present within the brain where they can interact with neurons and that lipoprotein transport by apoE is important for normal functioning of adult neurons.

Studies on apoE in the maintenance and repair of peripheral nervous system (PNS) and CNS nerves have provided clues to its function. In a rat model, the synthesis of apoE increases 250- to 350-fold following peripheral nerve injury (Ignatius et al., 1986; Snipes et al., 1986). This enormous increase of apoE is thought to play a key role in recycling lipids from the degenerating axonal and myelin membranes for use by regenerating axons and myelin sheaths. Similarly, induction of apoE synthesis has also been observed in the central nervous system following optic nerve and spinal cord injury (Ignatius et al., 1986; Messmer et al., 1996; Snipes et al., 1986). Studies on apoE deficient mice showed fewer synapses, vacuolized and swollen dendrites, and a reduced recovery following perforant pathway lesions (Masliah et al., 1995; Masliah et al., 1996; Masliah et al., 1997). Conversely, studies from other laboratories did not observe any significant morphological abnormalities in apoE gene KO mice (Gandy et al., 1995; Anderson et al., 1998). Another study found that apoE may not be required for the maintenance of the basal forebrain cholinergic neurons, but it may play a role in their repair following brain injury (Fagan et al., 1998). The reason for the discrepancy is not clear. However,

differences in the strain, age, and sex of mice used may have contributed to the data inconsistencies.

In contrast to the morphological studies, behavioral studies have consistently shown that the apoE-KO animals exhibit spatial learning deficits (Masliah et al., 1996; Gordon et al., 1996). Infusion of recombinant apoE into the lateral ventricles of apoE KO mice reversed behavioral and morphological anomalies (Masliah et al., 1996). Other studies involving apoE KO mice have suggested that apoE may be involved in protecting the brain against acute injury (Chen et al., 1997). These results provide convincing evidence that apoE plays a critical role in neuroprotection, preservation and plasticity within the CNS.

Evidence linking apoE to Alzheimer's disease:

Increased apoE immunoreactivity is present in the brains of patients with such neurological diseases as Creutzfeldt-Jacob disease, Down's syndrome and Alzheimer's disease (AD) (Namba et al., 1991). In addition, a recent study suggests the apoE4 allele may be involved with the onset of Parkinson's disease (Zareparsi et al., 2002). AD is characterized by the presence of senile (neuritic) plaques, amyloid angiopathy, and neurofibrillary tangles (Cotran et al., 1999). The neuritic plaques are focal, spherical collections of dilated, tortuous, silver-staining neuritic processes (dystrophic neurites) surrounding a central amyloid core, often surrounded by a clear halo. The dominant component of the plaque core is amyloid beta, a peptide of approximately 40- to 43-amino acid residues derived from a larger molecule, amyloid precursor protein (APP) (Cotran et al., 1999). The neuritic components often colocalize with both APP and growth associated protein (GAP) 43, suggesting that

they may represent aberrant sprouting of neurons (Masliah et al., 1992). Neurofibrillary tangles are intracellular and contain paired helical filaments along with some straight filaments that appear to have comparable composition. These filaments are composed of hyperphosphorylated tau (Cotran et al., 1999). ApoE fragments have been shown to induce intracellular neurofibrillary tangles in cultured neurons with truncated apoE4 inducing more inclusions than truncated apoE3 (Huang et al., 2001). The deposition of tau in the brains of Alzheimer's disease patients is related to the amount of Abeta40 and the presence of apoE4 allele (Mann et al., 2002). The role of the plaques and tangles in the etiology of AD is still unclear.

There are three forms of AD: early-onset familial, late-onset familial and late-onset sporadic. Early-onset AD represents approximately 5% of patients, whereas late-onset AD accounts for a majority of AD cases. Accumulating data demonstrates a strong association of the apoE4 allele with late-onset familial AD (Corder et al., 1993; Mayeux et al., 1993; Rebeck et al., 1993; Sanders et al., 1993). The apoE4 allele is greatly over-represented in late-onset familial AD subjects (52%) versus controls (16%), and the risk of AD in individuals homozygous for apoE4 is over five times that of homozygous apoE3 individuals (Coder et al., 1993). Moreover, AD patients with apoE4 alleles usually show an earlier age of onset and a more rapid progression of the disease (Bennet et al., 1995). These findings suggest a causal link between apoE polymorphism and the development of AD; again, the mechanism behind the pathological effects of the apoE polymorphism remains unknown (Roses et al., 1994).

ApoE and CNS plasticity:

ApoE might 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 (Ignatius et al., 1986). Axonal degeneration and myelin sheath alterations were observed in the optic nerve of apoE KO mice (Masliah et al., 1995). 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 (Masliah et al., 1995, 1996 and 1997). In contrast, two other studies have not observed any obvious neuropathology in apoE KO animals (Anderson et al., 1998 and Gandy et al., 1995). Thus the importance of apoE in the CNS is controversial, and warrants further studies.

The Olfactory System:

The OE of the mouse is a pseudostratified, columnar epithelium. The OE contains three major cell types of the sense organ, the sensory neurons (about 75-80% of the epithelial cell population), the supporting cells (about 15-17% of the cells), and the basal cells (most of the remainder). The oval nuclei of the supporting cells form a single layer near the epithelial surface, and the round nuclei of the sensory cells usually make up six to eight layers in the middle half of the epithelium. Some nuclei along the basement membrane are flat; others, the globose basal cells (GBC), are round or polyhedral. Bowman's glands lie in the connective tissue below the epithelial basement membrane; each gland acinus gives rise to a duct that penetrates through the epithelium and opens on to the surface. The unique feature of the adult

olfactory sensory neurons is the fact that they are continuously being replaced. The GBCs undergo mitosis to form new sensory neurons which move apically in the OE and then gives rise to a single basally directed axon and an apical dendrite. The axon extends through the basement membrane and projects to the OB. Final maturation occurs as the axon forms synapse in the OB and the dendrite reaches the epithelial surface and forms cilia from its knob like termination.

The olfactory bulb develops from the telencephalon, and is, therefore, a part of the cerebral hemispheres. It is the first CNS relay for olfactory input. The bulb is organized in concentric laminae each with characteristic cell types and synaptic connectivity. The most peripheral layer is the olfactory nerve layer. It is composed of the axons of the ORN, sheathing glia and microglia. The olfactory receptor axons terminate in the glomerular layer, synapsing on the dendrites of mitral cells in structures termed glomeruli. Internal to the glomerular layer is the external plexiform layer, which is a cell poor zone containing the dendrites of mitral cells and other interneuron's. The cell bodies of the mitral cells, the principal projection neurons of the olfactory bulb, are located in a distinct monolayer between the external plexiform layer and granular cell layer. The granular cell layer is principally composed of small interneurons, called granule cells, whose dendrites ramify in the external plexiform layer. Apart from the granule cells, the bulb also contains two other interneurons, tufted cells, in the external plexiform layer, and periglomerular cells, surrounding the glomeruli.

Goals and Hypothesis:

1. To examine the distribution of apoE in the mouse olfactory epithelium. I hypothesized that apoE would be expressed in the mouse OE and LP.
2. To examine the expression of apoE in the developing mouse olfactory system. I hypothesized that apoE would be expressed during the developmental stages of the mouse olfactory system.
3. To examine the deficiency of apoE on neuronal maturation. I hypothesized that apoE deficient KO mice would show delay in neuronal maturation.
4. To examine the effects of apoE in the olfactory epithelium following injury. I hypothesized that apoE levels in the OE would increase following unilateral bulbectomy.

Chapter1: Distribution of ApoE in the Mouse Olfactory Epithelium

Materials & Methods

Animals:

Breeding pairs of WT C57BL/6 strain and homozygous apoE KO mice were purchased from the Jackson Laboratories, Bar Harbor, ME. ApoE genotype of the litters were verified by PCR and confirmed by immunoblotting. Male 4 months old mice were used in this study.

Tissue preparation:

For fluorescence immunohistochemistry, mice were anesthetized with sodium pentobarbital (80 mg/kg) (Cat # 466-6161, Henry Schein, Indianapolis, IN) transcardially perfused with cold saline (0.9% NaCl) and then with 4% paraformaldehyde (Cat. # 441244-1KG, Sigma Aldrich, St. Louis, MO) in 0.1M PBS. The turbinates were fixed in 4% paraformaldehyde for an hour and then treated with Cal-EX Decalcifying Solution (Cat. # CS510-D, Fisher Scientific,) for an hour. Olfactory turbinates were removed and cryoprotected overnight in 30% sucrose (Cat. # S-9378, Sigma Aldrich, St. Louis, MO) in 0.1 M PBS. After cryoprotection, the turbinates were frozen with dry ice using Tissue freezing medium (Cat. # 15-183-13, Fisher Scientific) and sections were cut on a cryostat (Jung Frigocut 2000E) at 18 μm, and air dried for 2 h at room temperature.

Immunohistochemical Analysis:

OE sections on slides (Cat. # 12-544-15, Fisher Scientific, St. Louis, MO) were rinsed in 0.1 M PBS, and permeabilized with 0.2 % Triton X-100 (Cat # BP 151-500, Fischer Scientific, St. Louis, MO) in PBS for 30 minutes at room

temperature. The slides were rinsed once with PBS and treated with 70, 95, 100, 95, and 70% ethanol for two minutes each (Jang and Schwob 03). Non specific immunoreactivity was attenuated by incubation in 2.25% gelatin (Cat. # G-1890-500, Sigma, St. Louis, MO) in 0.1M PBS for 1 h, followed by overnight incubation at 4^0 C with primary antisera solution (see Table 1 for source and concentration used). The primary antibody was mixed with 4% normal donkey serum (Cat. # 017-000-121, Jackson Immunoresearch, West Groove, PA) in 0.1 M PBS. The sections were washed three times in PBS, and incubated for 1 hour at room temperature with secondary antibody solution as listed in Table 1. The sections were washed three times in PBS, mounted in Vectashield (cat# H-1000, Vector labs, Burlingame, CA). Double labeling immunohistochemistry was performed similar to single staining, except using cocktails of primary and secondary antisera at concentration indicated in Table 1. For apoE staining, KO mice were processed in parallel with WT mice. Specificity was determined by incubation with normal serum in place of the primary antisera which resulted in no staining.

Table 1: List of primary and secondary antibodies used in this study

Antibodies	Host	Source	Dilution
ApoE	Goat	Calbiochem, San Diego, CA (Cat. # 178479)	1:500
Sus-4	Mouse	Gift of Dr. Schwob	1:50
GBC-2	Mouse	Gift of Dr. Schwob	1:50
FITC-Anti goat	Donkey	Jackson Immunoresearch, West Groove, PA. (Cat. # 705-095-147)	1:4000
TRITC-Anti mouse	Donkey	Jackson Immunoresearch, West Groove, PA. (Cat. # 705-095-147)	1:1000

Microscopy and imaging

Immunohistochemistry was visualized with appropriate fluorescence excitation filters for fluorescent microscopy (Olympus BX-50). Tiff format images of the nasal septum of the turbinates were taken with a Pixera digital camera for microscopy at identical manual exposure settings for all experiments within each study.

Results:

Immunohistochemistry supported our study by localizing apoE in the OE and its underlying lamina propria. The OE is a pseudostratified epithelium, with three main layers (Figure 1A). The layer closest to the apical surface of the OE consists of the nuclei of the Sus cells. These cells have the bulk of their cytoplasm, including the nucleus, in a single row at the surface of the OE. Sus cells are capped by microvilli on the apical side.

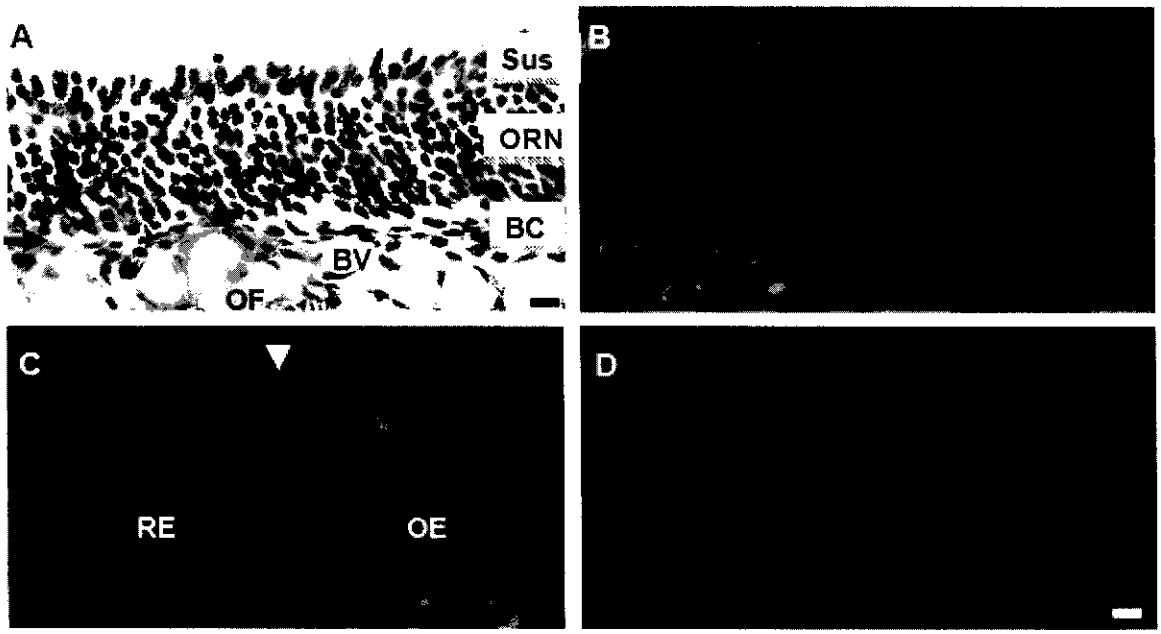

Fig1: ApoE expression in adult mouse olfactory epithelium. (A) Cresyl-violet stained section of olfactory epithelium and the underlying lamina propria. Sus, sustentacular cells, ORN, olfactory receptor neuron, BC, basal cell zone, BV, blood vessel, OF olfactory fascicle. Arrow indicates basal lamina. (B) ApoE immunoreactivity in WT mice was intense in the perikarya and end feet of sustentacular cells. (C) Low level of apoE expression in the respiratory epithelium of WT mice. ApoE immunoreactivity in the respiratory epithelium was highly reduced. The transition zone of the olfactory (OE) and respiratory (RE) is marked by an arrowhead. (D) Absence of apoE staining in the olfactory epithelium of KO mice. Scale bars = 10 μm in A and 15 μm in D (also for B and C).

Their cytoplasmic processes extend between ORN to reach the basal cell layer. There they expand to form knob like processes called endfeet. Just below the Sus cell nuclei are the ORN nuclei which occupy most of the thickness of the OE. The bipolar ORN extends a short basal dendrite that expands into a knoblike structure at the apical surface from which olfactory cilia extend into the mucus. In addition, the ORN extends a long basal axon that exits the OE and projects to the OB. Newly generated ORN are produced by basal cells found at the base of the OE. The lamina propria underlies the OE and is composed of connective tissue, unmyelinated axons of the ORN surrounded by processes

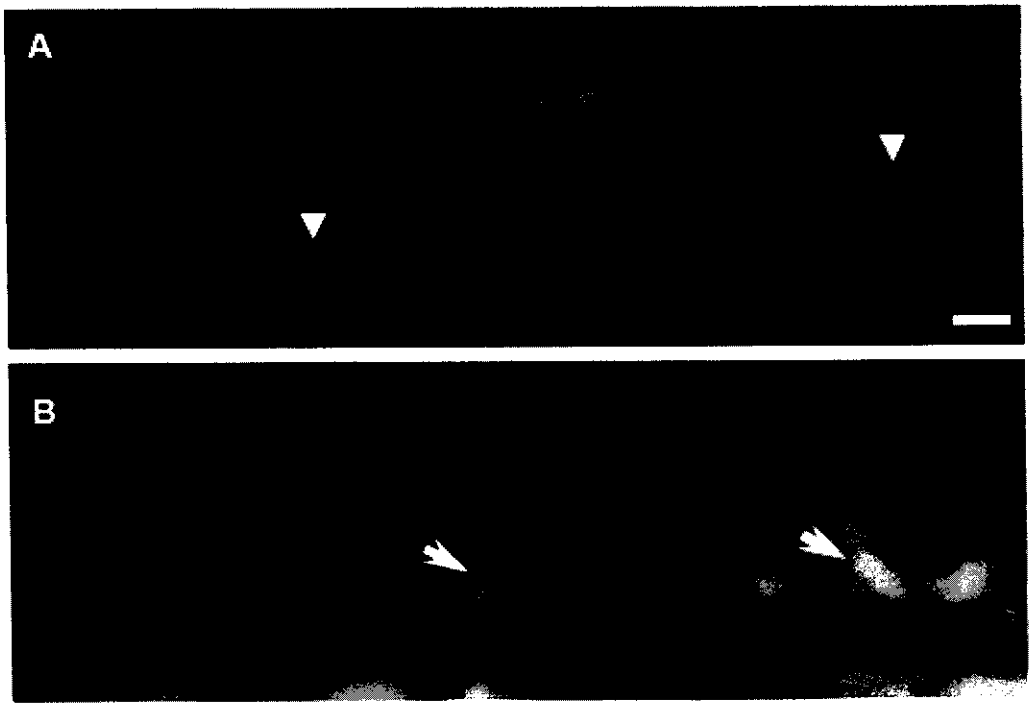

Fig 2: Perikarya and end feet of Sus cells express apoE. (A) A Higher magnification photographs show apoE immunoreactivity in the cytoplasm, but not the nucleus of Sus cells (arrow heads). (B) Endfeet of Sus cells (arrows) surrounding GBC intensely stain for apoE. Scale bars = 10 μm in A (also for B).

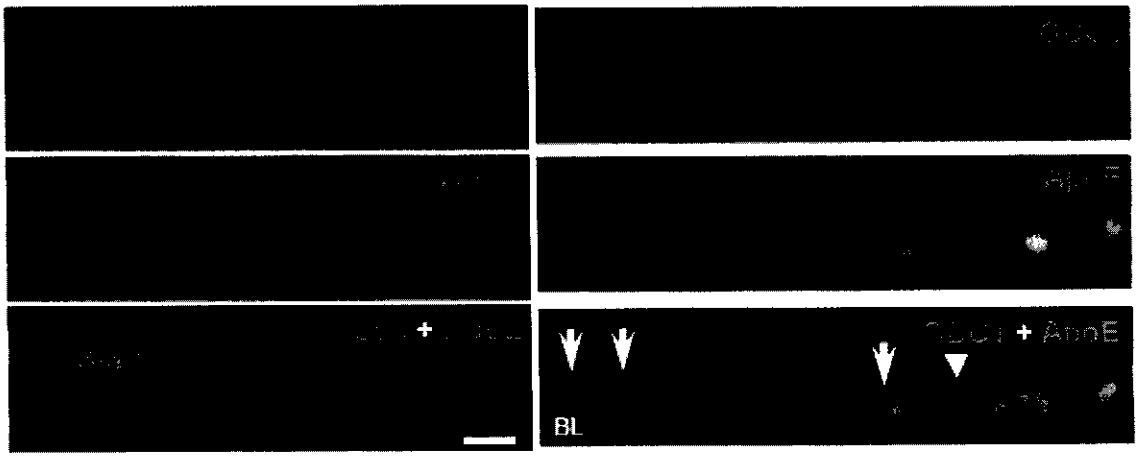

Fig 3: Double-labeling immunofluorescence of apoE and Sus-4 or GBC-1 in adult mouse OE. (A) Sus-4 immunoreactivity colocalized with apoE in the perikarya and villi of Sus cells. (B) ApoE positive processes were found mostly abutting on the surface of the GBC (arrows), although a few of these processes (arrow head) were found between the GBC to contact the basal lamina (BL). Scale bar = 10 μm.

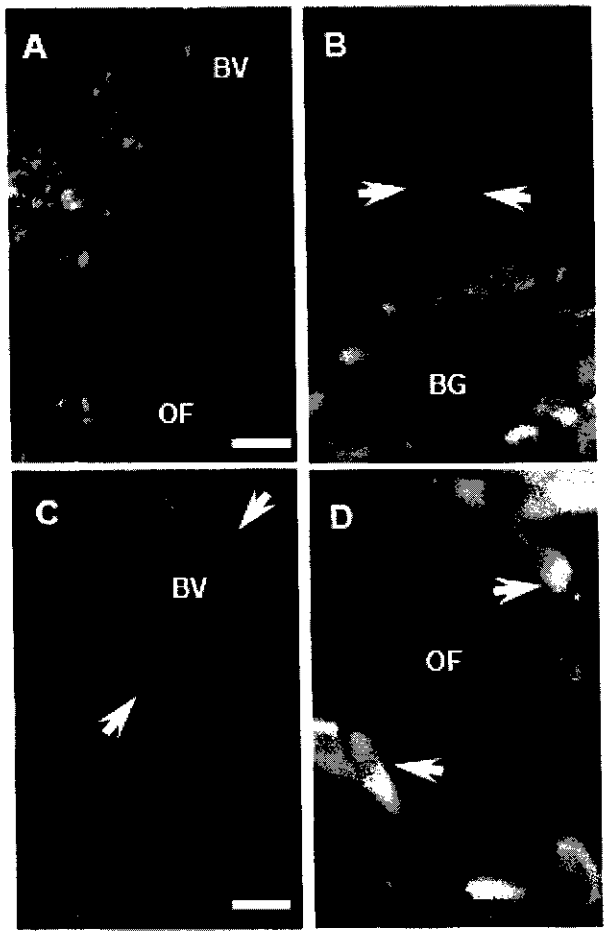

Fig 4: ApoE expression in adult mouse lamina propria. (A) A variety of cell types in the lamina propria expressed high levels of apoE. These cells were located around the blood vessels (BV) and olfactory fascicles (OF). (B) Cells forming the Bowman's gland also expressed low levels of apoE, and were associated with a diffuse staining of apoE extending from the gland to the OE surface (arrows). (C) A higher magnification view of the blood vessels illustrates apoE expression in the endothelial cells (arrows) forming the vessels. (D) Olfactory fascicles expressed low level of apoE and heavily stained cells, probably ensheathing glia (arrows), surrounded the nerve fascicles. Scale bars = 15 ⌠m in A (also for B) and 30 μm in C (also for D).

of the ensheathing glial cells, Bowman's gland with ducts that extend upwards to the epithelial surface, blood vessels, fibroblasts, and immune cells. Our results revealed that apoE immunoreactivity was present in most of the layers of OE and the lamina propria. Intense apoE immunoreactivity was observed in the olfactory epithelial

surface and in cytoplasmic processes of the Sus cells extending to the epithelial surface (Figure 1B). Immunoreactive processes also traversed between the ORN. Intense apoE immunoreactivity was clearly present in the basal cell region of the OE. At high magnification it was clear that the immunoreactivity was predominantly localized in the cytoplasm of the Sus cells (Figure 2A), and in knob like structures which appear to be Sus cells endfeet surrounding basal cells (Figure 2B). ApoE staining in the respiratory epithelium was considerably lower than that in the OE (Figure 1C) and the change was striking in contrast to the OE. A complete absence of apoE-like immunoreactivity in the KO mice confirmed that we were visualizing apoE immunoreactivity (Figure 1D). Double-labeling immunohistochemical studies were performed to concurrently examine apoE expression with SUS-4 and GBC-1. The results revealed that apoE immunoreactivity consistently colocalized with SUS-4 in the perikarya of Sus cells (Figure 3A). Double labeling studies with apoE and GBC-1 also revealed similar colocalization of apoE and GBC, except that some apoE-only positive processes were not in spatial register with the GBC (Figure 3B). Our impression of the spatial mismatch is that the apoE was in endfeet surrounding the Sus cells. These endfeet abutted the GBCs. However, in the absence of electron microscopy we cannot exclude the possibility that some immunoreactive endfeet-like profiles represent GBC. In the lamina propria, the endothelial cells of blood vessels were intensely stained for apoE (Figure 4). Cells forming Bowman's gland also stained for apoE. The apoE staining pattern in the nerve fascicles was composed of intensely immunostained cells surrounding, uniformly distributed less intensely

immunostained apoE throughout the core of the nerve bundles. The distribution and morphology of these intensely stained cells suggested they were ensheathing glia.

Discussion

The results from this study demonstrate that the lipid transporting protein apoE is expressed throughout the OE and its underlying lamina propria. In the OE, Sus cells expressed significant levels of apoE in their perikarya and villar processes, located in the surface of OE, and in their end feet, which were in close proximity to basal cells lying above the basal lamina. ApoE was only present at low levels surrounding the ORN. In the lamina propria, apoE was expressed at high levels in the endothelial cells of the blood vessels. Furthermore, ensheathing cells were intensely stained whereas the olfactory nerve fascicles showed faint staining of apoE. Occasionally we observed apoE expressing macrophage-like cells surrounding the olfactory fascicles. In addition, cells forming the Bowman's gland were also stained for apoE. Our study did not completely replicate a previous study of apoE expression in the OE of elderly humans (Yamagishi et al., 1998). They reported intense apoE staining in the blood vessels and diffuse staining in the olfactory nerve fascicles, which is consistent with our data. However, they did not report any detectable level of Sus staining. The reason for these discrepant findings could have several explanations. The absence of Sus staining might be due to age-related structural and physiological alterations within Sus cells (Loo, et al., 1996). Sus cells in aged rats are swollen and contain abundant eosinophilic material. Additionally, a linear decline in CSF apoE levels has been shown during aging in humans and apoE expression in the OE may similarly decrease as animals age (Fukuyama et al., 2000). Alternatively, the

absence of apoE in human Sus cells may represent post-mortem changes. It is possible that maintenance of Sus apoE is metabolically maintained. With death there is a general release of apoE to the neuropil. The dichotomy we find between olfactory and respiratory epithelia suggests a functional association between apoE and ORN. Both Sus cells and ensheathing glia produce apoE. Sus and ensheathing glia derived apoE could be critically involved in recycling of membrane components liberated from the senescing ORN population and recycling it to support basal cell division and differentiation and axonal elongation of the newly differentiated ORN. We propose that Sus derived apoE facilitates the various functions attributed to Sus and ensheathing glia (Barnett, 2004; Dahl et al., 1982; Getchell et al., 1984; Ramon-Cueto and Avila, 1998). Disposal of cellular debris in a variety of peripheral tissue is primarily carried out by macrophages. And a previous study has shown that apoE promotes clearance of cellular debris at the site of neural injury (Fagan et al., 1998). ApoE appears to be vital to phagocytotic function of macrophages (Grainger et al., 2004). Thus one potential role of apoE produced by Sus in a normal/uninjured OE is to support the continuous disposal and recycling of cellular debris as a result of the constant turnover of the receptor neurons in the OE. A hypothesis of apoE function in lipid recycling in the OE is also compatible with published studies that examined the impact of apoE deficiency on the olfactory system in apoE KO mice. We reported a striking qualitative and quantitative increase of apoE in the olfactory nerve and bulb concurrent with olfactory nerve degeneration (Nathan et al., 2001). In addition, we found that olfactory nerve regeneration and recovery in the OB following OE lesion were significantly delayed in mice lacking apoE (Nathan et al., 2005). Reappearance

of OMP in the OB following reversible lesion of OE in normal mice occurred between 7 and 21 days post lesion, reaching statistically normal levels by 42 days. In contrast, in KO mice, OMP remained indistinguishable from baseline for 42 days, but then recovered by 56 days. Although the exact mechanism for this delay is open to question, it is tempting to propose that the absence of apoE production by the Sus cells and ensheathing glia in the OE impairs re-innervation of the OB by disruption of the lipid recycling process. The absence of apoE in Sus cells may disrupt regenerative processes of basal cells. ApoE secreted by Sus cell endfeet, found in close proximity to basal cells of OE, may support efficient transport of lipids to facilitate basal cell division and differentiation to mature neurons. Absence of apoE in ensheathing glia may slow the growth of newly generated axons. This hypothesis was supported by compartmental culture paradigms which showed that apoE facilitated neurite growth, but only when added at the site of the growth cone (Hayashi et al., 2004). Hence, delayed regeneration in apoE KO mice could represent both slower regeneration of ORN and slower axonal growth. Our previous data tend to support that possibility. It is important to note that olfactory function shows early severe deficits in various chronic neurological disorders where apoE genotype is a major risk factor (Ansari and Johnson, 1975; Hawkes, 2006; Talamo et al., 1989). We propose that rather than a disease-specific effect of apoE on the course of the disease, dysfunctional apoE would delay repair and underlie abnormalities of olfactory function found in chronic neurological diseases. The olfactory system, with its unique ability to continuously regenerate, may be an ideal structure for determining the role of apoE in nervous system during normal and pathological condition.

Chapter 2: Distribution of ApoE in the Developing Mouse Olfactory System

Materials & Methods

Animals:

Breeding pairs of WT C57BL/6 strain and homozygous apoE KO mice were purchased from the Jackson Laboratories, Bar Harbor, ME. ApoE genotype of the litters were verified by PCR and confirmed by immunoblotting. Mouse Embryos were obtained from timed pregnant littermates. The male and the female were housed together for 12 hours and the day the male was separated from the female was considered the 0.5 day. The tissues were collected at embryonic 17 and 19 days and postnatal 2, 7, 14, & 21 days respectively. Males 3-4 months old mice were used in this study.

Tissue preparation:

For fluorescence immunohistochemistry, mice were anesthetized with sodium pentobarbital (80 mg/kg) (Cat # 466-6161, Henry Schein, Indianapolis, IN) transcardially perfused with cold saline (0.9% NaCl) and then with 4% paraformaldehyde (Cat. # 441244-1KG, Sigma Aldrich, St. Louis, MO) in 0.1M PBS. The heads of the embryo and postnatal mice were fixed in 4% paraformaldehyde for an hour and cryoprotected overnight in 30% sucrose (Cat. # S-9378, Sigma Aldrich, St. Louis, MO) in 0.1 M PBS. After cryoprotection, the whole heads of the embryo and postnatal mice were frozen with dry ice using Tissue freezing medium (Cat. # 15-183-13, Fisher Scientific) and sections were

cut on a cryostat (Jung Frigocut 2000E) at 18 μm, and air dried for 2 h at room temperature.

Immunohistochemical Analysis:

For both ApoE & OMP Immunoreactivity, the sections on slides (Cat. # 12-544-15, Fisher Scientific, St. Louis, MO) were rinsed in 0.1 M PBS, and permeabilized with 0.2 % Triton X-100 (Cat # BP 151-500, Fischer Scientific, St. Louis, MO) in PBS for 30 minutes at room temperature. The slides were rinsed once with PBS and treated with 70, 95, 100, 95, and 70% ethanol for two minutes each (Jang and Schwob 03). Non specific immunoreactivity was attenuated by incubation in 2.25% gelatin (Cat. # G-1890-500, Sigma, St. Louis, MO) in 0.1 M PBS for 1 h, followed by overnight incubation at 4^0 C with primary antisera solution (see Table 2 for source and concentration used). The primary antibody was mixed with 4% normal donkey serum (Cat. # 017-000-121, Jackson Immunoresearch, West Groove, PA) in 0.1 M PBS. In case of OMP Immunoreactivity the primary antisera incubation was carried out at room temperature overnight. The sections were washed three times in PBS, and incubated for 1 hour in dark at room temperature with secondary antibody solution as listed in Table 2. The sections were washed three times in PBS, mounted in Vectashield (cat# H-1000, Vector labs, Burlingame, CA).

Table 2: List of primary and secondary antibodies used in this study

Antibodies	Host	Source	Dilution
ApoE	Goat	Calbiochem, San Diego, CA (Cat. # 178479)	1:500
OMP	Goat	Gift of Dr. Margolis	1:500
FITC-Anti goat	Donkey	Jackson Immunoresearch, West Groove, PA. (Cat. # 705-095-147)	1:4000 (ApoE) 1:200 (OMP)

Microscopy and imaging

Immunohistochemistry was visualized with appropriate fluorescence excitation filters for fluorescent microscopy (Olympus BX-50). Tiff format images of the nasal septum of the turbinates were taken with a Pixera digital camera for microscopy at identical manual exposure settings for all experiments within each study.

Results:

ApoE Expression in the OE: ApoE expression in the embryonic and postnatal mouse olfactory system was analyzed by immunohistochemistry. Neurogenesis begins at E12 in the OE, and continues throughout the adult life, during which the epithelium is organized into proliferative and neuronal layers (Farbman, 1992). ApoE immunoreactivity was evident in the developing mouse olfactory system by E17 days (Fig 5). At E17 days, apoE immunostaining was concentrated throughout the perinuclear regions of the cells in the OE (Fig 6). At this time, the Sus cells, ORNs located at apical and basal levels of the OE expressed apoE. At this stage, apoE immunostaining is seen to follow a

"banded" pattern (not shown) within the olfactory neuroepithelium, as functional columns of columnar organization are set up among sus cells or supporting cells. Also, apoE positive ONFs are seen coursing back to form primary synapses within the newly formed OB. During the embryonic stages apoE immunostaining can be observed throughout the olfactory neuroepithelium. ApoE immunostaining decreased in neurons as they matured and migrated apically in the OE. A similar pattern of distribution was seen postnatally.

ApoE immunoreactivity was absent from mature and immature neurons, while Sus cells and basal cells expressed apoE immunostaining. However, apoE immunostaining was observed within the extracellular spaces of the ORNs in the olfactory neuroepithelium. Most of the apoE immunoreactivity was observed surrounding the blood vessels and the ONFs located in the LP (Fig 6), by P7 days and beyond, apoE immunostaining was observed around the neuronal cell bodies in the OE, and most immunostaining appeared to be associated with the endothelial cells (surrounding the blood vessels) and the ensheathing glial cells (surrounding the ONFs) in the LP. At all ages, Sus cells within the OE represented the vast majority of apoE expression within the epithelium proper. ApoE expression in the OE proper declines rapidly after birth (P2), indicating that this pattern of apoE immunoreactivity may subserve an important function in olfactory neuron development. To investigate further whether apoE influences synapse formation in the OB, we examined apoE expression in the developing OB during the embryonic and postnatal ages.

Fig 5: ApoE expression in the developing mouse olfactory system. The above low power (10X) figure shows apoE expression in the embryonic (E17 & E19) and postnatal (P2, P7 and P21) days mice. The OB is found attached to the OE in the developmental stages. Scale bar = 10 μm.

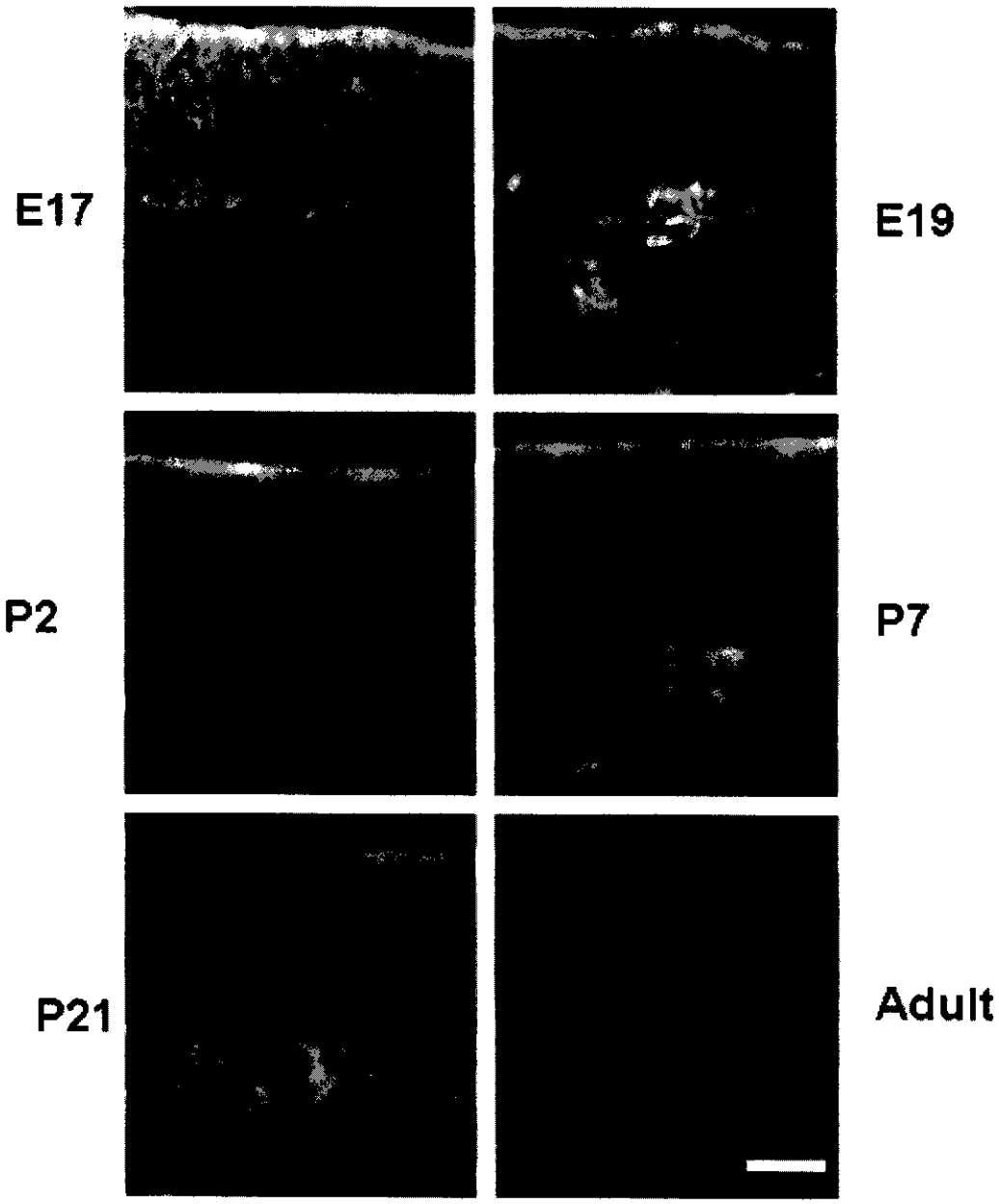

Fig 6: ApoE expression in the developing mouse OE. The above figure (40X) shows apoE immunostaining in the developing OE at E17, E19, P2, P7, P21 and 3-4 month old adult mice. Scale bar = 10 μm.

ApoE Expression in the OB: Since apoE is clearly expressed in the OE in a pattern that changes with development, I examined the expression of apoE in the OB, the target of ORNs in the OE. ApoE immunostaining in the OB was observed at embryonic ages (E17 & E19) and postnatal ages (P2, P7, P14 & P21) (Fig 7). ORN axons project through the basal lamina to the OB to form the olfactory nerve layer. These axons synapse with mitral and tufted cells in glomerular structures. Different layers of different cell types contribute to the lamellar structure of the OB (Mori et al., 1999). Most of the significant bulb development and ORN synaptogensis occurs postnatally (Davis & Reed, 1996; Bailey et al., 1999).

At E17, apoE was located in the olfactory bulb outer nerve layer; this localization is consistent with the detection of apoE in the OE. Expression of apoE in the OB was more organized during the later stages of the development. By E19 days, apoE staining was visualized in the developing mitral and glomerular cell layers (Fig 7). However, there was no distinct immunostaining observed at this time. As the development continued, there appeared a distinct immunostaining in different areas of the OB. By P7 days, apoE immunostaining extended to the mitral/tufted cell layer and to the granule cell layer. The glomeruli at this stage were very well developed and expressed apoE immunostaining. The intensity of apoE immunoreactivity increased between P2 and P7 days (Fig 7). ApoE expression was also observed in the ONL. However, there was less intensity of apoE immunostaining observed at P21, glomeruli, compared to P7 in the OB (Fig 7).

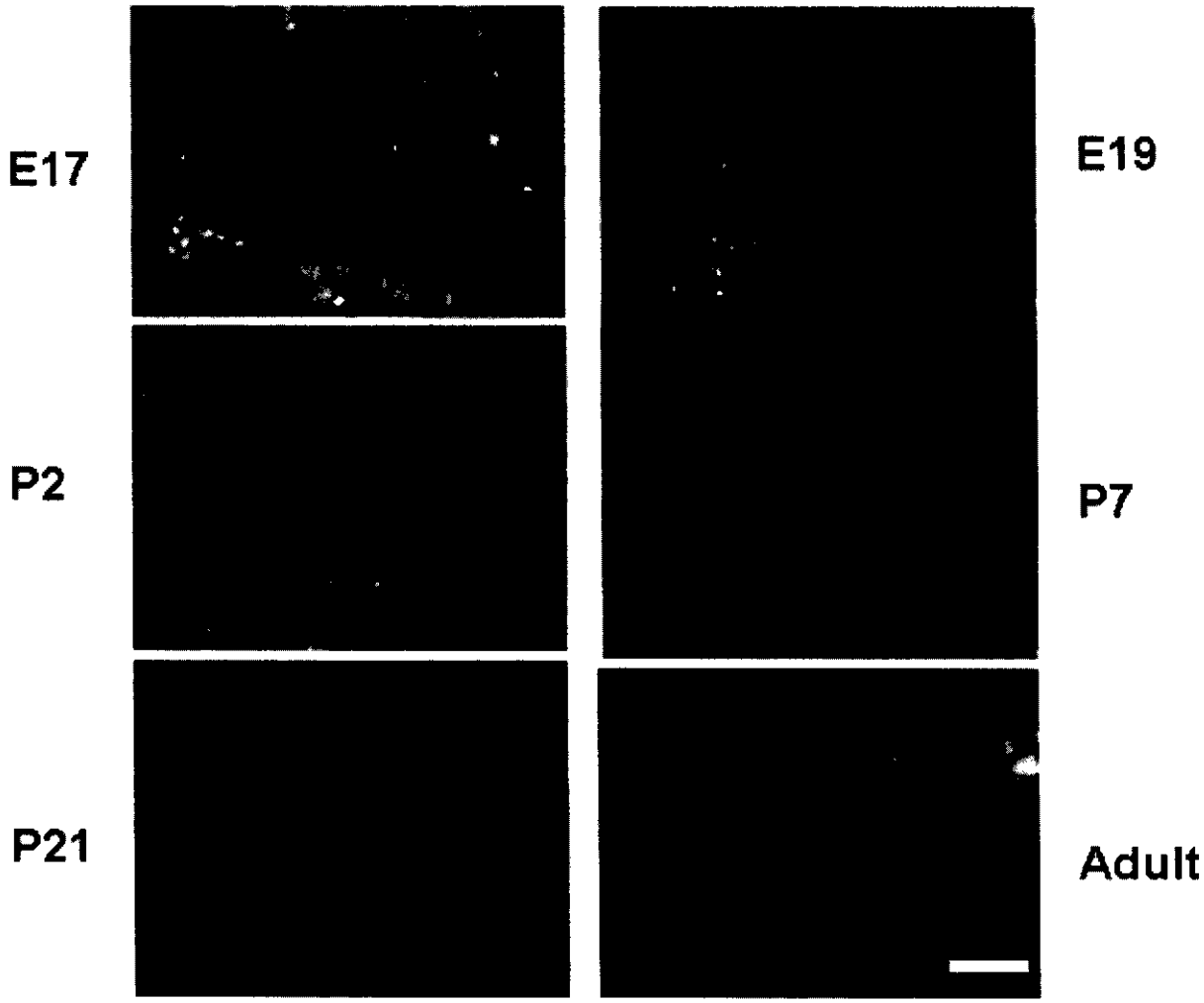

Fig 7: ApoE expression in the developing mouse OB. The above figure (40X) shows apoE immunostaining in the developing mouse OB at E17, E19, P2, P7, P21 and adult. Scale bar = 10 μm.

Previous results have shown that apoE expression in the adult mouse OB is confined to the ONL, around the glomeruli and the glial cells (Struble et al, 1999). This leads to the prediction that apoE levels in the OB decrease overtime

as the glomeruli reach their "mature" adult stage, indicating a role in synaptogenesis.

OMP expression in the OE: To verify that apoE expression correlates with neuronal maturation, we examined the expression of OMP during embryonic and postnatal stages in both apoE WT and KO littermates. The olfactory sensory neurons are morphologically identified at E10, become numerous between E10 and E13, and are known to express OMP on E14 (Graziadei et al., 1980). Neurons containing OMP, localized in the apical portion of the OE, were observed at E17 in both WT & KO littermates. The numbers of OMP^+ neurons were found to increase in number at E19 in WT littermates than their counterparts, i.e. KO littermates, and are scattered over most of the epithelium (Fig 8). Neurons expressing OMP immunostaining increased in numbers postnatally in both WT and KO littermates

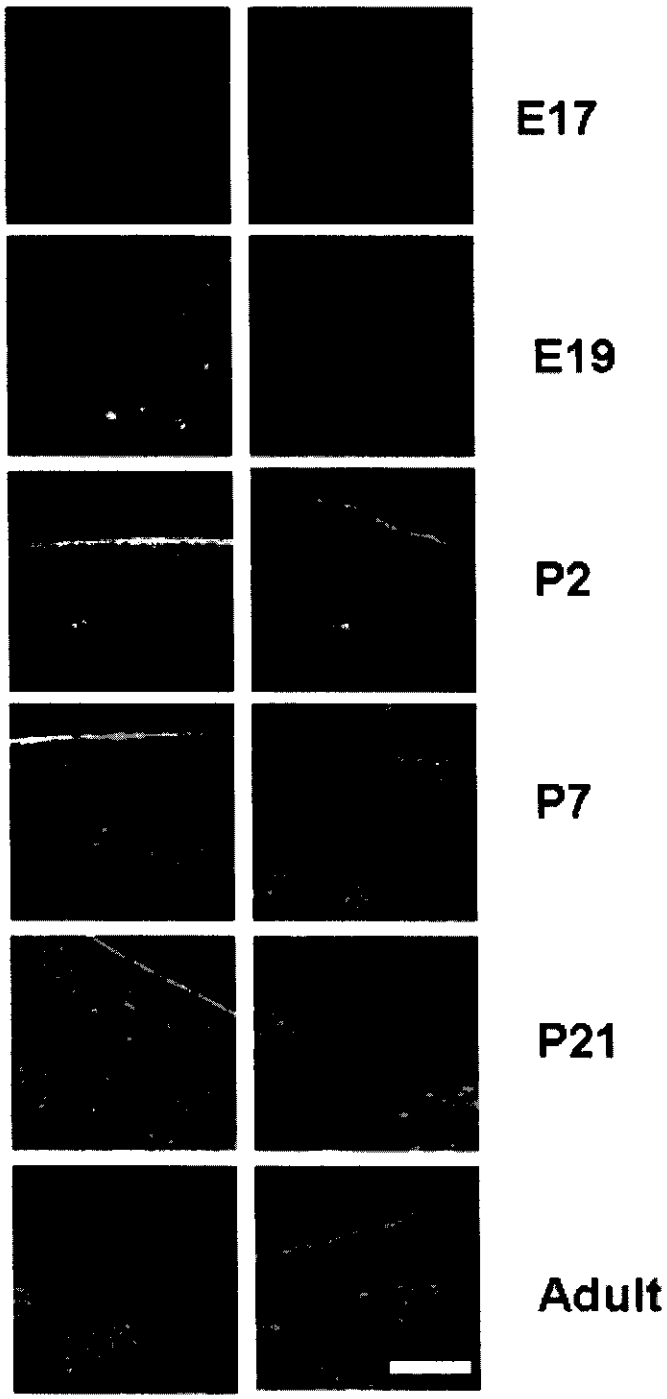

Fig 8: OMP immunostaining in the OE of WT and KO mice during their respective developmental stages. Scale bar = 10 μm.

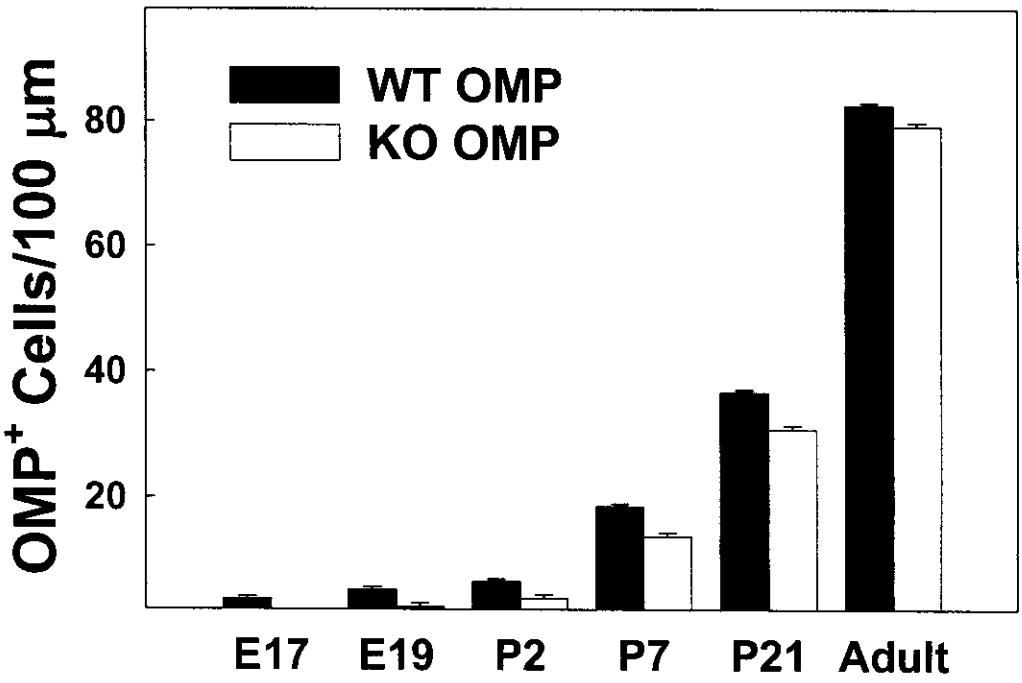

Fig 9: Graph showing the number of OMP^+ cells per 100µm in both WT and KO mice. OMP positive cells per 100 µm of linear length of OE. P<0.001 WT vs KO. P = 0.089 for interaction of days with WT/KO

compared to their prenatal stages. However, the number of neurons expressing OMP immunostaining in WT littermates was much higher than the KO littermates at this stage (Fig 9). The OMP^+ neurons appear as a band close to the epithelial surface and extend over the entire sensory area. The staining is very intense in the cell bodies, dendrites and ONFs at P7. The number of OMP^+ neurons continues to increase exponentially as the development continues. However, a conspicuous portion of the basal neuroepithelium is not stained in both WT and KO littermates, and this area being greater in KO littermates than the WT littermates. The numbers of stained neurons increase dramatically by

P21 days in WT mice and the stained elements extend from the layer of supporting cell nuclei to a narrow band of basal cells (Fig 8). The data are shown in graphical representation form in Fig 10.

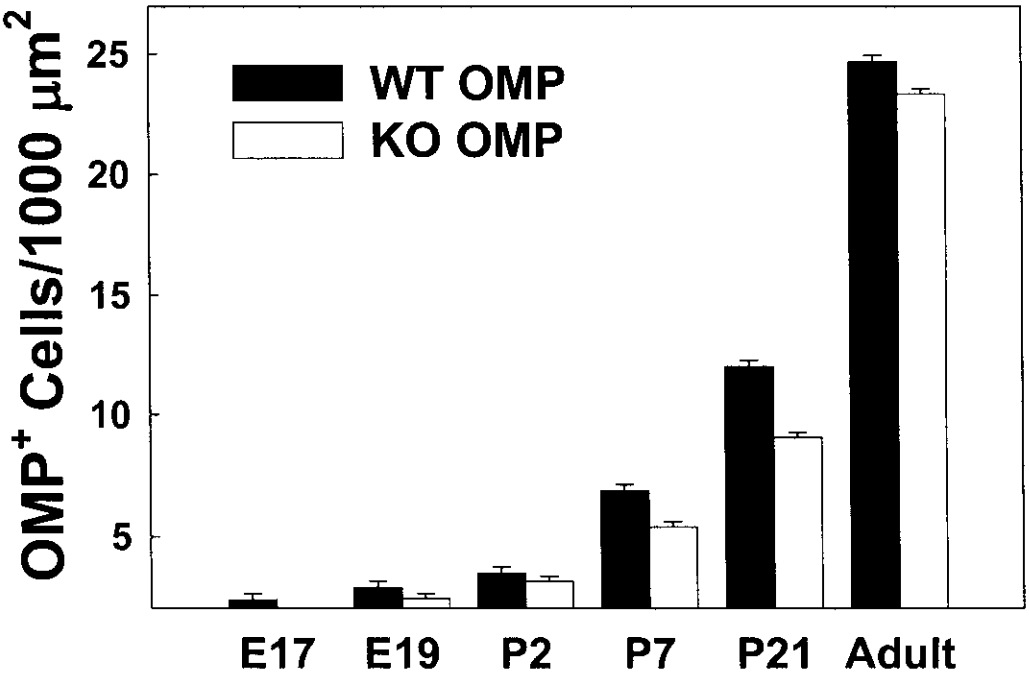

Fig 10: Graph showing the number of OMP^+ cells per 1000 μm^2 in WT and KO mice. OMP positive cells per 1000 square µm of OE. P<0.001 WT vs KO. P = 0.001 for interaction of days with WT/KO

At this stage of the olfactory neuroepithelium most of the neurons are stained while the supporting cells and the basal cells are not stained. The layer of unstained basal cells varies in thickness from one zone to the other. In the adult, most of the neurons express OMP immunostaining in both WT and KO littermates, other than the basal cell region where there are immature neurons

(Gap43^{+}). The number of neurons expressing OMP immunostaining in WT and KO littermates at this stage is almost equal.

OMP expression in the OB: OMP immunostaining was observed in a subpopulation of ORN perikarya and axons at E17 days in both WT and KO littermates. There was very faint OMP immunostaining observed at this stage of the bulb, indicating that most of the ORNs are immature or there are very few mature ORNs. ORN axons are confined to the olfactory nerve layer (ONL), except for a small population that extends into the superficial layers of the bulb proper. At E19, ORN axons form a dense band in the deepest part of the ONL (Fig 9). A small number of axons penetrate past the dense band into the external plexiform and mitral cell layer. At E19, axonal glomeruli are more prominent and form an orderly row in the rostral bulb. At this stage all distinct axonal glomeruli contain astrocytic processes, but there are still occasional glial glomeruli that lack axonal glomeruli in the caudal protoglomerular layer (Bailey et al., 1999).

At P2, in the OB, the terminal portions of the ORNs begin to arrange in discrete, globose structures resembling glomeruli. Through a well defined external plexiform layer, stained axons go from the glomeruli to the most superficial mitral cells. At P7, there is a significant rostral to caudal increase in the proportion of the glomeruli. The OB contains a well developed glomerular layer. However, the intensity of OMP immunostaining is greater in the WT littermates compared to the KO littermates. At P14 (not shown), the glomeruli are "adult like" in both size and distribution.

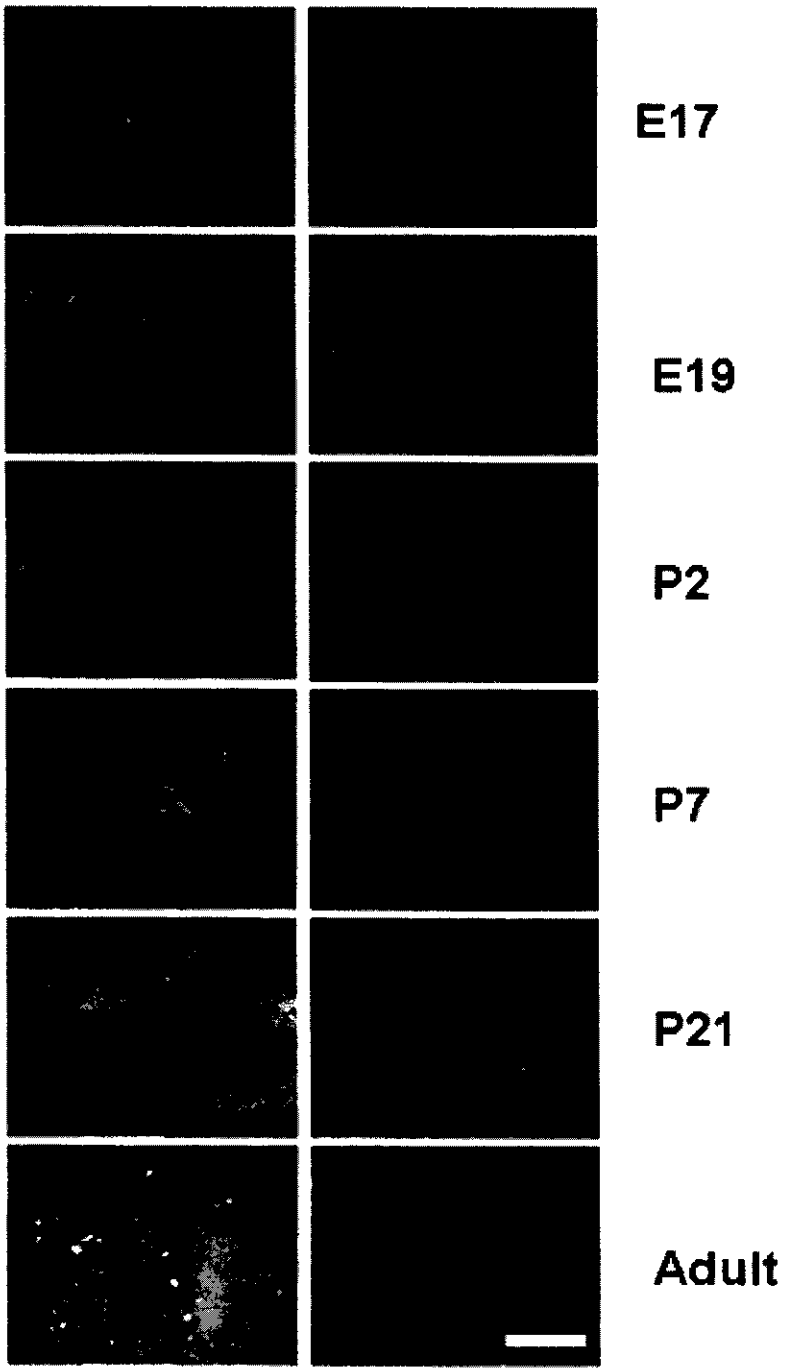

Fig 11: OMP expression in the OB of developing WT and KO mice. The above figure shows the OB at high power (40X). Scale bar = 10 μm.

There were still ORN axons coursing into the superficial external plexiform layer (Bailey et al., 1999). The OB has a layer of glomeruli whose pattern of immunostaining remained unchanged at the later stages. At P21, OMP immunostaining is restricted to the sensory axons and to their terminal portions in the glomeruli (Fig 9). The intensity of the stain was much higher when compared to the earlier stages of the development.

Discussion:

ApoE expression occurred during neuronal maturation prior to synaptogenesis and the maturation of ORN to express OMP. During early embryogenesis, when neurogenesis is quite robust and the OE lacks the cell layer organization present in the adult (Cuschieri & Bannister, 1975). ApoE immunostaining was detected at embryonic stages throughout the OE in a disorganized fashion. One possible explanation for the expression of apoE during early stages is that apoE might play a role in the maturation and extension of olfactory axons towards the bulb. Previous studies show that apoE plays a vital role in terminal axonal growth and differentiation (Nathan et al., 2001 and Struble, G. R., 1999). Postnatally, apoE expression decreased in the OE proper considerably in the maturing OE. Over postnatal ages, apoE protein expression remained associated with Sus cells and the basal cells. While apoE expression levels in the OE were steady through embryonic stages, they declined significantly during the postnatal stages. This reduction in apoE expression correlates well with the establishment of the mature adult OE. ApoE

expression was dynamic during ORN development, indicating a role for apoE in neuronal maturation and in neurogenesis.

In the developing OE, apoE was concentrated in the OE proper from early embryonic ages. During postnatal ages, especially P21, apoE immunostaining was observed to be high in the LP. As the OE acquired its cell layer organization, and OMP^+ cells occupied a greater proportion of the epithelium, apoE expression became restricted to the Sus cells, basal cells and LP. ApoE was highly expressed during the embryonic ages in the OE proper when the neurons are in their transition state from immature state to the mature state. As the number of neurons expressing OMP immunostaining increased, the apoE protein immunostaining decreased in the OE proper. This suggests a role for apoE during neurogenesis and maturation. Previous studies have shown apoE expression in the olfactory nerve and around the glomeruli in the OB of adult mice (Struble et al., 1999 and Nathan et al., 2000). Expression of apoE in the OB during the embryonic stages suggests a vital role in the formation and maintenance of synaptic connections between olfactory axons and terminal dendrites of mitral cells. This possibility is supported by the finding that the levels of apoE in the glomeruli were increased during development, as more axons reached the bulb and formed very well organized glomeruli. Previous studies have demonstrated that apoE expression is correlated with neurite outgrowth (Nathan et al., 2004). Lipids have been shown to be very important in synapse generation (Mauch et al., 2001). At the later stages, i.e. the adult, apoE immunostaining was confined to the outer margins of the glomeruli. This

indicates that apoE has a major role in synapse generation and maturation. However, the absence of synaptophysin (marker for synapses/glomeruli) staining does not permits detailed explanation.

From the results it is observed that the numbers of OMP cells in the OE of KO mice are less when compared to the WT mice during the developmental stages. There was delayed neuronal maturation in the KO mice compared to the WT mice. Previous studies have shown that there is delayed olfactory nerve regeneration in the apoE deficient mice (Nathan et al., 2005). The exact etiology of delay in neuronal maturation in the KO mice can be debated. ApoE alters differentiation of various cell types by regulating cell signaling pathways (Ishigami et al., 1998; Ishigami et al., 2000 and Ferreira, S., 2000). The delay in neuronal maturation in the KO mice compared to the WT mice might be because of the unavailability of the lipids at this stage. ApoE deficiency may slow axonal growth of ORN in the KO mice. Culture studies have shown that, in the absence of apoE, neurite outgrowth was slowed and that this slowing can be reversed by supplying exogenous apoE (Nathan et al, 1994; Nathan et al, 2002 and Teter et al, 1999). ApoE may be involved in supplying recycled lipids to growing axons. The absence of apoE in the KO mice could impair the lipid recycling process and delay axonal growth either at the periphery or within the OB. The other explanation would be that the ORN entrance into the OB might be delayed in the KO mice or newly born ORNs may enter the bulb, but then show regression or stasis until appropriate post-synaptic sites are available for connection and maintenance of the ON.

Chapter 3: ApoE Immunoreactivity in the OE following Unilateral OBX

Materials & Methods

Animals:

Breeding pairs of WT C57BL/6 strain and homozygous apoE KO mice were purchased from the Jackson Laboratories, Bar Harbor, ME. ApoE genotype of the litters were verified by PCR and confirmed by immunoblotting. Male 4 months old mice were used in this study.

Bulbectomy:

Adult WT & KO mice were anesthetized with ketamine/xylazine (Cat # K-113, 800/120 mg, Sigma Aldrich, St.Louis, MO) and fixed in a stereotaxic apparatus during surgery. A small midline incision was made to expose the skull and a small hole was drilled into the skull with the help of a drill bit. OBX was performed by exposing the right OB with the help of suction. Care was taken to avoid damage to the contralateral OB. The ablation cavity was filled with Gelfoam™ (Pharmacia & Upjohn, Kalamazoo, MI) to prevent blood loss and invasion of frontal cortex into this cavity that could provide an alternative target for regenerating olfactory axons. The skin above the lesion was then sutured, and the animals were allowed to recover from anesthesia under a heat lamp. The animals were given a subcutaneous dose of 0.9% NaCl saline solution as an electrolyte replenisher. After recovery from anesthesia, the mice were returned to the animal colony and maintained on normal diet until they were killed 3, 7, 14 & 21 days post OBX procedure.

Tissue preparation:

For fluorescence immunohistochemistry, mice were anesthetized with Ketamine/Xylazine (80 mg/kg), transcardially perfused with cold saline (0.9% NaCl) and then with 4% paraformaldehyde (Cat. # 441244-1KG, Sigma Aldrich, St. Louis, MO) in 0.1M PBS. The turbinates were fixed in 4% paraformaldehyde for an hour and then treated with Cal-EX Decalcifying Solution (Cat. # CS510-D, Fisher Scientific,) for an hour. Olfactory turbinates were removed and cryoprotected overnight in 30% sucrose (Cat. # S-9378, Sigma Aldrich, St. Louis, MO) in 0.1 M PBS. After cryoprotection, the turbinates were frozen with dry ice using Tissue freezing medium (Cat. # 15-183-13, Fisher Scientific) and sections were cut on a cryostat (Jung Frigocut 2000E) at 18 μm, and air dried for 2 h at room temperature.

Immunohistochemical Analysis:

OE sections on slides (Cat. # 12-544-15, Fisher Scientific, St. Louis, MO) were rinsed in 0.1 M PBS, and permeabilized with 0.2 % Triton X-100 (Cat # BP 151-500, Fischer Scientific, St. Louis, MO) in PBS for 30 minutes at room temperature. The slides were rinsed once with PBS and treated with 70, 95, 100, 95, and 70% ethanol for two minutes each (Jang and Schwob 03). Non specific immunoreactivity was attenuated by incubation in 2.25% gelatin (Cat. # G-1890-500, Sigma, St. Louis, MO) in 0.1 M PBS for 1 h, followed by overnight incubation at 4^0 C with primary antisera solution. The primary antibody (Goat Anti- ApoE, Cat. # 178479, Calbiochem, San Diego, CA) was mixed with 4% normal donkey serum (Cat. # 017-000-121, Jackson Immunoresearch, West

Groove, PA) in 0.1 M PBS at a concentration of 1:500. The sections were washed three times in PBS, and incubated for 1 hour at room temperature with secondary antibody (FITC conjugated Donkey Anti- Goat, Cat. # 705-095-147, Jackson Immunoresearch, West Groove, PA) in 4% normal donkey serum in 0.1 M PBS at a concentration of 1:4000. The sections were washed three times in PBS, mounted in Vectashield (cat# H-1000, Vector labs, Burlingame, CA). For apoE staining, KO mice were processed in parallel with WT mice. Specificity was determined by incubation with normal serum in place of the primary antisera which resulted in no staining.

Microscopy and imaging

Immunohistochemistry was visualized with appropriate fluorescence excitation filters for fluorescent microscopy (Olympus BX-50). Tiff format images of the nasal septum of the turbinates were taken with a Pixera digital camera for microscopy at identical manual exposure settings for all experiments within each study.

Results:

3 Days Post OBX: The contralateral unoperated side of each and every animal served as the control. The thickness of the OE ipsilateral to the operated side following 3 days OBX was noticeably thinner. The apically located Sus cells appeared to be normal. In the LP the blood vessels appeared to be dilated and the ONF's reduced in diameter (Fig 12).

7 Days Post OBX: The thickness of the OE Ipsialteral to the operated side was observed to be much reduced compared to 3 days post OBX. The LP was noticed

to be damaged, increase in diameter of the large blood vessels, and/or smaller blood vessels dilation was the other observation. The ONFs were seen to be shrunken in size or became fragmented into smaller bundles (Fig 12).

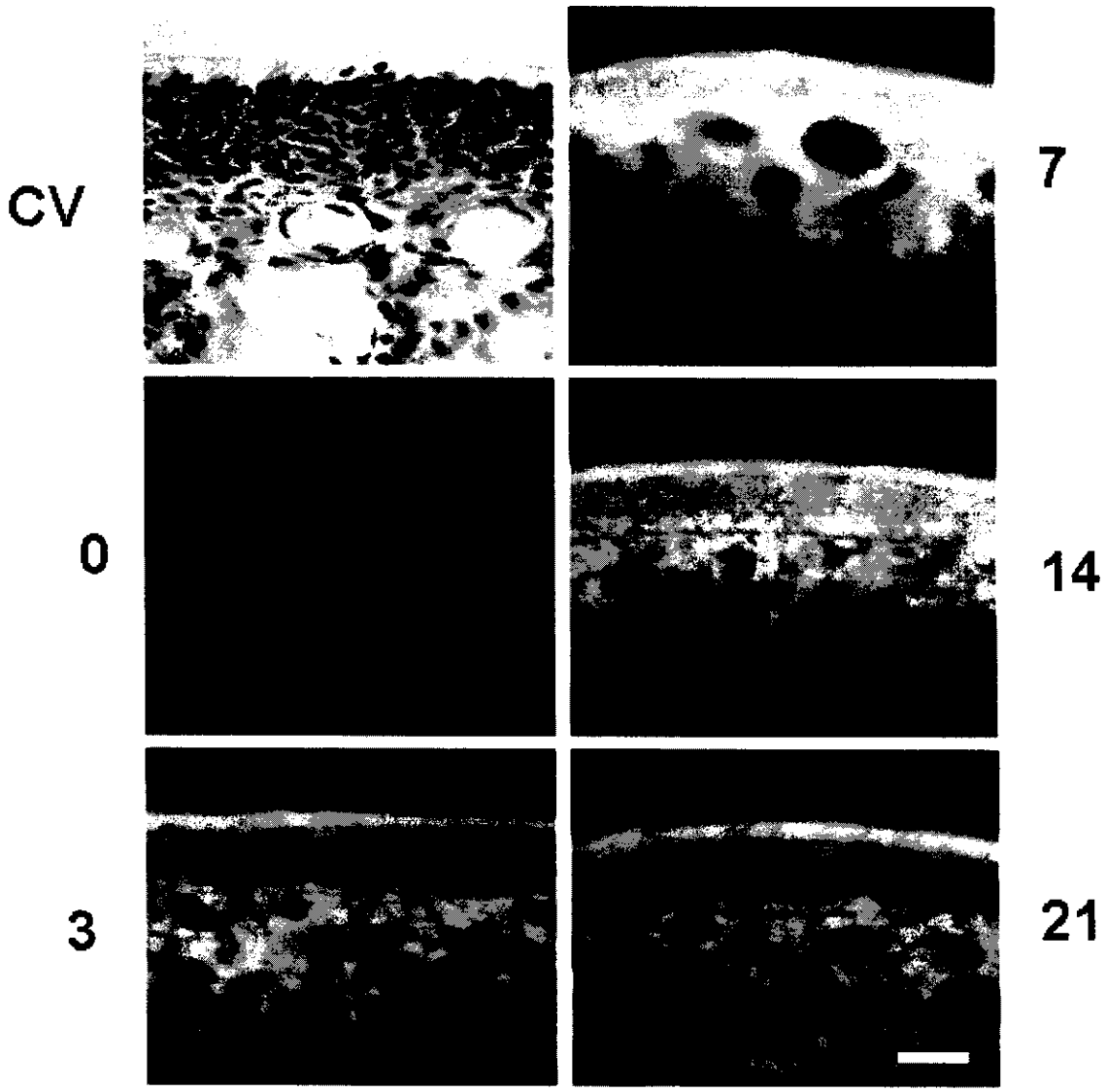

Fig 12: Apolipoprotein E immunoreactivity in the OE following OBX. The above figure shows apoE immunostaining in the OE following OBX at 0, 3, 7, 14 and 21 days post OBX. A sample Cresyl Violet stain of the OE is also shown. Scale bar = 10 μm.

14 Days Post OBX: The thickness of the OE Ipsilateral to the operated side was found to be increasing in thickness compared to 7 days post OBX. The LP was also seen to be getting its conventional morphological features. The ONF's were seen to be getting organized with the glial cells surrounding the ONFs (Fig 12).

21 Days Post OBX: The thickness of the OE ipsilateral to the operated side was found to be much increased in thickness compared to 7 & 14 days post OBX. The intensity of the stain (apoE expression) was also observed to be minimal compared to the previous time courses. The LP is observed to be retaining its original morphological features (Fig 12).

Control OE: The control side or the un-operated side of the OB was found to be normal. There were no significant changes observed in the OE. The three main types of cells; Sus cells, ORNs & basal cells were found to be normal and the thickness of the un-operated side was found to be much thicker than the ipsilateral side of the OBX. The intensity of the stain on the un-operated side, however, was found to be minimal compared to the ipsilateral side. The LP was very much in shape consisting of BVs, ONF, glial cells and the endothelial cells (Fig 12).

Discussion:

From the above results it can be noticed that the apoE expression has increased in the OE post OBX until day 7 and then started to decrease following day 7 through 21 days post OBX. However, the expression of apoE in the OE ipsilateral to OBX was observed to be maximal compared to the un-operated side. The thickness of the OE on the ipsilateral side has decreased following OBX. Previous work from a number of different laboratories has shown that when the OB is removed (unilateral

bulbectomy) from an adult animal, nearly all ORNs in the ipsilateral OE die (Costanzo & Graziadei, 1983). The OE then decreases in thickness as cells degenerate. The results of this study show that in the absence of normal target tissue (OB), the sensory neurons of the OE are still capable of replacement and subsequent formation of new axonal processes which grow centrally towards the brain. The normal target tissue is not a prerequisite for neuronal replacement (Verhaagen et al, 1990). This does not mean that a target is unimportant. In the absence of the bulb tissue, there are more iORNs (Gap 43^{+}) compared to mORNs (OMP^{+}). A normal target may be necessary for recovery to reach control levels and maintain a normal population of cells. It is possible that the amount of target readily available could be a factor in determining the size of the neuronal population that will be sustained. Despite the fact that cells in the basal compartment of the OE then proliferate and many new ORNs are generated (Costanzo & Graziadei, 1983 and Schwartz-Levey et al., 1991), in the absence of the OB, the OE never reaches its original thickness. The proliferation rate of the neuronal precursors is very high at this stage and the life span of the ORNs in the OE is very short. This is due to the fact that newly generated ORNs are able to survive for only a short period of time due to the absence of the OB (Schwob et al., 1992; Carr & Farbman, 1992, 1993). It is also been reported that the rate of generation of new ORNs is elevated tremendously following OBX, which suggests that the ongoing ORN death might somehow play a role in regulating the proliferation of neuronal precursors in the OE (Carr & Farbman, 1992). At this stage, apoE expression was very high in the OE. Bulbectomy induces apoptosis of mainly the ORNs, but does not affect two other cell types; Sus cells or supporting cells and

basal cells (Holcomb et al., 1995). Interestingly, very prominent apoE immunostaining was seen in the Sus cells after bulbectomy. Published results indicate that supporting cells in the OE play a significant role in phagocytosis in both acute and chronic periods of cell death after bulbectomy (Suzuki et al, 1996). This suggests the role of Sus cells in neuronal regeneration involving gene expression changes in response to injury, increased apoE expression might be necessary for ORN repair. This provides support to our study in that the Sus cells play a vital role in regards to producing apoE as well as clearing off the excess debris (phagocytosis). Disposal of cellular debris in a variety of peripheral tissue is primarily carried out by macrophages and apoE modulates the uptake of apoptotic cells by macrophages, and consequently regulates macrophage population dynamics in multiple tissues by a mechanism independent of lipoprotein transport (Grainger et al, 2004). And a previous study has shown that apoE promotes clearance of cellular debris at the site of neural injury (Fagan et al., 1998). ApoE appears to be vital to phagocytotic function of macrophages (Grainger et al., 2004). Thus one potential role of apoE produced by Sus in a normal/uninjured OE is to support the continuous disposal and recycling of cellular debris as a result of the constant turnover of the receptor neurons in the OE (Nathan et al., 2006). The hypothesis that reduced apoE expression following day 7 post OBX is due to the fact that as the OE starts to regain its thickness, i.e., as the rate of cell proliferation increases, more number if immature ORNs are in place leading to the usage of the excess lipoprotein present in the OE for their survival. However, it should be noted that the complete recovery of the OE thickness is not achieved at 21 days post OBX compared to the control OE.

Conclusions:

From the above results, it can be concluded that: (1) apoE is expressed in the adult OE and lamina propria by different cell types 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; (2) apoE is expressed during the developing mouse olfactory system at the early embryonic and postnatal ages, and the expression pattern changed along with the maturation of the ORNs in the OE. ApoE expression in the OB suggests that, as the synapses are developing, apoE assists in synapse formation, and once formed, migrates away towards the periphery of the glomeruli. The results on OMP data revealed that there was difference in the maturation of ORNs between WT and KO mice. This leads to the conclusion that apoE deficiency in the KO mice might have caused this delay and suggests the role for apoE in neuronal maturation; (3) the unilateral OBX studies suggested that apoE is expressed in the OE following OBX and that the expression levels increased in the OE ipsilateral to the OBX compared to the contralateral side of the epithelium. However, the intensity of apoE immunostaining decreased in the ipsilateral OE to OBX as the OE starts to gain its thickness. This suggests that apoE is being used by the regenerating neurons.

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