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Role of apoE in regeneration, maturation and synaptogenesis of olfactory receptor neurons in mice

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Role of apoE in regeneration, maturation and synaptogenesis of olfactory receptor neurons in mice

BY

Salina Gairhe

THESIS

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Department of Biological Sciences
Eastern Illinois University
Fall Semester 2006

DEDICATION

I wish to dedicate this dissertation to my swami Sree Sathya Sai Baba, my grandmothers Uttara Devi Adhikari , and Padmawati Gairhe, my parents Damodar Gairhe and Kalpana Gairhe.

To my swami who was there all through my life as a guiding star and as a shield for a tortoise. Baba it's only because of your blessings, only because of your blessings.

To my grandmothers who understood the value of education and let me follow the dream, I wished to follow.

To my parents, who introduced me to the love of science. It is through their support, love and guidance that made this endeavor possible. Thank you very much for your never ending love, patience and encouragement.

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ABSTRACT

Previous studies have shown that apolipoprotein E (apoE), a lipid transporting protein, is involved in the growth and regeneration of injured neurons. apoE is a key molecule involved in lipid transport system associated with compensatory sprouting and synaptic remodeling after neuronal injury. Also, transport of cholesterol and phospholipids in brain is regulated by the coordinated expression of apoE and its main Low Density Lipoprotein (LDL) receptors during the different phases of the neuronal reinnervation processes. The discovery of apolipoprotein E4 genotype, a major risk factor for Alzheimer's disease (AD), is suggestive of involvement of dysfunctional apoE lipid transport system in the progression of AD. We used mouse olfactory system, olfactory epithelium (OE) and olfactory bulb (OB), as a model to study the role of apoE in the neuronal regeneration, maturation and synaptogenesis. Olfactory epithelium provides "window" on the neuronal development because it is a site for continual neurogenesis. Although the underlying mechanism(s) is incompletely understood, previous studies have shown that olfactory neurons reinnervate following lesioning. Recent studies including studies in our lab have shown that apoE is expressed at high concentrations in the olfactory nerve as well as in the olfactory bulb and that the apoE levels in these regions increase substantially following lesioning of the source of the olfactory nerves, the olfactory epithelium. However, the mechanism(s) underlying the contribution of apoE to the neuronal regeneration, maturation, and synaptogenesis are not clearly understood. We therefore hypothesize that apoE is essential for regeneration, maturation and synaptogenesis of mice olfactory epithelial neurons.

In this study, we have advanced a series of experiments to understand the role of apoE in regeneration, maturation and synaptogenesis of mice olfactory epithelial neurons. Aim 1 was used to test the hypothesis that apoE is involved in neuronal regeneration and maturation following olfactory epithelial lesioning. This hypothesis was tested by using immunoblotting technique to demonstrate time dependent change in levels of expression of apoE and Olfactory Marker Protein (OMP) at several stages of post OE lesioning induced by nasal irrigation of Triton X-100. The result revealed that apoE expression

levels initially down-regulated at day 7 post-lesioning and reached to basal level at day 21 and significantly exceeded basal level at days 42 and 56. Whereas, OMP expression levels were down-regulated for 21 days post lesioning followed by a significant up-regulation at day 42 post lesioning in WT mice as compared to apoE KO mice. These collectively suggest that apoE is involved in neuronal regeneration and maturation of olfactory epithelium following olfactory epithelium lesioning.

Aim 2 was used to test the hypothesis that apoE promotes synaptogenesis of Olfactory Receptor Neuron (ORN) with OB neurons post OE lesioning. In this study, immunoblotting technique was used to examine the role of apoE on the rate of synaptic recovery following OE lesioning. The result revealed that Synaptophysin (Syn) levels declined sharply between 3 and 7 days post lesioning in both WT and KO mice. Following this precipitous decline, OB Syn levels steadily increased to about 80% of the normal levels by 56 days post lesion in WT mice. In contrast to WT mice, Syn density did not increase significantly in KO mice and was significantly less than that of WT mice on day 56. Since the rate of Syn recovery was delayed in apoE KO mice, suggesting apoE facilitates synaptogenesis of olfactory nerve post injury.

Aim 3 was used to test the hypothesis that apoE is involved in regeneration and maturation of ORN neurons following removal of the synaptic targets of ORN by bilateral olfactory bulb ablation. This was assessed by studying the level of expression of apoE and OMP in OE following day post bulbectomy. Olfactory bulbectomy (OBX) resulted in a significant increase in the expression of apoE level 3 days post bulbectomy, which further increased by 7 days and remained at high levels thereafter. Whereas, olfactory bulbectomy resulted in a significant decrease in the expression of OMP level for 14 days post bulbectomy and it increased slightly at day 21. This collectively suggests that apoE is involved in regeneration and maturation of OE neurons following removal of synaptic targets of ORN by OBX.

Taken together, these results indicate that apoE is involved in regeneration and maturation as well as in the synaptic formation of ORN.

ABBREVIATIONS

ApoE	Apolipoprotein E
APOER2	ApoE Receptor 2
AD	Alzheimer's Disease
CNS	Central Nervous System
CSF	Cerebrospinal Fluid
GAP-43	Growth Associated Protein 43
HRP	Horseradish Peroxide
KO	Knock-Out
LDL	Low Density Lipoprotein
LRP	LDL receptor-related protein
OB	Olfactory Bulb
OBX	Olfactory Bulbectomy
OE	Olfactory Epithelium
OMP	Olfactory Marker Protein
ON	Olfactory Nerve
ORN	Olfactory Receptor Neuron
PNS	Peripheral Nervous System
SDS	Sodium Dodecyl Sulfate
SYN	Synaptophysin
TBST	Tris Buffer Saline with Tween
T-TBS	Tris – Tween Buffer Saline
VLDL	Very Low Density Lipoprotein
WT	Wild-Type

1. INTRODUCTION

1.1. Characteristics of Apolipoprotein E

Apolipoprotein E (apoE) is a 34 kDa glycosylated protein and is classically associated with transport of lipids and redistribution of cholesterol [70, 73, 94, 100]. Circulating apoE is synthesized in many peripheral organs particularly in liver. Glial cells specifically astrocytes [60] and possibly microglia [48] are the second most important sites for apoE biosynthesis in brain [40]. The major function of apoE is in regulation of lipid metabolism during development and is involved in growth and regeneration of injured neurons [62]. Studies have shown that the increase level of apoE after brain lesions [58, 64] which contribute to the regeneration of neurons [54].

1.1.2. ApoE Polymorphism

Genotype of apoE has been clearly demonstrated to be the risk factor for Alzheimer Disease (AD) [12]. Human apoE is a polymorphic protein. ApoE exists in three major isoforms, designated as apoE2, apoE3, apoE4, that are that are products of the three alleles (designated ε2, ε3, and ε4) located at a single gene locus on the long proximal arm of chromosome 19 [40]. The molecular basis of this polymorphism of the apoE gene results from cysteine-arginine interchanges at two positions in the apoE protein [94]. These single amino acid substitutions are found at residues 112 and 158 [66]. The most common isoform, apoE3, contains cysteine at residue 112 and arginine at position 158. ApoE2 has cysteine at both positions and apoE4 contains arginine at both positions [94].

1.2. ApoE and Neurobiology

With the discovery of ApoE in the early 1970's as a component of triglyceride rich lipoprotein, researchers begin to speculate its potential role in normal functioning of brain after two decades around mid 1980's [40]. In order to understand the role of apoE in neurobiology, it is essential to explore about the function of apoE in neuronal repair, remodeling, or protection [40]. After the neuronal injury and damage of neurons by injurious agents like β-amyloid deposition, reactive oxygen species and exitotoxic amino acids neurons requires repair. Now, clinical and experimental studies have shown protective effects of apoE2 and apoE3 isoforms against neurodegeneration/AD, whereas the apoE4 isoform have been implicated in the development of AD as a major genetic risk factor [8, 12, and 40] (Figure 1.1.).

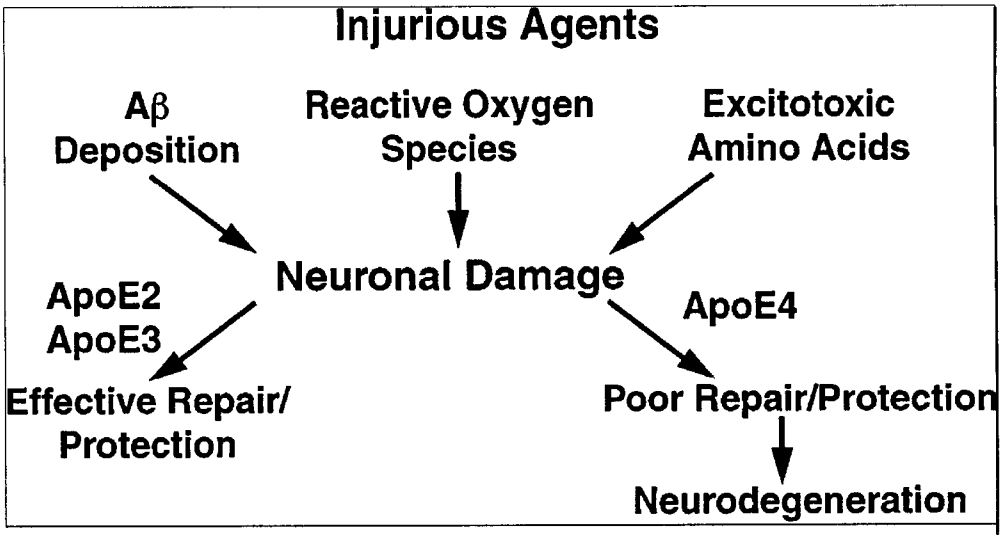

Figure 1.1. Effect of apoE: Differential effect of apoE isoforms on repair, remodeling and protection

However, the mechanisms by which the apoE4 allele convey the progression of AD is poorly understood. But many studies have showed isoform specific effects on neurodegeneration, formation of NFTs and β-amyloid. Studies have shown that plastic responses in brain are impaired in apoE4 carriers [3] and *in vitro* studies have shown that apoE3 promotes comparatively high neurite outgrowth than apoE4 [50]. More severe astrogliosis have been documented in apoE4 carriers compared to apoE4 non-carriers [57]. These data implicates the role of apoE in the development of neurofibrillary and amyloid pathology. ApoE3 firmly binds with microtubule-associated proteins [30] and promotes microtubule polymerization whereas apoE4 was found to depolymerize microtubules [51]. Also, apoE3 has a stabilizing effect which may prevent the abnormal phosphorylation of the tau protein [81]. However, mice have only one form of apoE, which is similar to human apoE3 in its structural and functional properties, including receptor binding and lipoprotein preferences [6, 93]. Therefore, apoE deficit mice mimic the status of human apoE4 [8, 77].

1.2.1. AopE in Nervous System

ApoE is the major apolipoprotein in the brain and cerebrospinal fluid (CSF) [6, 60, 61]. Previous studies have shown that apoE is the only apolipoprotein in the CSF that can interact with lipoprotein receptors [6, 61]. 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) [27]. 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 [5]. 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.

1.2.2. Evidence linking apoE to Chronic Neurological Disorders

Genetic studies have revealed that inheritance of apoE4 alleles increases the risk and rate of progression of late-onset Alzheimer's disease [12, 70, 81]. 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 [73, 56]. 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 [3].

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 [84, 102]. ApoE4 inheritance increases the risk of developing dementia following head trauma [32, 46, 85]. Recovery following stroke or head trauma is prolonged and significantly decreased in apoE4 carriers as compared to apoE3 individuals [1, 16, 36]. This phenomenon is also observed in football players and boxers who are prone to head injury [37]. 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.

1.2.3. ApoE and Peripheral Nerve Regeneration

Peripheral nerve maintenance and repair are suggested as possible roles of apoE in the PNS [40, 32, 17, 31]. In rats, the synthesis of apoE increases several-fold within 3-weeks post-injury of peripheral nerve [7, 32, 79]. 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 [7]. 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 [21, 40, 63]. Deficiency of apoE in apoE KO mice leads to impaired blood-sciatic nerve barrier and to loss of unmyelinated fibers in the sciatic nerve [17, 18]. However, regenerating sciatic nerves in both apoE KO and WT mice were morphologically indistinguishable at 2 and 4 weeks post nerve injury [21, 65]. In 1990, Verhaagen and his colleagues postulated two stage of regeneration of olfactory epithelium following peripheral deafferentaton of olfactory epithelium with Triton-X 100 or olfactory bulbectomy. First stage was characterized by the formation of large population of immature ORN. These newly formed neurons expressed GAP-43 which is a phosphoprotein related to neuronal growth and plasticity. Second stage was characterized by maturation of newly formed ON. As a result there was decrease in expression of GAP-43 and increase in expression of OMP. Important point they noted was second stage of regeneration occurred only if developing ORN have access

to the target olfactory bulb [90]. In the light of these facts, we studied the role of apoE in peripheral nerve regeneration, maturation and synaptogenesis after reversible injury made to ORN on 3,7,14, 21, 42, 56 day post lesion. Also, we studied regeneration and maturation process of ORN after 3, 7, 14, 21 day post bulbectomy.

1.2.4. ApoE and CNS plasticity

ApoE may be important in plasticity and repair in the CNS although the data are equivocal. Newly synthesized apoE increases following optic nerve injury in rats, but absolute levels do not [33]. Axonal degeneration and myelin sheath alterations were observed in the optic nerve of apoE KO mice [42]. 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 [42, 43, 44]. In contrast, two other studies have not observed any obvious neuropathology in apoE KO animals [2, 20]. Thus the importance of apoE in the CNS is controversial, and warrants further studies.

1.3. The Olfactory System

The olfactory system includes the nasal cavity and many other parts of the brain. Our prime interest is olfactory epithelium and olfactory bulb. The mechanism involved in olfaction is that, the olfactory receptor neuron (ORN) present in the olfactory epithelium senses the smell. The olfactory neuron regenerates from division of stem cells throughout the life of organism [2, 3, 43, 54]. These divisions give rise to many immature ORN that gradually differentiate into mature neuron [55, 39]. These ORN in OE (peripheral nervous

system) project axon to olfactory bulb (central nervous system) to terminate in olfactory glomeruli. In both mice and rat, soon after the start of axon genesis growing axon first reaches the presumptive bulb region and establishes contact with target cell [14]. Neurogenesis in olfactory system is highly regulated in order to maintain an equilibrium between basal cell mitosis, cell death and cell survival in OE [55, 39].

The olfactory epithelium can be easily damaged by any physical injury to interior of nasal cavity either through inhalation of toxic fume or by use of nasal spray [23, 28, 69]. However, olfactory epithelium has unique capacity to regenerate [23, 24] because of that these injury are temporal but permanent condition like anosmia can occur in extreme cases [23, 28, 69]. These advantages of olfactory system is extensively used to study events involved in formation of new neuron (neurogenesis) [14] and neuronal differentiation [13]. In olfactory system, the continual occurrence of neurogenesis has been proposed to respond to constant neurodegeneration resulting from direct exposure of ORN to environmental toxins or physical injury [13]. The development of olfactory system is similar to developing CNS. Features like basal orientation of mitotic cells and their differentiation into neurons are shared by olfactory epithelium as well as many developing CNS [13]. Therefore, olfactory system is a suitable and comparable model to study processes that regulate neuronal development.

1.3.1. 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 [50, 51, 52, 53] (Figure 1.2). First, the ORN regenerate from stem cells throughout the life of the organism [23, 24]. 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 [31, 76, 90, 91]. Second, the cytoarchitecture and connectivity of the bulb are relatively simple, permitting reasonably simple interpretations of experimental manipulations [25, 78]. 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.

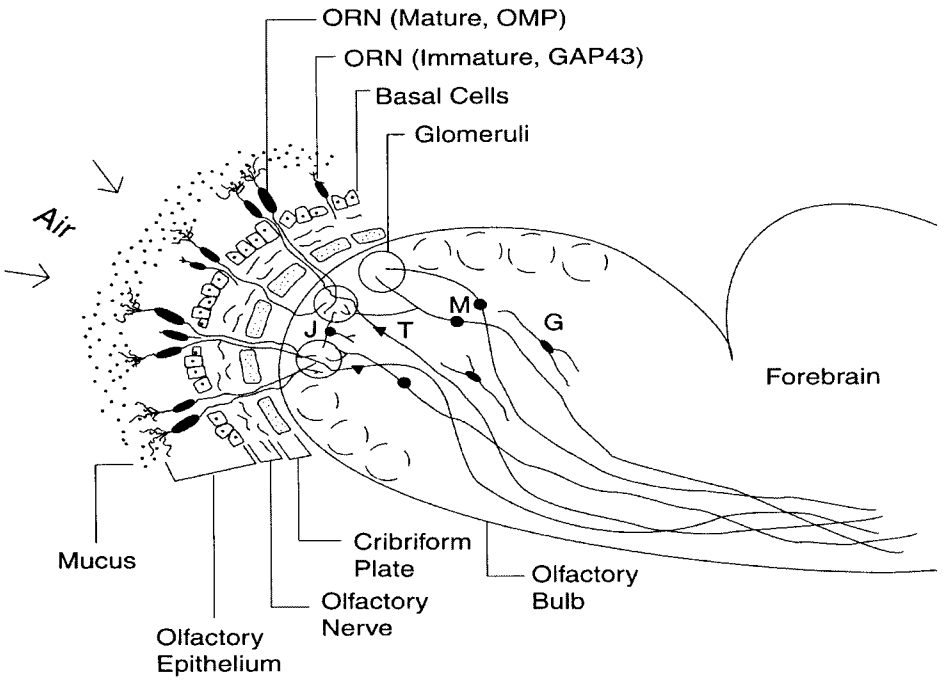

Figure 1.2. Organization of the olfactory system. The cell bodies of the olfactory receptor neurons (ORN) are located in the olfactory epithelium.ORN continuously regenerate from the basal cells. Their axons pass through the cribriform plate and terminate in the glomeruli of the olfactory bulb. In the 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.

1.4. Biochemical Cell markers unique to Olfactory System.

Study of olfactory system and identification of cell in the olfactory epithelium is made easy with the discoveries of many biochemical markers. Expression of many proteins in olfactory system is limited to subset of cells/stage within an organism and act as cell/stage specific marker. For example, one of the marker for the immature olfactory neuron is Growth Associated Protein 43 (GAP-43/B50) [90], marker for mature olfactory neuron are Microtubule Associated Protein-5 (MAP5) [92] and Olfactory Marker Protein (OMP), a 19-kDa, acidic, soluble protein found abundantly in mature olfactory sensory neurons [15, 11]. Similarly, marker for synaptogenesis in bulb is synaptophysin [96]. Two stage specific marker proteins are of our interest, particularly, OMP and Synaptophysis (Syn).

1.5. AIMS OF THE STUDY

Previous studies from our laboratory suggest that apoE, a lipid transporting protein, facilitates olfactory nerve regeneration. We have shown that apoE is enriched in the olfactory nerve and around the glomeruli of the olfactory bulb (OB). Also, demonstrated that apoE promotes synapse development in neuronal cultures. These observations suggest a critical role of apoE in ORN plasticity; however the mechanism whereby apoE mediates these processes is unknown. This thesis tests the overall hypothesis that apoE is important for the regeneration, maturation and synaptogenesis of ORN in olfactory epithelium. Therefore, the study was designed to addresses nerve regeneration and subsequent maturation and synaptogenesis 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.

The specific aims of the present study were:

1) **To investigate the role of apoE in neuronal regeneration and maturation following olfactory epithelial lesioning**

In this specific aim, we assessed level of expression of apoE and OMP in OE post –OE injury by immunoblotting techniques. Both the processes was evaluated at multiple stages post injury to follow the time related regeneration and maturation of ORN.

2) **To study the effect of apoE in synaptogenesis between ORN dendrites and terminals in the OB post OE lesioning.**

In order to understand whether apoE support synapse development of ORN with bulbular neurons following OE lesioning, we evaluated Syn expression in whole OB from KO and WT mice post OE lesioning by using immunoblotting analysis.

3) **To investigate the role of apoE in neuronal regeneration and maturation following bulbectomy**

This aim was fulfilled by studying, the rate of apoE and OMP recovery after removal of its target OB by bulbectomy or CNS injury we studied the expression of apoE and OMP in WT mice in OE.

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. Olfactory Bulbectomy

Mice used for olfactory bulbectomy were of the same strain as the ones used for nasal irrigation. Ketamine/Xylazine (Cat. # 026K46031) was purchased from Sigma-Aldrich (St. Louis, MO). Driller used Dremel Cordless Shaft, Drill bit of size 2.4mm was purchased from harware shop. Gelfoam used were of size 4 (Pharmacia & Upjohn, Kalamazoo, MI) and was purchased from dentist.

2.1.3. Sacrifice and Tissue Preparation

TMN buffer (Tris-HCl, $MgCl_2$, NaCl, phenylmethylsulfonyl fluoride) all the chemicals were purchased from Sigma-Aldrich (St. Louis, MOS). Triton-X100 (Cat # BP 151-500), deoxycholate (Cat. # NC985-78-02), and SDS (Cat. # O26-74-25) were purchased from Fisher Scientific (St. Louis, MO).

2.1.4. Protein Assya

Bradford Coomassie Assay (BCA), protein assay kit (Cat # PI-23240) was purchased from Pierce (Rockford, IL). Protein A-Sepharose CL-4B (Cat. # P-3391) and Bovine Serum Albumin (BSA) (Cat. # A-9418) were obtained from Sigma-Aldrich (St. Louis, MO).

2.1.5. Western Immunoblot

Materials used for immunoblots like pre-cast 4-20% gradient gels (Cat. # FB3435), Tris (Cat. # BP154-1), Glycine (Cat. # BP381-1), SDS (Cat. # BP166-100), Tween 20 (Cat. # BP337-500) and Sodium Bromophenol Blue (Cat. # BP-114-25) were purchased from Fisher Scientific (Chicago, IL). Kaleidoscope prestained standards (Cat #161-0324) was obtained from Bio-Rad Laboratories, Hercules, CA. Immobilon-P (Cat #IPVH000 10), Stripping buffer (Cat #21059), SuperSignal West Pico Chemiluminescent Substrate (Cat #34080) and chemiluminescent clear-blue x-ray film (Cat # PI-34091) were purchased from Sigma-Aldrich (St. Louis, MO).

2.1.6. Immunoblot for apoE

Goat anti-human apolipoproteinE (apoE) (Cat. # 178479) was purchased from Calbiochem (San Diego, CA). HRP conjugated secondary antibody rabbit anti-goat IgG (Cat. # AP106P) was obtained from Chemicon, Temecula, CA.

2.1.7. Immunoblot for OMP

Goat anti-rat olfactory marker protein (OMP) was obtained as a generous gift from Dr. Margolis, (University of Maryland, MD). Rabbit anti-goat IgG-HRP (Cat #AP106P) was purchased from Chemicon, Temecula, CA.

2.1.8. Immunoblot for Synaptophysin

Rabbit anti-Synaptophysin (Cat. # CMC 109) was purchased from Cell Marque Corporation (Hot Springs, AR). HRP-conjugated anti-rabbit IgG (Cat #AP132P) were purchased from Chemicon, Temecula, CA.

2.1.9. Immunoblot for Actin

Mouse anti-actin (Cat # JLA20) was obtained from Developmental Studies Hybridoma Bank, Department of Biological Sciences, The University of Iowa, Iowa City, IA. Goat anti-mouse IgG-HRP (Cat # AP124P) was purchased from Chemicon, Temecula, CA.

2.2. Methods

2.2.1. Nasal Irrigation

Two to four months wild type C57BL6J and apoE KO mice , were obtained from the departmental mouse colony and were lesioned as previously described [62, 67, 91]. The time course used for TX treated and SA treated WT and KO mice were 0, 1, 3, 7, 14, 21, 42 and 56 days. For each time point, an equal number of wild type (n=5) and apoE KO mice (n=5) 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 [74]. Animals were placed under a heat lamp while recovering from anesthesia and then were housed in the animal care facility until sacrificed.

2.2.2. Olfactory bulbectomy

Two to four month old WT and KO male mice were either bulbectomized (OBX) or sham operated, depending on the treatment group. They were anesthetized with intraperitoneal injection of Ketamine/Xylazine [100mg/kg/50mg/kg (0.15 cc/20–25 g body weight-[73, 74, 75)]. Animals were placed in a stereotaxic apparatus, and the frontal bones over the olfactory bulbs were exposed by a midline incision. For OBX group, complete bilateral bulbectomies were performed by drilling a small hole in the bone over

the left and right bulb. The olfactory bulb was removed by gentle aspiration with a curved glass pipette connected to a suction apparatus. The bulb cavity was filled with sterile Gelfoam (size 4, Pharmacia & Upjohn, Kalamazoo, MI) to control bleeding and the incision was sutured. After recovery from anesthesia, animals were returned to home cages and they were allowed to survive for 3, 7, 14, 21days. A total of twenty four experimental WT and KO animals were used, three for each postoperative survival time. For Sham treated group same procedure was followed except removal of olfactory bulb.

All procedures were carried out in accordance with protocols approved by institutional animal care and utilization committees.

2.2.3. Surgery and sacrifice

2.2.3.1. Nasal irrigation

Mice were deeply anesthetized with pentobarbital (80 mg/kg, i.p.) and perfused transcardially with 4°C phosphate buffered saline (PBS) until the perfusate was free of blood. Olfactory epithelium and olfactory bulb were harvested, washed with ice cold 0.1M PBS preserved at 4^0C and processed for tissue preparation as described below.

2.2.3.2. Olfactory bulbectomy

Mice were deeply anesthetized with ketamine (100 mg/kg)/ xylazine (50 mg/kg) and perfused transcardially with 4°C PBS. Olfactory epithelium were harvested washed with ice cold 0.1M PBS preserved at 4^0C and processed for tissue preparation as described below.

2.2.4. Tissue preparation

Isolated epitheliums and bulb were homogenized in ice-cold TMN buffer (25 mM Tris-HCl [pH 7.6] 3 mM $MgCl_2$, 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride) [99]. The homogenate was lysed with lysis buffer (20 % Triton X-100, 10 % deoxycholate, and 10 % SDS on ice for 5 min) [98]. The homogenate was then centrifuged for 2 min in a microcentrifuge (g = 13,000) [98]. The supernatant was saved for protein assay and western immunoblot analysis.

2.2.5. Protein Assay

Protein assay was performed by BCA protein assay method as described by the manufacturer (Pierce). The protein contents of the eluted lysates were determined by a BCA protein assay kit from Pierce (Rockford, IL). Briefly, 1 µl of homogenized sample was diluted to 99 µl with double distilled water, and 30 µl of diluted samples (in triplicates) were assayed. 20 µg of protein from each sample was electrophoresed in a PAGE Gold Precast Gels (Cambrex , Rockland, ME) and transferred to a nitrocellulose membrane.

2.2.6. SDS-Polyacrylamide Gel Electrophoresis

Proteins in the olfactory epithelium and bulb were resolved by SDS-PAGE as previously described [5]. Briefly, 20 µg of olfactory epithelium protein was mixed with an equal volume of 2X Lammeli sample buffer [pH 6.8], 5 ml glycerol, 1g SDS, 0.5 ml 2-mercaptoethanol, bromophenol, 13.25 ml dH_2O). Samples were boiled for 5 min and then centrifuged at 14,000 g for 5 min. The gel cassettes were inserted into the buffer tank of

an EC120 Mini gel vertical system (E-C Apparatus Corporation, St. Petersburg, FL) containing 1X running buffer, pH 8.3 [250 ml of 5X running buffer (15 g Tris-base, 72 g glycine, 5 g SDS, 750 ml dH_2O].

The samples and 5 µl of kaleidoscope prestained standards were electrophoresed through a pre-cast 4-20% gradient gel. Samples were electrophoresed at 80 volts until separation began, and then at 140 volts until the dye front reached the bottom of the gel.

2.2.7. SDS-Polyacrylamide Gel Electrophoresis-Protein Transfer

Following electrophoresis, the gel was placed in transfer buffer (3.03 g Tris-base, 14.4 g glycine, 200 ml methanol, 800 ml dH_2O) on a shaker. The transfer membrane (Immobilon-P IPVH00010, Millipore, Bedford, MA) was soaked in methanol for 5 sec and then washed in dH_2O for 5 min. The gel was placed on presoaked filter paper in the holder and the transfer membrane was placed on top of the gel. Using a trans-blot transfer cell (170-3930, Bio-Rad), proteins from the gel were transferred onto the membrane by passing 100 volts for an hour.

2.2.8. ApoE/OMP/Syn Western Immunoblotting

2.2.8.1 ApoE

ApoE was quantified as previously described [99, 98]. Briefly, the protein blots were first probed with polyclonal goat antiserum against human apoE (1:1,000 dilution in T-TBS [pH 7.6] 0.1M Tris, 0.15M NaCl, 0.1% Tween-20) for 30 min on a shaker at room temperature. The membrane was then washed 5 times (5 min each) in T-TBS. The blot

was then incubated in the secondary antibody solution (rabbit anti-goat IgG-HRP-1:1,250dilution) for 30 min on a shaker at room temperature. Blots were washed with Tris – Tween Buffer Saline (T-TBS) 6 times (5 min each) [99, 98]. Antibody binding was detected by the addition of chemiluminescent substrate (SuperSignal Kit, Pierce, Rockford, IL) and then exposed to clear blue X-ray film (Pierce).

2.2.8.2. OMP

The same protocol was used as above, except dilutions differ. Primary antibody solution consists of goat anti-rat OMP [1:10,000 dilutions in Tris Buffer Saline with Tween (TBST)]. Secondary antibody solution is rabbit anti-goat IgG-HRP (1:5,000 dilutions in TBST).

2.2.8.3. Synaptophysin

The same protocol was used as above to quantitate synaptophysin. Primary antibody solution consists of Rabbit anti-Synaptophysin was purchased from Cell Marque Corporation (Hot Springs, AR). at 1:2,000 dilution in TBST. The secondary antibody was goat anti-rabbit IgG-HRP (AP132P, Chemicon, Temecula, CA) (1:1,000 dilution in TBST).

2.2.9. Quantification of Actin

To quantitate actin, blots were then washed 2 times (6 min each) in TBST, pH 7.5 [20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20, 0.1 g BSA]. Later, blots were incubated in mouse anti-actin (Developmental Studies Hybridoma Bank, Department of Biological Sciences, The University of Iowa, Iowa City, IA) (1:10,000 dilution in TBST)

for on hour on an orbital shaker at room temperature. Blots were then washed 4 times (6 min each) in TBST, pH 7.5 [20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20, 0.1 g BSA]. Blots were incubated in secondary antibody solution (goat anti-mouse IgG-HRP) [AP124P, Chemicon, Temecula, CA] 1:5,000 dilution in TBST) on an oribital shaker for one hour at room temperature. The blots were then washed 5 times (10 min each) in TBST. Visualization of the bands was done using the same protocol as previously described for apoE.

2.2.10. Experimental Quantitation

All experiments were repeated three-five times to assure reproducibility of the results. Bands were quantified by densitometry (Scion Image). As an internal control, the blots also contained an olfactory bulb and olfactory epithelium extract from unlesioned animals in case of OE injury whereas SHAM treated animal were used in OBX. .

2.2.11. Statistical analysis

Statistical analysis was performed using Systat © analysis of variance software. The data in individual experiments were presented as mean ± standard error. A one-way analysis of variance (ANOVA, Repeated Measures ANOVA) was used to compare treatment means. If a significant F value was found, a Duncan's Multiple Range mean comparison test was used to differentiate significance between and among means Significance was considered at $p<0.05$ level. Post-hoc testing was performed with a Bonferrroni-corrected t-test for selected contrasts.

3. RESULTS

3.1 ApoE is expressed both in olfactory epithelium and olfactory bulb

In this study, the olfactory epithelium and olfactory bulb from 2-4 months old mice were analyzed. Immunoblotting techniques were used to confirm apoE expression in mouse OE (Figure 3.1) by evaluation of olfactory turbinates and OB tissues from adult WT and KO mice. Immunoblotting revealed similar bands for apoE at about 35 kDa in both the OE and OB extracts from WT mice. The observed molecular weight of apoE in this blot is similar to that reported previously for apoE [93]. The absence of the apoE band in both OE and OB extracts from KO mice confirmed the specificity of the antibody used in this study. Actin immunoblotting was used as a loading standard.

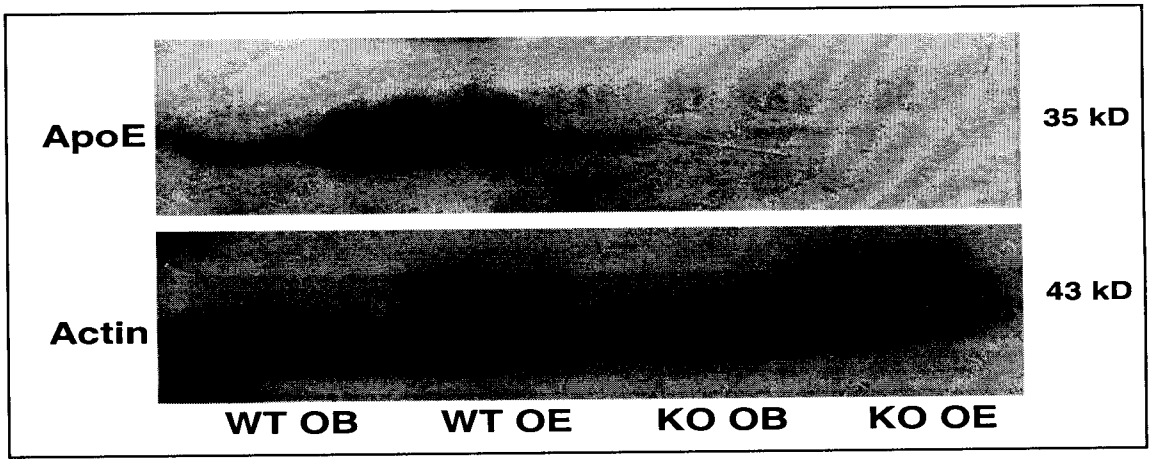

Fig. 3.1. Immunoblot analysis of OB and OE extracts from WT and KO mice. Twenty micrograms of tissue extracts were immunoblotted with polyclonal apoE antibody. A single 35 kDa band was detected in OB and OE extracts from WT, but not from KO mice. Actin was used as internal standard for protein loading.

3.2. Expression of ApoE is up-regulated in the OE following OE lesioning

The nasal passages of WT and KO mice were lesioned by intranasal irrigation with Triton X-100 in saline. Following the lesioning ApoE expression was quantified to test the relationship of apoE to injury and regeneration. Immunoblotting techniques were used to study the time course of apoE expression in OE following triton (TX) lesioning. Immunoblotting revealed bands for apoE at about 35 kDa in the OE extracts from WT mice. The observed molecular weight of apoE in this blot is similar to that reported previously for apoE [93]. The expression of apoE was markedly reduced at day 7 post lesioning compared to control (saline treated) mice and it reached to basal level at day 21 and significantly exceeded basal level at days 42 and 56 (Figure 3.2a).

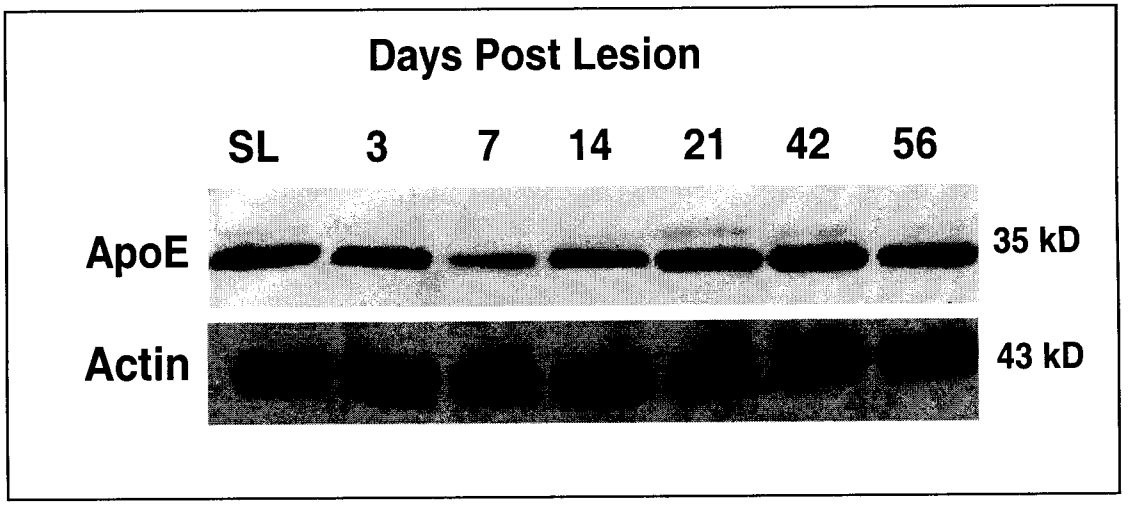

Fig. 3.2a. Immunoblotting of apoE in the OE following lesioning in mice. OE homogenates collected at indicated times from WT mice following nasal irrigation of TX or saline (SL) were immunoblotted using a polyclonal antibody to apoE as described under Materials and Methods. Actin was used as internal standard for protein loading.

Statistical analysis of apoE levels was performed with a one Way ANOVA on days post lesion. Initial testing was followed with Bonferroni-corrected t-tests for pair-wise comparisons. Days post Lesion was significant ($F_{6, 14}=144.55$; $p<0.001$). Post-hoc testing disclosed that apoE declined from initial levels after TX-100 treatment at seven days returned to "normal" levels at 21 days and then significantly exceeded initial levels at days 42 and 56 (Figure 3.2b).

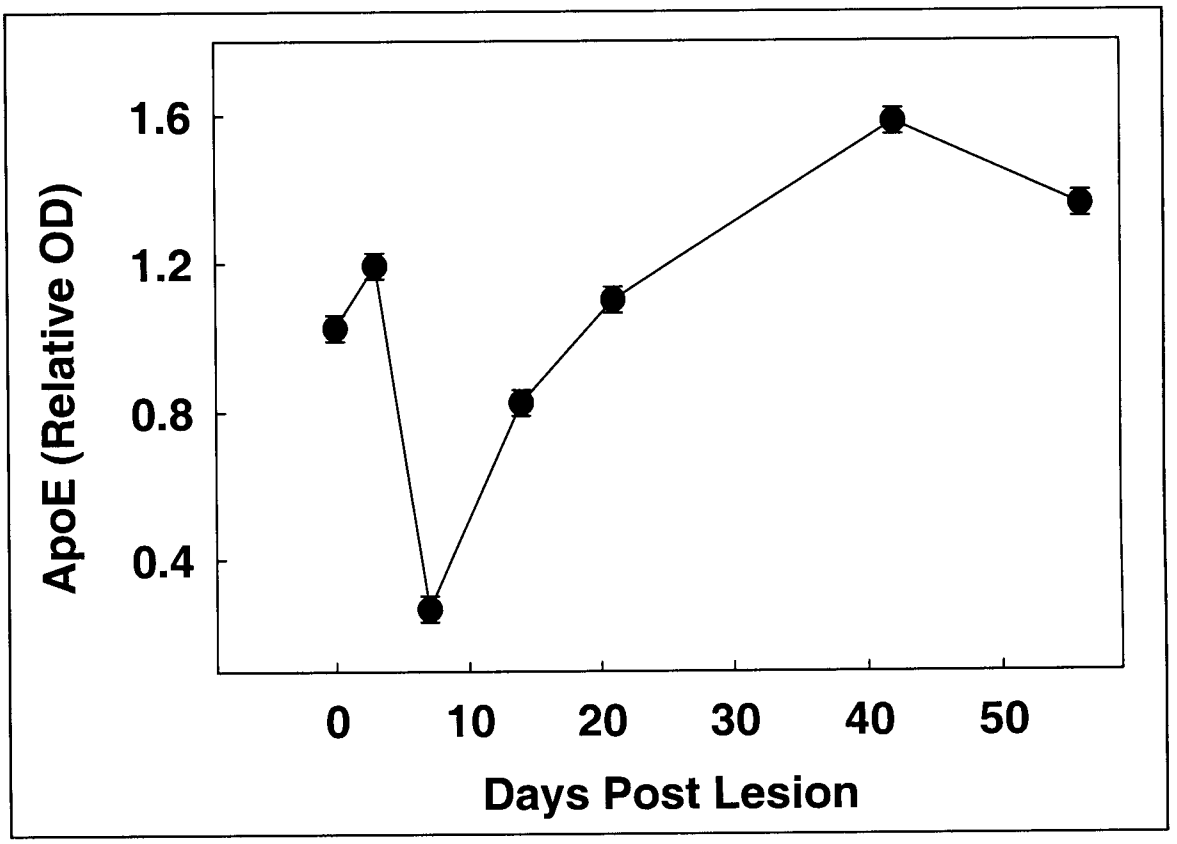

Fig. 3.2b. The relative changes (optical density; means +/- SEM) in the apoE levels post nasal irrigation. All data points were generated by densitometric scans of immunoblots in three separate experiments.

3.3 Expression of OMP is down-regulated in the OE following OE lesioning

Previous studies have shown that OMP level is a reliable marker of ORN maturation following OE lesioning [36, 45]. Therefore, we examined the level of OMP in WT and KO mice OE by immunoblotting. The pattern of expression of OMP in the olfactory epithelium of WT and KO mice following TX-100 lesioning is shown in Figure 3a. Immunoblotting revealed bands for OMP at about 19 kDa in the OE from both WT and KO mice. The observed molecular weight of OMP in this blot is similar to that reported previously for OMP [11, 15]. OMP expression levels were down-regulated for 21 days post lesioning followed by a significant up-regulation at day 42 post lesioning in WT mice as compared to ApoE KO mice (Figure 3.3a). Quantitative assessment of OMP levels in both WT and KO mice olfactory epithelium following TX-100 treatment demonstrated a sharp initial decline in the amounts of OMP and it was significantly up-regulated to exceed the basal level at day 42 in WT mice only (Figure 3.3b).

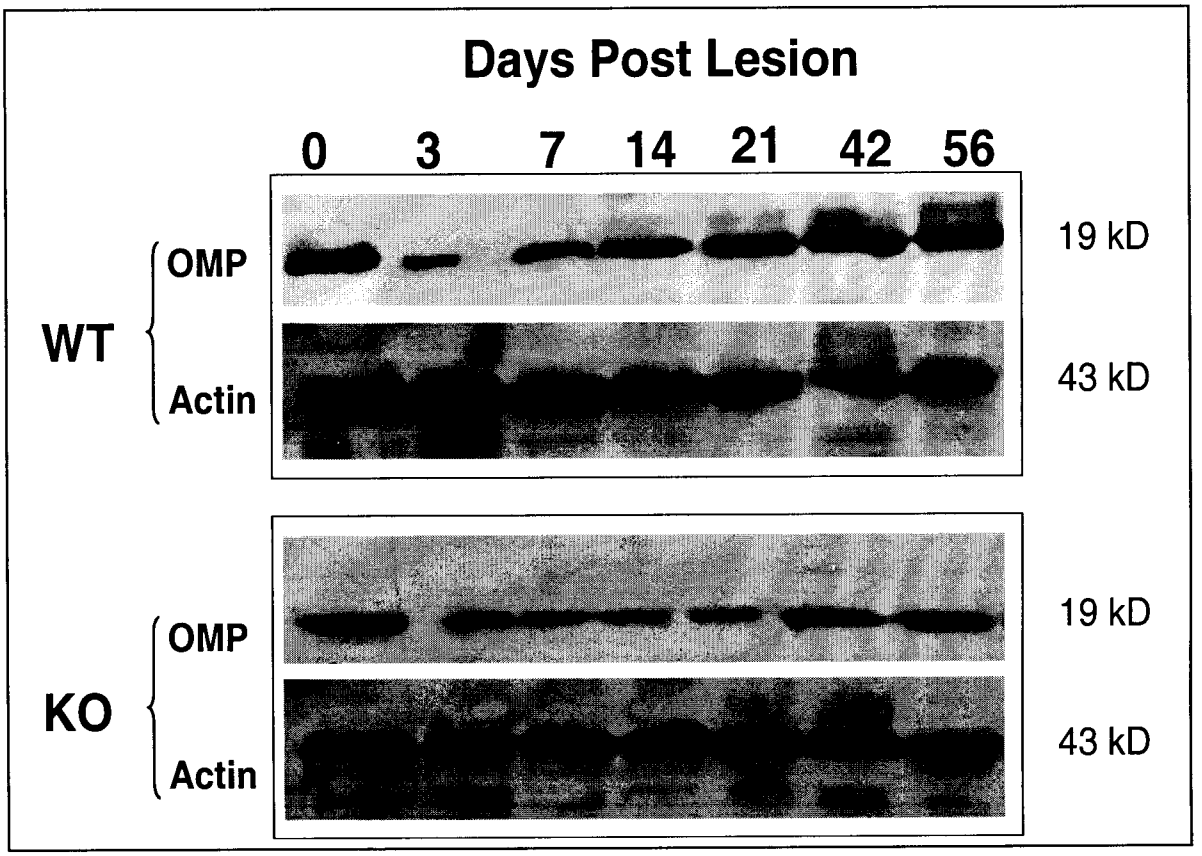

Fig. 3.3a. Time course of OMP in OE following lesioning in mice. OE homogenates collected at indicated times from WT and KO mice following nasal irrigation of TX or saline (SL) were immunoblotted using a polyclonal antibody to OMP as described under Materials and Methods. Actin was used as internal standard for protein loading.

The statistical analysis, Post Hoc pair wise t testing of each data revealed no differences between the level of OMP in OE of WT and KO mice in between individual time point except at day 42 (Figure 3.3b). There was significant effect of genotype at 42 day of WT OE compare to KO OE. However, OMP recovery was significantly more rapid in WT mice compared to KO mice.

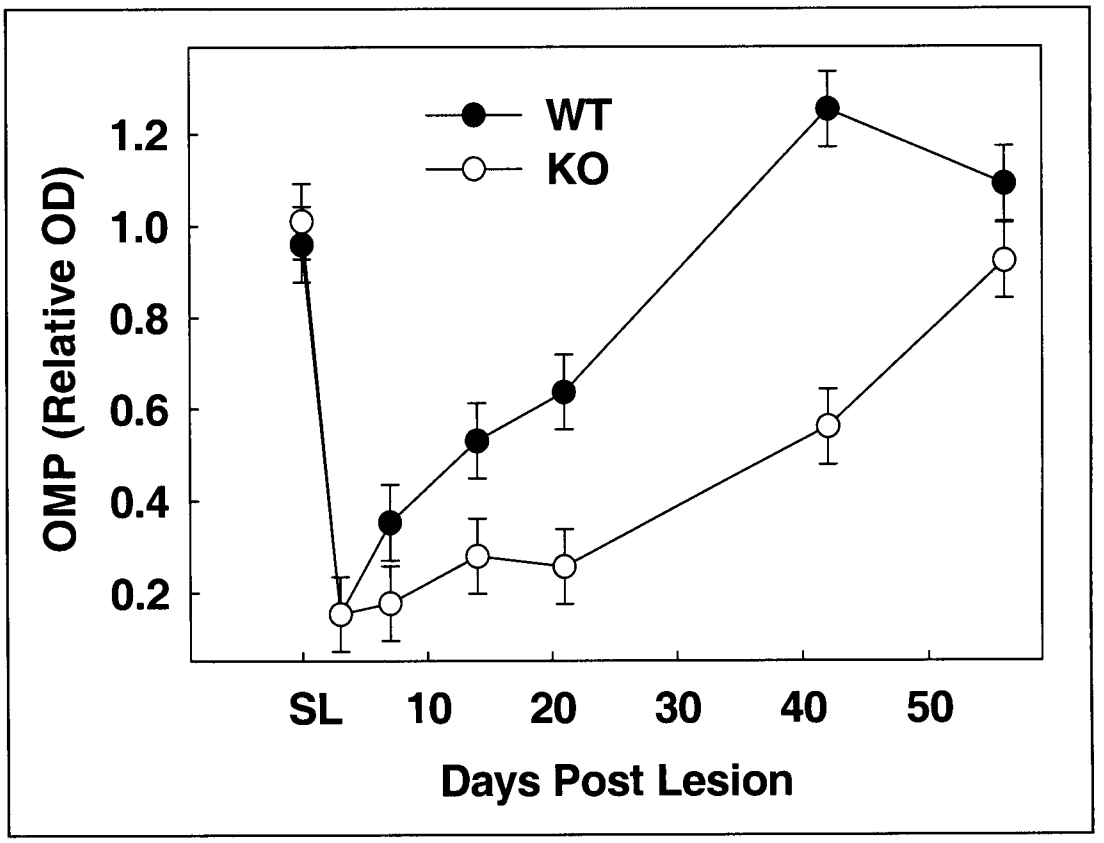

Fig. 3.3b. Post Hoc pair wise t testing of each data survival period found a significant effect of genotype at day 42. The WT showed significantly more OMP than did the KO mice. Other days were not significantly different from one another. The data show that recovery of OMP in the KO mouse is delayed compared to the WT mouse. [Sample size (n) =3, bar indicates Standard Error Mean (SEM)]

3.4. Synaptogensis of ORN with OB neurons is delayed in the olfactory bulb of ApoE deficient mice

In order to mature and survive ORN requires trophic substances. Formation of synapsis by ORN in the olfactory bulb facilitates the uptake of trophic substances. It was hypothesized that apoE is very crucial for the rate of synaptogensis in olfactory bulb. We collected whole OB of both WT and apoE KO mice separately and assayed for levels of expression of synaptophysin day post lesion. We speculated that the rate of synaptogensis would be delayed in OB of apoE KO mice. Immunoblotting revealed bands for Syn at about 48 kDa in the OB from both WT and KO mice (Figure 3.4a). The observed molecular weight of Syn in this blot is similar to that reported previously for Syn [96]. The bands of Syn appeared in immunoblots of WT mice and KO mice were compared at various time points. The result revealed that Syn levels down-regulated sharply between 3 and 7 days post lesioning in both WT and KO mice which is followed by up-regulation towards the basal levels by 56 days post lesion in WT mice only (Figure 3.4b).

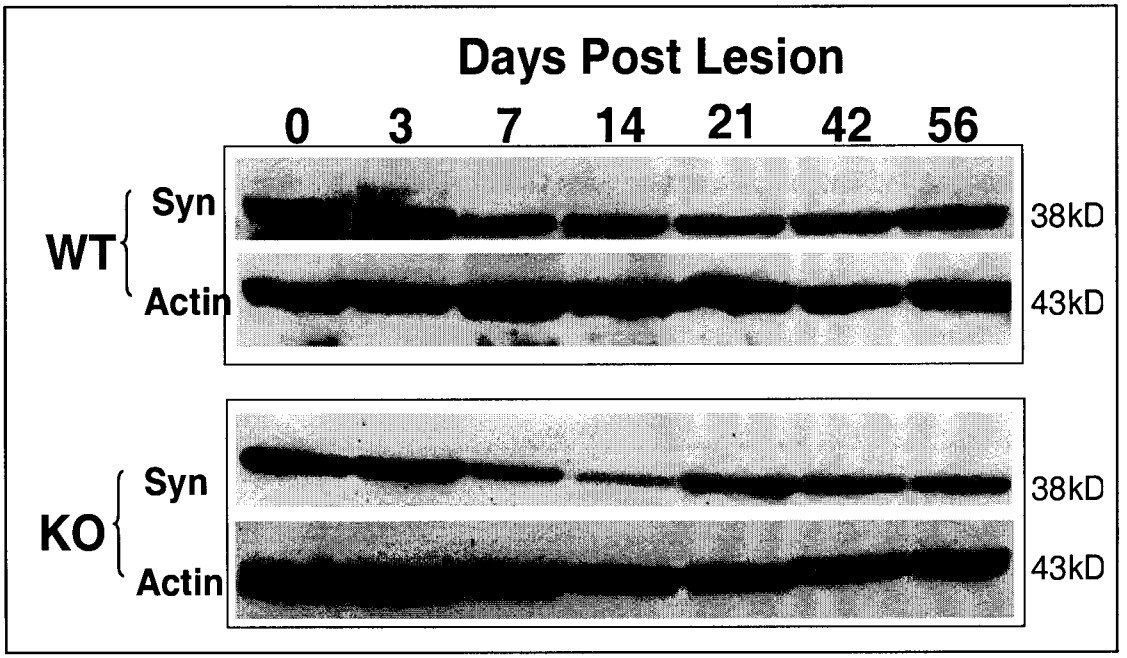

Fig. 3.4a. Immunoblotting of Syn in the OB following OE lesioning in WT and KO mice. OB homogenates (20 μg) collected at the indicated times following nasal irrigation of Triton X-100 were immunoblotted using a polyclonal antibody to Syn as described under Materials and Methods. Actin was used as a loading standard.

Statistical analysis of whole bulb expression of Syn measured by immunoblot revealed a significant interaction between day after lesion and genotype ($F_{6,28}=13.84$; $p<0.001$). Syn levels declined sharply between 3 and 7 days post lesioning in both WT and KO mice. Following this precipitous decline, OB Syn levels steadily increased to about 80% of the normal levels by 56 days post lesion in WT mice. This difference was not significantly different from levels at 0 or 3 days post lesion. In contrast to WT mice, Syn density did not increase significantly in KO mice and was significantly less than that of WT mice on day 56 (Figure 3.4b).

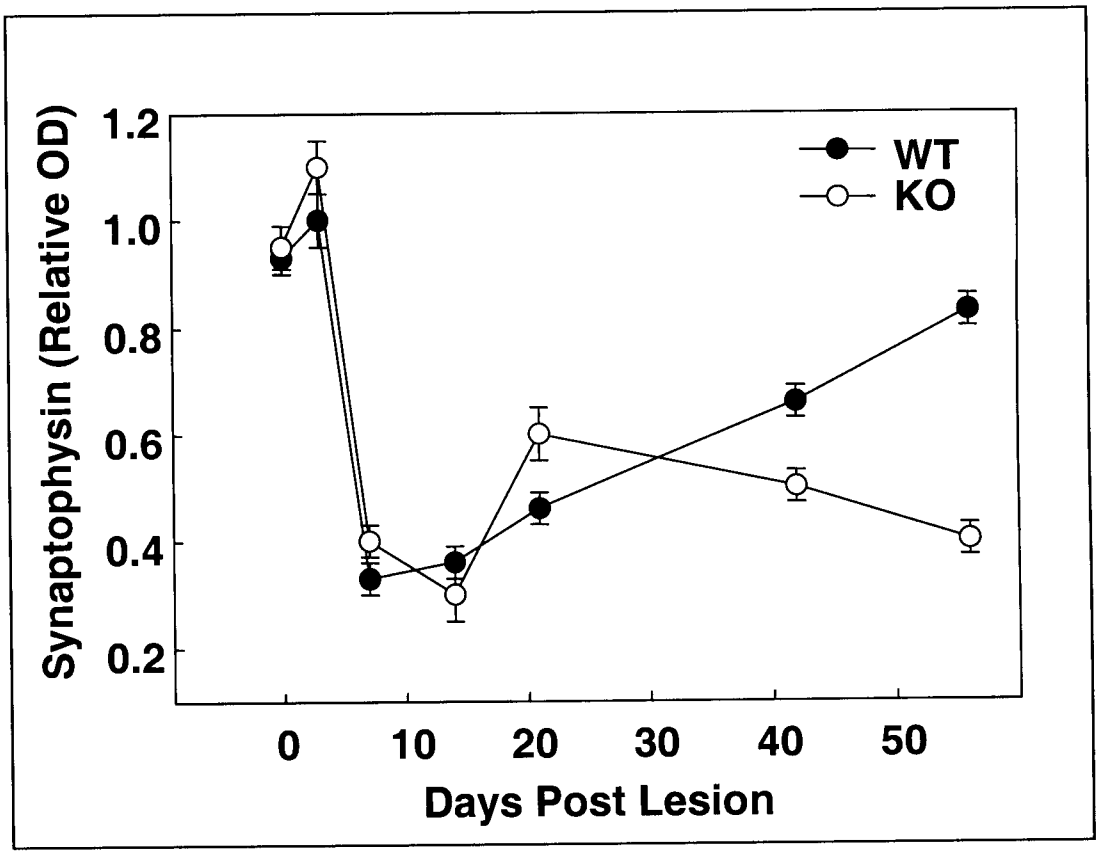

Fig. 3.4b. The relative changes (optical density, OD) in the Syn levels (Mean +/- SE). All data points were generated by densitometric scans of immunoblots in three separate experiments.

3.5. ApoE expression is up-regulated in the olfactory epithelium after bilateral bulbectomy

Our result already expressed the presence of apoE in both OE and OB. We know that in absence of apoE there is delay in regeneration process. We also know that apoE has significant role in Synaptogenesis. To verify whether apoE is dependent on its target for its function we performed bilateral bulbectomy and observed the change in expression of apoE using immunoblotting techniques. Immunoblotting revealed bands for apoE at about 35 kDa in the OE extracts from WT mice. The observed molecular weight of apoE in this blot is similar to that reported previously for apoE [93]. The changes in the expression of apoE following day post bilateral bulbectomy in the mice OE is illustrated in figure 3.5a. Olfactory bulbectomy resulted in a significant increase in the expression of apoE level 3 days post bulbectomy, which further increased by 7 days and remained at high levels thereafter.

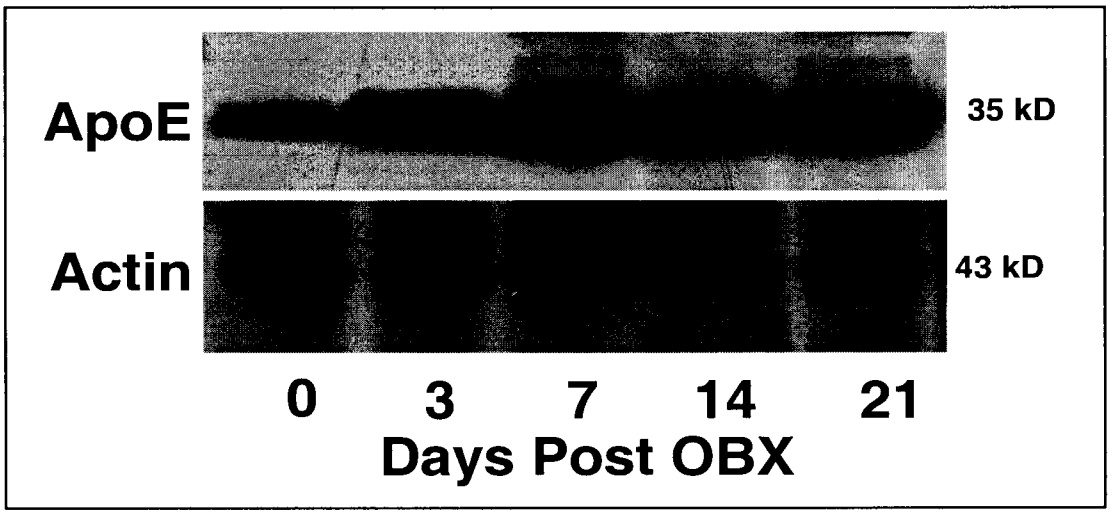

Fig 3.5a. Immunoblotting of apoE in the OE following OBX in mice. (A) OE homogenates collected at indicated times from WT mice following OBX were immunoblotted using a polyclonal antibody to apoE as described under Materials and Methods. Actin was used as internal standard for protein loading.

Days post bulbectomy was significant (F6, 14=144.55; $p<0.05$). Post-hoc testing disclosed that apoE highly upregulated from initial levels after OBX treatment at three days. It increases even more by seven days, and stays high thereafter (Figure 3.5b).

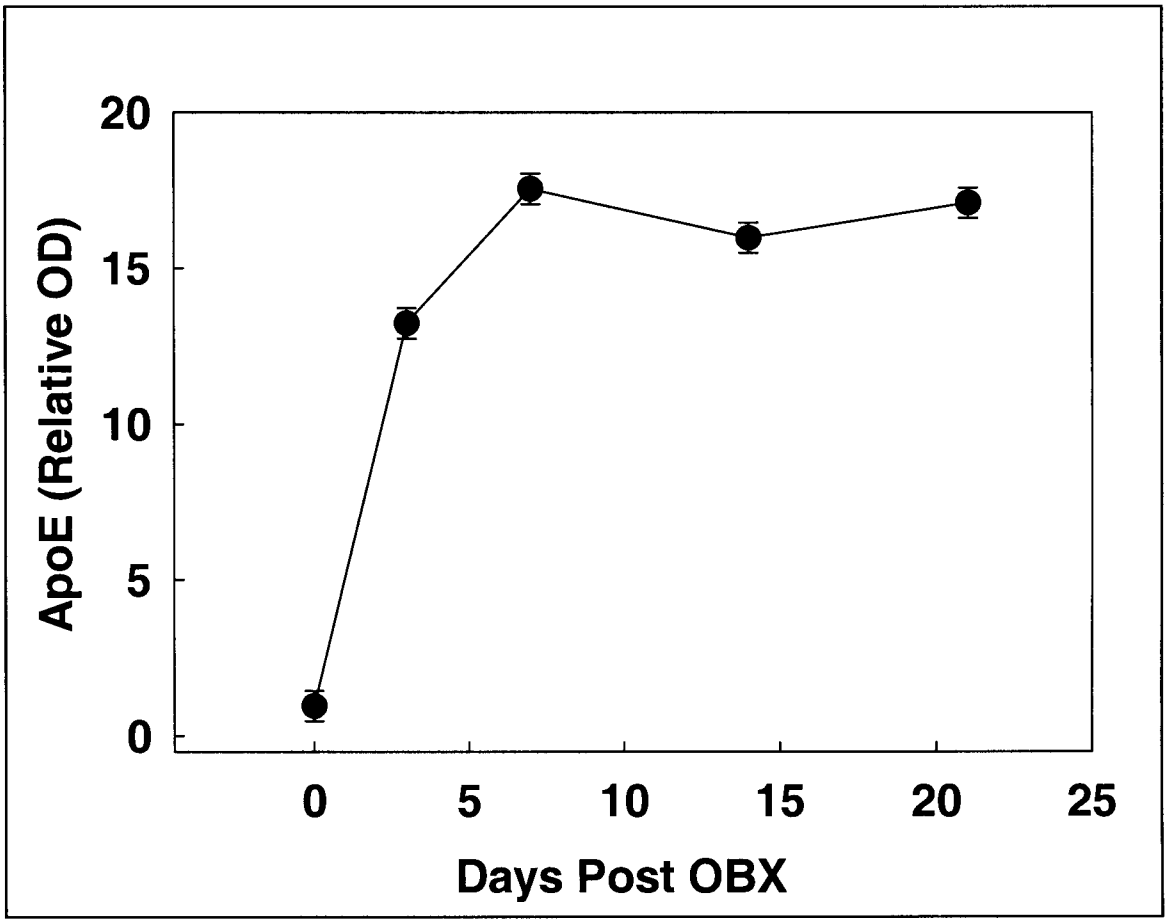

Fig 3.5b. The relative changes (optical density; means +/- SEM) in the apoE levels post OBX. All data points were generated by densitometric scans of immunoblots in three separate experiments.

3.6. Down-regulation of expression of OMP mice olfactory epithelium after bulbectomy

In order to study the level of maturation of ORN after OBX we performed immunoblotting of OE from WT mice. We observed the change in expression of maturation marker (OMP) following day post bulbectomy. Immunoblotting revealed bands for OMP at about 19 kDa in the OE extracts from WT mice. The observed molecular weight of OMP in this blot is similar to that reported previously for OMP [11, 15]. The changes in the expression of OMP following day post bilateral bulbectomy in the mice OE is illustrated in Figure 6a. Olfactory bulbectomy resulted in a significant decrease in the expression of OMP level for 14 days post bulbectomy and it increased slightly at day 21 (Figure 3.6a).

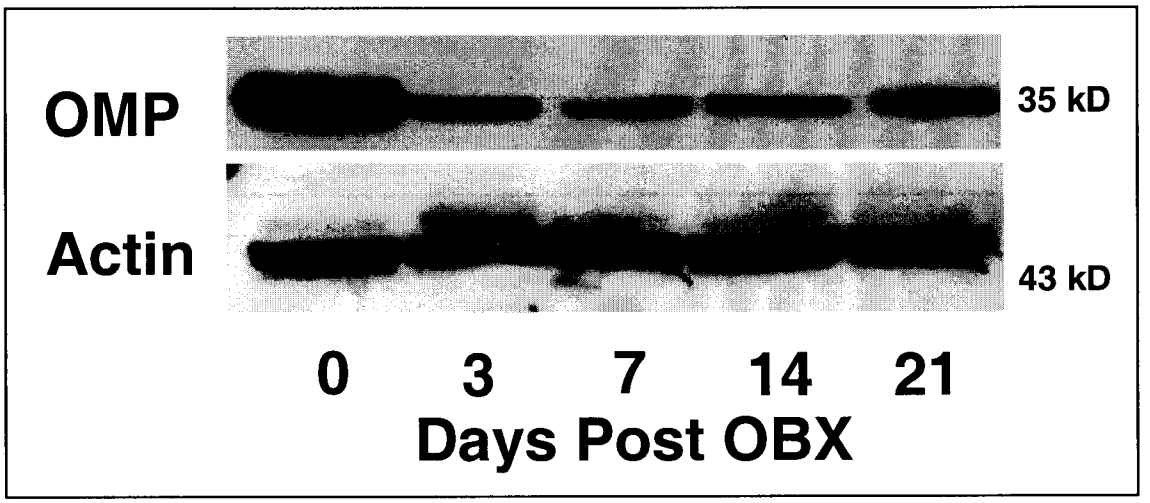

Fig. 3.6a. Immunoblotting of OMP in the OE following OBX in mice. (A) OE homogenates collected at indicated times from WT mice following OBX were immunoblotted using a polyclonal antibody to OMP as described under Materials and Methods. Actin was used as internal standard for protein loading.

Statistical analysis of OMP levels was performed with a one way ANOVA on days post bulbectomy. Initial testing was followed with Bonferroni-corrected t-tests for pair-wise comparisons. Days post bulbectomy was significant ($F_{6, 14}=144.55$; $p<0.05$). Post-hoc testing disclosed that OMP significantly downregulated for 14 days post bulbectomy and it increased slightly at day 21 following OBX compared to basal level (Figure 3.6b).

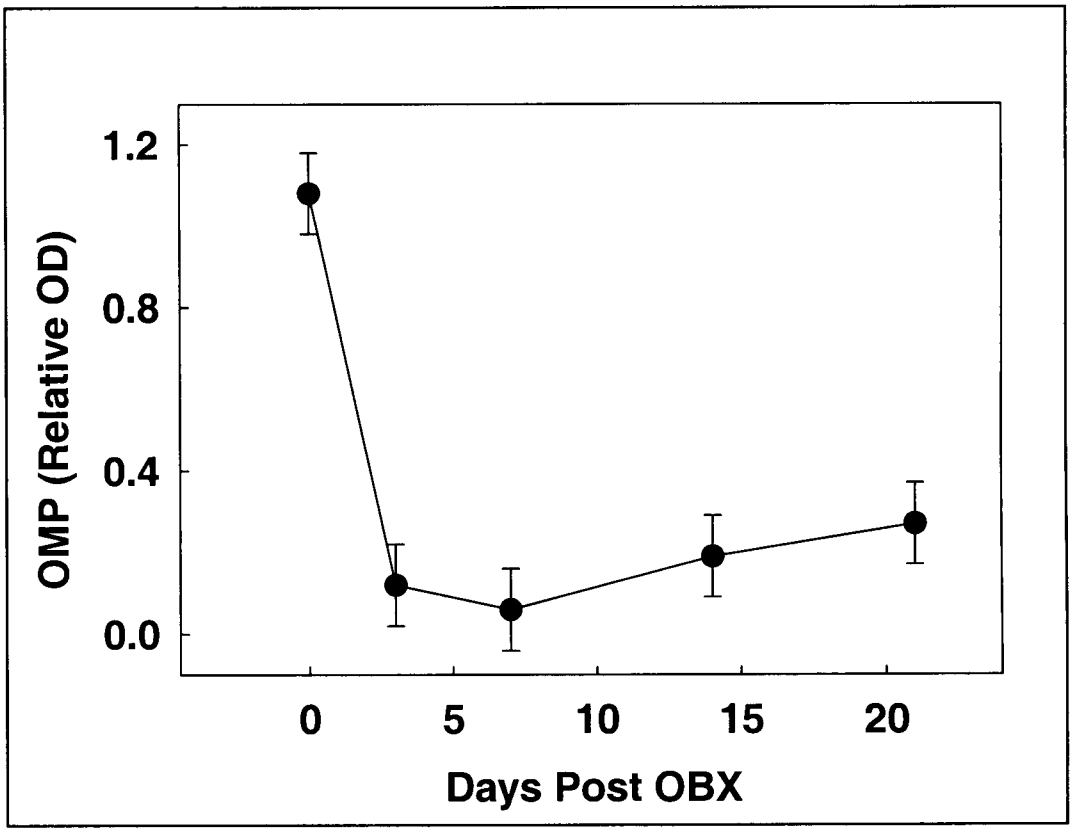

Fig. 3.6b. The relative changes (optical density; means +/- SEM) in the OMP levels post OBX. All data points were generated by densitometric scans of immunoblots in three separate experiments.

4. DISCUSSION

4.1. ApoE is involved in neuronal regeneration and maturation following olfactory epithelial lesioning

Studies done in WT and KO mice for OB localization and time course expression of apoE have revealed the presence of apoE in the OE [34, 100]. Immunohistochemistry studies from our lab on apoE localization and time course expression in the OE of WT and KO have revealed the presence of apoE in OE. In the present study, utilizing immunoblotting techniques it was shown that apoE is also expressed in OE and OB (Figure 3.1), which is supported by previous studies [34, 100]. ApoE was expressed at higher level in OE than in OB of WT mice (Figure 3.1) but as expected was absent in both OE and OB of the apoE KO mice suggesting high level of expression of apoE in OE in WT mice. ApoE and OMP have been routinely used in several labs including our lab as the markers of neuronal regeneration and maturation respectively [11, 15, 41, 52]. In consistent with these data, on OE lesioning the expression of apoE and OMP was evident figure 3.2a and 3.3b. which suggests that the expression of apoE and OMP acts as a function of neuronal regeneration and maturation. The OE regeneration over a period of several weeks has also been reported by others following the intranasal lesioning with triton TX-100, in WT and KO mice [41, 91]. ApoE expression level initially decreased at day 7 post-lesioning and reached to basal level at day 21 and significantly exceeded basal level at days 42 and 56 (Figures 3.2a and 3.2b) which is suggestive of the time dependent neuronal regeneration following lesioning. Statistically significant increase of apoE was observed at days 42 and 56 post lesioning (Figure 3.2b).

This is further supported by the time dependent down-regulation of OMP for 21 days post lesioning followed by a significant increase at day 42 post lesioning in WT mice as compared to ApoE KO mice (Figures 3.3a and 3.3b), which is suggestive of the time dependent maturation of newly formed neurons post lesioning. Statistically significant increase of OMP in WT mice compared to KO mice was observed at day 42 post lesioning (Figure 3.3b). This suggests that the mature ORN had been mostly destroyed and are making a return as the newly formed immature ORN and gets matured by the day 42 post leisioning in WT mice only. As KO mice did not produce significant increase of OMP as compared to WT mice showed that there is delayed recovery in KO mice than in WT mice. Since the function of OMP is yet unknown, it is not tangible to postulate on the possible interaction of apoE and OMP in the regenerating OE. Expression of OMP simply serves as a marker or indicator of a cell type – mature ORN [52, 53, 54].

Initial decrease in level of apoE (as a transporting ligand) in lesioned OE is likely to enhance the recycling of membrane components from degenerating cells, which is supported by previous studies on role of apoE in recycling membrane of degenerating axons and supplying cholesterol to growing axons [26, 32, 40, 94, 95]. ApoE binds to LDL receptors present in neuronal axons, cell bodies and dendrites and internalizes cholesterol molecules packaged as lipoproteins by glial cells for neurite growth [35, 60, 97]. High level of apoE expression was found in OE of WT mice (Figure 3.2a and 3.2b). The significant increase in expression of apoE post OE lesioning suggests increased secretion of apoE in the ORN. Collectively, these corroborative evidences indicate apoE may not have direct role in the rate of maturation of ORN in OE but plays important role in neuronal regeneration.

4.2. ApoE promotes synaptogenesis of ORN with OB neurons post OE lesioning

As olfactory system provides a simple model to quantify synaptic regeneration, we used this model system to evaluate the role of apoE in synaptic reinnervation of the OB following reversible lesioning of the OE. Immunohistochemical studies from our lab and others have shown that apoE is expressed in the olfactory nerve and around the glomeruli in the OB of adult mice [54, 52]. Concurrent with this, using immunoblotting techniques, the expression of apoE in OB was observed (Figure 1.1). The function of apoE in the olfactory system during normal and injury induced remodeling is unclear; however, studies suggest a role for apoE in the synaptogenesis of the CNS [80]. Studies done previously in our lab found that apoE levels in the OB were two fold higher than normal immediately following OE lesion, and remained elevated over a 3-week period when the newly differentiated ORN grow axons and reestablish the synaptic connection with cells in the glomerular [45, 50]. Measures of olfactory nerve regeneration, including OMP and glomerular area recovery were delayed in KO mice [42, 52]. The present study examined the time course of Syn recovery in the OB of WT and apoE KO mice post OE lesioning and it was found that synaptogenesis was delayed in KO mice.

Results from the present study demonstrates that apoE significantly modulates recovery of whole bulb Syn following a reversible OE lesion. After a rapid decline in Syn expression level between 3 to 7 days post lesioning, the WT mice showed a steady recovery that reached statistically significant (80% of the normal) Syn levels by 56 days post lesioning (Figures 3.4a and 3.4b). This difference was not significantly different from Syn levels at 0 or 3 days post lesion (Figure 3.4b), indicating that a significant

recovery occurred at day 56 post lesioning. In contrast, Syn recovery in the KO mice was significantly delayed (Figures 3.4a and 3.4b). KO mice also exhibited rapid decline in Syn expression between 3 to 7 days post lesioning, but it did not show any statistically significant recovery of Syn level for as long as 56 days post leisioning (Figure 3.4b). This corresponds to our previous studies showing slower recovery in OMP and glomerular area in KO mice when compared to WT mice. Hence, in the absence of apoE, synaptic recovery in whole bulb samples is substantially delayed compared to WT mice. This is corroborated by other studies that have found apoE KO mice to be neuropathologically normal, however showing numerous CNS defects, including reduced levels of Syn [87, 86, 88].

Culture studies have shown that the absence of apoE decreases neurite outgrowth and this decrease can be reversed by supplying exogenous apoE [52]. Delay in nerve regeneration has been demonstrated in KO mice using a variety of lesion models [54]. ApoE levels in the OB increases two-fold at 3 days post OE lesion and stay elevated for 21 days post injury. Recovery OMP in the OB of KO is slower than WT post OE lesioning, strongly suggesting a role for apoE in ORN regeneration. Lack of apoE may delay Syn recovery by delaying axonal growth from the newly differentiated ORN in the injured OE. Transynaptic changes known to occur following ON lesion [83] may result in down-regulation of whole bulb Syn. The absence of apoE in KO mice could impair ORN regeneration, which will then indirectly affect Syn recovery in the OB. Overall equivalence of Syn in bulb probably reflects this critical aspect.

The significance of delay in synaptogenesis post injury in apoE KO mice may explain apoE association to several chronic neurological diseases. We have proposed

that the increased risk for these diseases in individuals with dysfunctional apoE isoforms may represent, not a direct effect on the disease, but a slowing of repair and regenerative processes in these individuals. Hence, expression of the disease occurs earlier.

4.3. ApoE is involved in neuronal regeneration and maturation of ORN following removal of the synaptic targets of ORN by OBX

There is an important window of 7-21 days post lesion for ORN in the regenerating OE to differentiate or mature [31, 91, 75]. Taking this into account and the data from lesioning study, the study was conducted to evaluate the expression of apoE and OMP following 0, 3, 7, 14 and 21 day post bulbectomy in WT mice. The regeneration of the olfactory epithelium following OBX was studied using immunobloting techniques, which resulted in a significant increase in the expression of apoE level 3 days post bulbectomy. It further increased by 7 days and remained at high levels thereafter (Figures 3.5a and 3.5b). This significantly increased level of apoE expression is indicative of olfactory nerve regeneration in OE post OBX. The initial decline of apoE is likely to enhance the recycling of membrane components from degenerating cells. Statistically significant up-regulation of apoE was first observed at day 3 post OBX and it increased even more by day 7 and stayed high thereafter (Figure 3.5b). This may be explained by the fact that neurogenesis is independent of the presence of the OB as the ORN target and is expressed excessively to balance the injury made on the whole bulb [91]. The data is in consistent with studies of chronically bulbectomized mice that have not lost the ability of OE regeneration (though it does not fully reconstitute) [75]. Therefore, the study coincides with other studies that have shown

higher rates of apoptosis (cell death) and neurogenesis in OE when ORN from the OE fail to make synaptic connections [9, 10, 28, 76].

It is well documented in bulbectomy studies that there is unusual decline in mature ORN indicated by low level of expression of OMP and high level of expression of GAP-43 indicative of presence of immature ORN [28, 59, 75, 91]. This study found OBX resulting in a significant decrease in the expression of OMP level for 14 days post bulbectomy and it increased slightly at day 21 (Figures 3.6a and 3.6b). Statistically significant down-regulation of OMP was observed for 14 days post OBX and it increased slightly at day 21, which is not statistically significant indicating reappearance of matured ORN greatly delayed following removal of the olfactory bulb (Figure 3.6b). Initial sharp decrease in OMP level indicates that the mature ORN had been mostly destroyed by 14 days post OBX. Slight reappearance of OMP level after day 21 suggests that very few mature ORN are making a return as the new immature ORN begun maturing by the day 21 post bulbectomy but it is not statistically significant suggesting that a delayed maturation is taking place. The data is corroborated by the study that showed the production of OMP mRNA by newly formed olfactory neurons even in absence of its olfactory target [68].

Significant increase in apoE level in the OE following bulbectomy indicates that removal of synaptic targets of ORN by OBX could result in increased rate of regeneration of ORN. Also, a significant decrease of OMP expression was observed, however, significant up-regulation of OMP expression following OBX was failed to observe suggesting that matured ORN are destroyed following OBX but they are not fully recovered as matured neurons over time. This is in congruence with the previously

published studies on bulbectomy [75]. Therefore, suggesting the importance of olfactory bulb in maintenance of basal level of OMP in ORN [11]. Since function of OMP is yet unknown, it is not tangible to postulate the possible interaction of apoE and OMP in the regenerating OE. Therefore, expression of OMP simply serves as a marker for mature ORN. This collectively suggests that although maturation of ORN is delayed, apoE is involved in regeneration of ORN following removal of it synaptic targets by OBX.

5. CONCLUSION

The data revealed that: (1) ApoE is expressed both in olfactory epithelium as well as in olfactory bulb; (2) expression of ApoE is up-regulated in the OE following OE lesioning suggesting that apoE is involved in regeneration of ORN; (3) maturation of ORN, as measured by OMP is significantly large in WT mice as compared to KO mice post lesioning; (4) 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; (5) expression of ApoE is up-regulated in the OE following bulbectomy suggesting that apoE is involved in regeneration of ORN; (6) maturation of ORN, as measured by OMP is significantly delayed in the mice receiving bulbectomy.

In conclusion, our data suggest that apoE is an important factor in the regeneration, maturation and synaptic formation of ORN by (1) enhancing the neurogenesis and maintenance of ORN in the OE and (2) facilitating unimpeded synaptogenesis of ORN in the OB after injury by increasing transport of essential and rate-limiting cholesterol and lipid molecules to the site of synaptic formation. Based upon this, we can propose a model system of neuronal reinnervations in which the ability of an injured or regenerating ORN to complete the process of repair or growth is highly dependent upon availability of exogenous lipids or cholesterol. This is because intracellular availability of lipids and cholesterol molecules are highly limited during the stress of repair or synaptic remodeling. In this condition, apoE, a very efficient transporter of cholesterol and lipoproteins, would play a crucial role by rapidly internalizing cholesterol in to the reinnervating neurons. Absence of apoE or its failure to perform (isoforms defect) its function would hinder the crucial task of rapidly

internalizing lipids and cholesterol, which would ultimately result in delayed regeneration, maturation and synaptic formation of stressed ORN.

REFERENCES

1) Alberts, M.J., Graffagnino, C., McClenny, C., DeLong, D., Strittmatter, W., Saunders, A.M. and Roses, A.D., ApoE genotype and survival from intracerebral haemorrhage, *Lancet*, 346 (1995) 575.

2) Anderson, R., Barnes, J.C., Bliss, T.V., Cain, D.P., Cambon, K., Davies, H.A., Errington, M.L., Fellows, L.A., Gray, R.A., Hoh, T., Stewart, M., Large, C.H. and Higgins, G.A., Behavioural, physiological and morphological analysis of a line of apolipoprotein E knockout mouse, *Neuroscience*, 85 (1998) 93-110.

3) Arendt, T., Schindler, C., Bruckner, M.K., Eschrich, K., Bigl, V., Zedlick, D. and Marcova, L., Plastic neuronal remodeling is impaired in patients with Alzheimer's disease carrying apolipoprotein epsilon 4 allele. *J Neurosci*, 17 (1997) 516-529.

4) Bauer, S., Rasika, S., Han, J., Mauduit, C., Raccurt, M., Morel, G., Jourdan, F., Benahmed, M., Moyse, E. and Patterson, P.H., Leukemia inhibitory factor is a key signal for injury-induced neurogenesis in the adult mouse olfactory epithelium, *J Neurosci*, 23 (2003) 1792-1803.

5) Bellosta, S., Nathan, B.P., Orth, M., Dong, L.M., Mahley, R.W. and Pitas, R.E., Stable expression and secretion of apolipoproteins E3 and E4 in mouse neuroblastoma cells produces differential effects on neurite outgrowth, *J Biol Chem*, 270 (1995) 27063-17071.

6) Borghini, I., Barja, F., Pometta, D. and James, R.W., Characterization of subpopulations of lipoprotein particles isolated from human cerebrospinal fluid, *Biochim Biophys Acta*, 1255 (1995) 192-200.

7) Boyles, J.K., Zoellner, C.D., Anderson, L.J., Kosik, L.M., Pitas, R.E., Weisgraber, K.H., Hui, D.Y., Mahley, R.W., Gebicke-Haerter, P.J., Ignatius, M.J. and et al., A role for apolipoprotein E, apolipoprotein A-I, and low density lipoprotein receptors in cholesterol transport during regeneration and remyelination of the rat sciatic nerve, *J Clin Invest*, 83 (1989) 1015-1031.

8) Buttini, M., Orth, M., Bellosta, S., Akeefe, H., Pitas, R.E., Wyss-Coray, T., Mucke, L., and Mahley, R.W., Expression of human apolipoprotein E3 or E4 in the brains of Apoe−/− mice: isoform-specific effects on neurodegeneration, *J. Neurosci*, 19 (1999) 4867–4880.

9) Calof, A.L. and Murray, R.C., Neuronal regeneration: Lessons from the olfactory system, *Cell & Dev Bio,* 10 (1999) 421-431.

10) Calof, A.L., Hagiwara, N., Holocomb, D.J., and Mumm, J.S., Neurogenesis and cell death in olfactory epithelium, *J Neurobio,* 30 (1996) 67-81.

11) Chuah M.I., and Farbman, A.I., Olfactory bulb increase marker protein in olfactory receptor cells. *Journal of Neuroscience*, 3 (1983) 2197-2205.

12) Corder, E.H., Saunders, A.M., Strittmatter, W.J., Schmechel, D.E., Gaskell, P.C., Small,G.W., Roses, A.D., Haines, J.L., and Pericak-Vance, M.A., Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families., *Science,* 261 (1993) 921-923.

13) Crews,L., and Hunter, D., Neurogenesis in olfactory epithelium. *Prospectives on Developmental Neurobiology*, 2 (2) (1994) 151-161.

14) Farbman A.I., Developmental neurobiology of the taste system. In smell and taste in health and disease .*Raven Press, New York*, (1991) 19-34

15) Farbman, A.I., and Margolis, F.L., Olfactory marker protein during ontongeny: Immunohistochemical localization. *Developmental Biology,* 74 (1980) 205-215.

16) Friedman, G., Froom, P., Sazbon, L., Grinblatt, I., Shochina, M., Tsenter, J., Babaey, S., Yehuda, B. and Groswasser, Z., Apolipoprotein E-epsilon4 genotype predicts a poor outcome in survivors of traumatic brain injury, *Neurology*, 52 (1999) 244-248.

17) Fullerton, S.M., Shirman, G.A., Strittmatter, W.J. and Matthew, W.D., Impairment of the blood-nerve and blood-brain barriers in apolipoprotein e knockout mice, *Exp Neurol*, 169 (2001) 13-22.

18) Fullerton, S.M., Strittmatter, W.J. and Matthew, W.D., Peripheral sensory nerve defects in apolipoprotein E knockout mice, *Exp Neurol*, 153 (1998) 156-163.

19) G.W., Roses, A.D., Haines, J.L. and Pericak-Vance, M.A., Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families. *Science*, 261 (1993) 921-923.

20) Gandy, S., Stafstorm, C., Turner, B., Sweeney, D., Breslow, J., Greengard, P., Smith, J., Ouimet, C., Aged apoE deficient mice: Abnormal learning and glial markers but no obvious neuropathology., *Soc. Neur. Abstr.*, 21 (1995) 5.

21) Goodrum, J.F., Bouldin, T.W., Zhang, S.H., Maeda, N. and Popko, B., Nerve regeneration and cholesterol reutilization occur in the absence of apolipoproteins E and A-I in mice, *J Neurochem*, 64 (1995) 408-416.

22) Goodrum, J.F., Weaver, J.E., Goines, N.D. and Bouldin, T.W., Fatty acids from degenerating myelin lipids are conserved and reutilized for myelin synthesis during regeneration in peripheral nerve, *J Neurochem*, 65 (1995) 1752-1759.

23) Graziadei, P.P. and Graziadei, G.A., Neurogenesis and neuron regeneration in the olfactory system of mammals. I. Morphological aspects of differentiation and structural organization of the olfactory sensory neurons, *J Neurocytol*, 8 (1979) 1-18.

24) Graziadei, P.P. and Monti Graziadei, G.A., Neurogenesis and plasticity of the olfactory sensory neurons, *Ann N Y Acad Sci*, 457 (1985) 127-42.

25) Greer, C.A., Structural Organization of the Olfactory System. In T.V. Getchell, R.L. Doty, L.M. Bartoshuk, J.B. Snow, C. Pfaffmann and H.B. P. (Eds.), *Smell and Taste in Health and Disease.*, Raven Press NY, NY, 1991, pp. 65-81.

26) Hayashi H., Igbavboa U., Hamanaka H., Kobayashi M., Fujita S.C., Wood W.G. and Yanagisawa K., Cholesterol is increased in the exofacial leaflets of synaptic plasma membranes of human apolipoprotein E4 knock-in mice, *NeuroReport* 13 (2002) 383-386.

27) Herz, J., Lipoprotein receptors: beacons to neurons?, *Trends Neurosci*, 24 (2001) 193-195.

28) Holcomb, J.D., Mumm, J.S. and Calof, A.L., Apoptosis in the neuronal lineage of the mouse olfactory epithelium: regulation in vivo and in vitro, *Dev Biol*, 172 (1995) 307-23.

29) Holtzman, D.M., Pitas, R.E., Kilbridge, J., Nathan, B., Mahley, R.W., Bu, G. and Schwartz, A.L., Low density lipoprotein receptor-related protein mediates apolipoprotein E-dependent neurite outgrowth in a central nervous system-derived neuronal cell line, *Proc Natl Acad Sci U S A*, 92 (1995) 9480-9484.

30) Huang, D.Y., Goedert, M., Jakes. R., Weisgraber, K.H., Garner, C.C., Saunders, A.M., Pericak-Vance M.A., Schmechel, D.E., Roses, A.D., and Strittmatter W.J., Isoform-specific interactions of apolipoprotein E with the microtubule-associated protein MAP2c: implications for Alzheimer's disease. *Neurosci Lett,* 182 (1994) 55-58.

31) Hurtt, M.E., Thomas, D.A., Working, P.K., Monticello, T.M. and Morgan, K.T., Degeneration and regeneration of the olfactory epithelium following inhalation exposure to methyl bromide: pathology, cell kinetics, and olfactory function, *Toxicol Appl Pharmacol*, 94 (1988) 311-28.

32) Ignatius, M.J., Gebicke-Haerter, P.J., Pitas, R.E. and Shooter, E.M., Apolipoprotein E in nerve injury and repair, *Prog Brain Res*, 71 (1987) 177-84.

33) Ignatius, M.J., Gebicke-Harter, P.J., Skene, J.H., Schilling, J.W., Weisgraber, K.H., Mahley, R.W. and Shooter, E.M., Expression of apolipoprotein E during nerve degeneration and regeneration, *Proc Natl Acad Sci U S A*, 83 (1986) 1125-9.

34) Ignatius, M.J., Shooter, E.M., Pitas, R.E. and Mahley, R.W., Lipoprotein uptake by neuronal growth cones in vitro, *Science*, 236 (1987) 959-62.

35) Ji Z.S., Dichek H.L., Miranda R.D. and Mahley R.W. Heparan sulfate proteoglycans participate in hepatic lipaseand apolipoprotein E-mediated binding and uptake of plasma lipoproteins, including high density lipoproteins. *J Biol Chem.* 272 (1997) 31285-31292.

36) Jordan, B.D., Relkin, N.R., Ravdin, L.D., Jacobs, A.R., Bennett, A. and Gandy, S., Apolipoprotein E epsilon4 associated with chronic traumatic brain injury in boxing, *Jama*, 278 (1997) 136-40.

37) Kutner, K.C., Erlanger, D.M., Tsai, J., Jordan, B. and Relkin, N.R., Lower cognitive performance of older football players possessing apolipoprotein E epsilon4, *Neurosurgery*, 47 (2000) 651-7; discussion 657-8.

38) Lopez-Sanchez, E., Frances-Munoz, E., Diez-Juan, A., Andres, V., Menezo, J.L. and Pinazo-Duran, M.D., Optic nerve alterations in apolipoprotein E deficient mice, *Eur J Ophthalmol*, 13 (2003) 560-5.

39) Mackay-Sim, A., and Kittel, P. On the life span of olfactory receptor neurons. *European Journal of neuroscience*, 3 (1991) 209-215.

40) Mahley, R.W., Apolipoprotein E: Cholesterol transport protein with expanding role in cell biology. *Science*, 240 (1988) 622-630.

41) Margolis, F.L., Roberts, N., Ferriero, D. and Feldman, J., Denervation in the primary olfactory pathway of mice: biochemical and morphological effects, *Brain Res*, 81 (1974) 469-483.

42) Masliah, E., Mallory, M., Ge, N., Alford, M., Veinbergs, I. and Roses, A.D., Neurodegeneration in the central nervous system of apoE-deficient mice, *Exp Neurol*, 136 (1995) 107-122.

43) Masliah, E., Mallory, M., Veinbergs, I., Miller, A. and Samuel, W., Alterations in apolipoprotein E expression during aging and neurodegeneration, *Prog Neurobiol*, 50 (1996) 493-503.

44) Masliah, E., Samuel, W., Veinbergs, I., Mallory, M., Mante, M. and Saitoh, T., Neurodegeneration and cognitive impairment in apoE-deficient mice is ameliorated by infusion of recombinant apoE, *Brain Res*, 751 (1997) 307-314.

45) Mauch, D.H., Nagler, K., Schumacher, S., Goritz, C., Muller, E.C., Otto, A. and Pfrieger, F.W., CNS synaptogenesis promoted by glia-derived cholesterol, *Science*, 294 (2001) 1354-1357.

46) Mayeux, R., Ottman, R., Maestre, G., Ngai, C., Tang, M.X., Ginsberg, H., Chun, M., Tycko, B. and Shelanski, M., Synergistic effects of traumatic head injury and apolipoprotein-epsilon 4 in patients with Alzheimer's disease [see comments], *Neurology*, 45 (1995) 555-557.

47) Morrison, E.E., Graziadei, P.P., Transplants of olfactory mucosa in the rat brain I. A light microscopic study of transplant organization. *Brain Res,* 279 (1983) 241-245.

48) Nakai, M., Kawamata, T., Taniguchi, T., Maeda, K. and Tanaka, C., Expression of apolipoprotein E Mrna in rat microglia. ***Neurosci Lett,*** 211 (1996) 41-44.

49) Nakai, M., Kawamata, T., Taniguchi, T., Maeda, K. and Tanaka, C., Expression of apolipoprotein E Mrna in rat microglia. ***Neurosci Lett,*** 211 (1996) 41-44. Mahley, R.W., Apolipoprotein E: Cholesterol transport protein with expanding role in cell biology. *Science*, 240 (1988) 622-630.

50) Nathan, B.P., Bellosta, S., Sanan, D.A., Weisgraber, K.H., Mahley, R.W., and Pitas, R.E., Differential effects of apolipoprotein E3 and E4 on neuronal growth in vitro. *Science,* 264 (1994) 850-852.

51) Nathan, B.P., Chang, K.C., Bellosta, S., Brisch, E., Ge, N., Mahley, R.W., and Pitas, R.E., The inhibitory effect of apolipoprotein E4 on neurite outgrowth is associated with microtubule depolymerization. *J Biol Chem*, 270 (1995) 19791-19799.

52) Nathan, B.P., Nisar, R., Beckman-Randall, S., Short, J., Sherrow, M. and Struble, R.G., Role of apoE in olfactory nerve regeneration in mice, *Soc. Neurosci. Abstr.*, 26 (2000).

53) Nathan, B.P., Nisar, R., Randall, S., Short, J., Sherrow, M., Wong, G.K. and Struble, R.G., Apolipoprotein E is upregulated in olfactory bulb glia following peripheral receptor lesion in mice, *Exp Neurol*, 172 (2001) 128-36.

54) Nathan, B.P., Nisar, R., Short, J., Randall, S., Grissom, E., Griffin, G., Switzer, P.V., Struble RG., Delayed olfactory nerve regeneration in ApoE-deficient mice. *Brain Res,* 11; 1041(1) (2005):87-94.

55) Newman, M.P., Feron, F., and Mackay-Sim A., Growth factor regulation of neurogenesis in adult olfactory epithelium. *Neuroscience,* 99(2) (2000) 333-350.

56) Ohm, T.G., Scharnagl, H., Marz, W. and Bohl, J., Apolipoprotein E isoforms and the development of low and high Braak stages of Alzheimer's disease-related lesions, *Acta Neuropathol (Berl)*, 98 (1999) 273-80.

57) Overmyer, M., Helisalmi, S., Soininen, H., Laakso, M., Riekkinen, P., Sr. and Alafuzoff, I., Astrogliosis and the ApoE genotype. an immunohistochemical study of postmortem human brain tissue. *Dement Geriatr Cogn Disord,* (1999) 252-257.

58) Page. K.J., Hollister, R.D., and Hyman, B.T., Dissociation of apolipoprotein and apolipoprotein receptor response to lesion in the rat brain: an in situ hybridization study. *Neuroscience*, 85 (1998) 1161-1171.

59) Pasterkamp, R.J., De Winter, F., Holtmaat, A.J., Verhaagen, J., Evidence for a role of the chemorepellent semaphorin III and its receptor neuropilin-1 in the regeneration of primary olfactory axons, *J Neurosci,* 18 (1998) 9962-76.

60) Pitas, R.E., Boyles, J. K., Lee, S. H., Foss, D. & Mahley, R. W., Astrocytes synthesize apolipoprotein E and metabolize apolipoprotein E- containing lipoproteins., *Biochim Biophys Acta*, 917 (1987) 48-61.

61) Pitas, R.E., Boyles, J.K., Lee, S.H., Hui, D. and Weisgraber, K.H., Lipoproteins and their receptors in the central nervous system. Characterization of the lipoproteins in cerebrospinal fluid and identification of apolipoprotein B,E(LDL) receptors in the brain, *J Biol Chem*, 262 (1987) 14352-60.

62) Poirier, J., Apolipoprotein E in animal models of CNS injury and in Alzheimer's disease. Trends *Neurosci*, 17 (1994) 525-530.

63) Poirier, J., Baccichet, A., Dea, D. and Gauthier, S., Cholesterol synthesis and lipoprotein reuptake during synaptic remodelling in hippocampus in adult rats, *Neuroscience*, 55 (1993) 81-90.

64) Poirier, J., Hess, M., May, P.C. and Finch, C.E., Astrocytic apolipoprotein E mRNA and GFAP mRNA in hippocampus after entorhinal cortex lesioning. *Brain Res Mol Brain Res*, 11 (1991) 97-106.

65) Popko, B., Goodrum, J.F., Bouldin, T.W., Zhang, S.H. and Maeda, N., Nerve regeneration occurs in the absence of apolipoprotein E in mice, *J Neurochem*, 60 (1993) 1155-1158.

66) Rall, S.C., Jr., Weisgraber, K.H., and Mahley, R.W., Human apolipoprotein E. The complete amino acid sequence. *J Bio. Chem,* 257 (1982) 4171-4178.

67) Rochel, S. and Margolis, F.L., The response of ornithine decarboxylase during neuronal degeneration and regeneration in olfactory epithelium, *J Neurochem*, 35 (1980) 850-860.

68) Rogers, K. E., Dasgupta, P., Gubler, U., Grillo, M., Khew-Goodall, Y. S., and Margolis F. L., *PNAS,* 84 (1987)1704-1708

69) Ronnett, G.V., Pevsner, J., Jeon, O., Tu, Y., Palmer, A.M., Matarazzo, V., Cohen, D.S., Expression of MeCP2 in olfactory receptor neurons is developmentally regulated and occurs before synaptogenesis, *Mol & Cell Neuro,* 22 (2003) 417-429.

70) Roses, A.D., Apolipoprotein E, a gene with complex biological interactions in the aging brain, *Neurobiol Dis*, 4 (1997) 170-185.

71) Roskams, A.J., Bethel, M.A., Hurt K.J., Ronnett, G.V., Sequential expression of Trks A, B, and C in the regenerating olfactory neuroepithelium, *J Neurosci.*, 16 (1996) 1294-1307.

72) Saunders, A. M., Strittmatter, W. J., Schmechel, D., St. George-Hyslop, P. H., Pericak-Vance, M. A., Joo, S. H., Rosi, B. L., Gussella, J. F., Crapper-McLachlan, D., Alberts, M. J., Hulette, C., Crain, B., Goldgaber, D. and Roses, A.

D., Association of apolipoprotein E allele 4 with late-onset familial and sporadic Alzheimer's disease. *Neurology,* 43 (1993) 1467–1472.

73) Schmechel, D.E., Saunders, A.M., Strittmatter, W.J., Crain, B.J., Hulette, C.M., Joo, S.H., Pericak-Vance, M.A., Goldgaber, D. and Roses, A.D., Increased amyloid beta-peptide deposition in cerebral cortex as a consequence of apolipoprotein E genotype in late-onset Alzheimer disease, *Proc Natl Acad Sci U S A*, 90 (1993) 9649-9653.

74) Schwob J.E., Stages in the differentiation of olfactory sensoryneurons, *Chem Senses* 16 (1991) 579-594.

75) Schwob, J.E., Mieleszko-Szumowski K.E. and Stasky, A.A., The Olfactory sensory neurons are trophically dependent on the olfactory bulb for their prolonged survival, *J Neurosci*, 12 (1992) 3896-3919.

76) Schwob, J.E., Youngentob, S.L. and Mezza, R.C., Reconstitution of the rat olfactory epithelium after methyl bromide- induced lesion, *J Comp Neurol*, 359 (1995) 15-37.

77) Sheng, H., Laskowitz, D.T., Bennett, E., Schmechel, D.E., Bart, R.D., Saunders, A.M., Pearlstein, R.D., Roses A.D. and Warner, D.S., Apolipoprotein E isoform-specific differences in outcome from focal ischemia in transgenic mice, *J. Cereb. Blood Flow Metab*, 18 (1998) 361–366.

78) Shepherd, G.M. and Greer, C.A., Olfactory Bulb. In G.M. Shepherd (Ed.), *The Synaptic Organization of the Brain*, Oxford Univ. Press, New York, 1998, pp. 159-203.

79) Snipes, G.J., McGuire, C.B., Norden, J.J., and Freeman, J.A., Nerve injury stimulates the secretion of apolipoprotein E by nonneuronal cells. *Proc Natl Acad Sci USA* 83 (1986) 1130-1134.

80) Stone, D.J., Rozovsky, I., Morgan, T.E., Anderson, C.P., and Finch, C.E., Increased synaptic sprouting in response to estrogen via an apolipoprotein mechanism: implications for Alzheimer's disease. *J Neuroscience* 18 (1998) 3180-3185.

81) Strittmatter, W.J. and Roses, A.D., Apolipoprotein E and Alzheimer's disease, *Annu Rev Neurosci*, 19 (1996) 53-77.

82) Strittmatter, W.J., Weisgraber, K.H., Goedert, M., Saunders, A.M., Huang, D., Corder, E.H., Dong, L.M., Jakes, R., Alberts, M.J., Gilbert, J.R. and et al. Hypothesis: microtubule instability and paired helical filament formation in the Alzheimer disease brain are related to apolipoprotein E genotype. *Exp Neurol*, 125 (1994) 163-171; discussion 172-164.

83) Struble, R.G., Beckman, S.L., Fesser, E. and Nathan, B.P., Volumetric and horseradish peroxidase tracing analysis of rat olfactory bulb following reversible olfactory nerve lesions, *Chem Senses*, 26 (2001) 971-81.

84) Tang, G., Xie, H., Xu, L., Hao, Y., Lin, D. and Ren, D., Genetic study of apolipoprotein E gene, alpha-1 antichymotrypsin gene in sporadic Parkinson disease, *Am J Med Genet*, 114 (2002) 446-9.

85) Teasdale, G.M., Nicoll, J.A., Murray, G. and Fiddes, M., Association of apolipoprotein E polymorphism with outcome after head injury, *Lancet*, 350 (1997) 1069-71.

86) Teter, B. and Ashford, J.D., Review: Neuroplasticity in Alzhemier's Disease, *J Neurosci Res*, 70 (2002) 402-437.

87) Veinbergs, I. and Masliah, E., Synaptic alterations in apolipoprotein E knockout mice, *Neuroscience*, 91 (1999) 401-403.

88) Veinbergs, I., Mante, M., Jung, M.W., Van Uden, E. and Masliah, E., Synaptotagmin and synaptic transmission alterations in apolipoprotein E-deficient mice, *Prog Neuropsychopharmacol Biol Psychiatry*, 23 (1999) 519-531.

89) Verhaagen, J., Greer, C.A., and Margolis, F.L., B50/GAP43 gene expression in the rat olfactory system during postnatal development and aging. *European Journal of Neuroscience*, 2 (1989) 397-407.

90) Verhaagen, J., Oestreicher, A.B., Gispen, W.H. and Margolis, F.L., The expression of the growth associated protein B50/GAP43 in the olfactory system of neonatal and adult rats, *J Neurosci*, 9 (1989) 683-91.

91) Verhaagen, J., Oestreicher, A.B., Grillo, M., Khew-Goodall, Y.S., Gispen, W.H. and Margolis, F.L., Neuroplasticity in the olfactory system: differential effects of central and peripheral lesions of the primary olfactory pathway on the expression of B-50/GAP43 and the olfactory marker protein, *J Neurosci Res*, 26 (1990) 31-44.

92) Viereck,C., Tucker, RP., and Matus, A., The adult rat olfactory system expresses microtubule-associated protein found in the developing brain. *Journal of Neuroscience*, 9 (1989) 3547-3557

93) Weisgraber, K.H. and Mahley, R.W., Human apolipoprotein E: The Alzhiemers disease connection. *FASEB J,* 10 (1996) 1485-1494.

94) Weisgraber, K.H., Apolipoprotein E: structure-function relationships, *Adv Protein Chem,* 45 (1994) 249-302.

95) White, F., Nicoll, J. A. R. and Horsburgh, K., Alterations in apoE and apoJ in relation to degeneration and regeneration in a mouse model of entorhinal cortex lesion, *Exp Neurology*, 169 (2001) 307-318.

96) Wiedenmann, B., and W. W. Franke., Identification and localization of synaptophysin, an integral membrane glycoprotein of Mr 38,000 characteristic of synaptic vesicles. *Cell.* 41(1985)1017-1078.

97) Willnow, T.E., Goldstein, J.L., Orth, K., Brown, M.S. and Herz, J., Low density lipoprotein receptor-related protein and gp330 bind similar ligands, including plasminogen activator-inhibitor complexes and lactoferrin, an inhibitor of chylomicron remnant clearance, *J Biol Chem*, 267 (1992) 26172-261780.

98) Xu, P.T., Schmechel, D., Qiu, H.L., Herbstreith, M., Rothrock-Christian, T., Eyster, M., Roses, A.D.,Gilbert, J.R. Sialylated Human apolipoprotein E is preferentially associated with neuron-enriched cultures from apoE transgene mice. *Neurobiology of Disease,* 6, (1998) 63-75.

99) Xu, P.T., Schmechel, D., Rothrock-Christian, T., Burkhart, D.S., Qiu, H.L., Popko, B., Sullivan, P., Maeda, N., Saunders, A.M., Roses, A.D., and Gilbert, J.R. Human apolipoprotein E2, E3, and E4 isoform-specific transgenic mice: human-like pattern of glial and neuronal immunoreactivity in central nervous system not observed in wild-type mice. *Neurobiology of Disease,* 3 (1996) 229-245.

100) Yamagishi, M., Getchell, M.L., Takami, S. and Getchell, T.V., Increased density of olfactory receptor neurons immunoreactive for apolipoprotein E in patients with Alzheimer's disease, *Ann Otol Rhinol Laryngol,* 107 (1998) 421-426.

101) Youngentob, S.L., Schwob, J.E., Sheehe, P.R. and Youngentob, L.M., Odorant threshold following methyl bromide-induced lesions of the olfactory epithelium, *Physiol Behav*, 62 (1997) 1241-1252.

102) Zareparsi, S., Camicioli, R., Sexton, G., Bird, T., Swanson, P., Kaye, J., Nutt, J. and Payami, H., Age at onset of Parkinson disease and apolipoprotein E genotypes, *Am J Med Genet,* 107 (2002) 156-161.