2002

Effects of Apolipoprotein E on Olfactory Nerve Plasticity in Mice

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A Thesis
Presented To The
Faculty Of
Department of Biological Sciences
At
Eastern Illinois University

In Partial Fulfillment
Of The Requirement For Degree
Masters of Science
In
Biological Sciences

By
Jody L. Short, DO
Summer Semester 2002

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Introduction

Apolipoprotein E

Apolipoprotein E (apoE) is a 34-kDa protein component of many lipoproteins including very low-density lipoproteins (VLDL), chylomicrons, and high density lipoproteins. ApoE plays a major role in their metabolism by mediating high-affinity binding of lipoproteins to receptors (Mahley, 1998). Receptor-lipoprotein binding initiates cellular uptake and degradation of the lipoproteins, making the lipid available for intracellular metabolism. ApoE therefore, serves as a ligand for the receptor-mediated clearance of lipoproteins from the plasma (Rall et al., 1982).

The apoE gene is located on chromosome 19 and contains 3597 nucleotides and four exons. ApoE is encoded by an 1163 nucleotide mRNA (Mahley, 1988). In humans, there are three common isoforms of apoE. The most common isoform is apoE3, which contains cysteine and arginine at positions 112 and 158, respectively. Both positions contain cysteine in apoE2 and arginine in apoE4 (Weisgraber, 1994). The allele frequencies found in several populations are as follows: 0.07 for apoE2, 0.78 for apoE3, and 0.14 for apoE4 (Hallman et al., 1991).

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 produce 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 of 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 knockout (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 suggest 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).

Effect of apoE on Neurite Outgrowth

Previous findings suggest that apoE4 may contribute to AD by inhibiting neurite outgrowth (Bellosta et al., 1995; Mahley et al., 1996; Nathan et al., 1994; Nathan et al., 1995). Incubation of primary dorsal root ganglion neurons or Neuro-2a cells, a murine neuroblastoma cell line, with apoE3 or apoE4 together with either plasma lipoproteins or CSF lipoproteins, resulted in dramatic isoform-specific effects. In both neuronal cell culture systems, apoE3 increased neurite outgrowth, whereas apoE4 stunted outgrowth (Nathan et al., 1994; Nathan et al., 1995). In these studies, the apoE isoforms also affected the cytoskeleton. Cells treated with lipoproteins and apoE4 showed fewer microtubules as compared with cells grown in medium alone, in medium containing lipoproteins, or in medium containing lipoproteins and apoE3 (Nathan et al., 1995). These effects of apoE on neurite outgrowth appear to be lipoprotein receptor-mediated. Free apoE, which is not a ligand for lipoprotein receptors, does not mediate the effect in the absence of lipoproteins (Bellosta, 1995; Nathan et al., 1995). The *in vitro* data therefore demonstrate that apoE has a tremendous impact on the structure and function of cultured PNS neurons.

Basic Structure of the Olfactory System

The olfactory epithelium is made up of an epithelium and a mucosal layer (figure 1). The olfactory receptor neurons (ORN) reside in a pseudostratified epithelium on the nasal turbinates. Basal cells lie at the base of the epithelium and are critical because they divide and form new olfactory neurons. Sustentacular cells are distributed throughout the epithelium. They help detoxify chemicals that come into contact with the epithelium. The axons from the olfactory neurons collect in bundles and form the olfactory nerve.

The nerve exits the nasal cavity, passes through the cribriform plate, and terminates in the glomeruli of the olfactory bulb.

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 (figure 2). 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 interneurons. 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.

Olfactory Nerve Regeneration

Verhaagen and coworkers induced reversible lesions of the olfactory epithelium and olfactory nerve with Triton X-100 (TX-100) and characterized the process of regeneration in mice (Verhaagen et al., 1989; Verhaagen et al., 1990). Intranasal irrigation of TX-100 in mice causes rapid retrograde degeneration of the ORN in the epithelium, with orthograde degeneration of their synaptic terminals in the bulb while sparing the basal cells. The degenerative process is completed during the first week

following lesioning. Verhaagen and coworkers identified two stages in the regeneration process of olfactory neurons. The first stage starts with the basal cells proliferating at about three days post-lesioning. These newly formed neurons expressed GAP 43, a phosphoprotein related to neuronal growth and plasticity. New axons reach the olfactory nerve layer at one to two weeks, and at three weeks, neurons are visible in the glomeruli. The second stage begins when the neurons reach the glomeruli. This stage is marked by a decrease in the expression of GAP43 and an increase in the expression of olfactory marker protein (OMP), a protein of unknown function present only in mature neurons. The entire process is complete at six to seven weeks (Cummings et al., 2000).

Goals and Hypotheses

1. To examine the distribution of apoE in the mouse olfactory bulb. I hypothesized that apoE would be expressed in areas of nerve degeneration and regeneration.
2. To examine the distribution of apoE in the olfactory bulb following olfactory nerve lesion. I hypothesized that apoE would be transiently increased following olfactory nerve lesion.
3. To examine the effects of apoE deficiency in olfactory nerve regeneration. I hypothesized that apoE deficient mice would have delayed olfactory nerve regeneration as compared to wild type mice.
4. To investigate the effects of human apoE4 and human apoE3 genes in olfactory nerve regeneration. I hypothesized nerve recovery would follow the order: 1. wild-type, 2. apoE3, 3. apoE4, 4. apoE-KO.

EXPERIMENT I: APOLIPOPROTIEN E IMMUNOREACTIVITY IN MOUSE OLFACTORY BULB

Materials and Methods

To examine the distribution of apoE-like immunoreactivity in mice, wild-type (WT) and apoE KO mice (C57BL/6J, males, 4 months old) were purchased from Jackson, Bar Harbor, ME. Mice were anesthetized with sodium pentobarbital (80 mg/kg), transcardially perfused with cold saline (0.9% NaCl) and then with 4% paraformaldehyde in 0.1 M phosphate buffered saline, pH 7.4 (PBS). Olfactory bulbs were removed and post-fixed in buffered fixative for two hours. Following the fixation, olfactory bulbs were cryoprotected overnight in 30% sucrose in 0.1 M phosphate buffer. After cryoprotection, the bulbs were frozen with dry ice and sections were cut on a cryostat at 30 µm. Sections were incubated in 5% dried milk in 0.1 M PBS for 1 h, then incubated for 48 h with anti-apoE polyclonal goat primary antisera (Calbiochem) at 1:16,000 dilution in PBS. The sections were then incubated in rabbit-anti-goat solution (1:200 dilution; Cappel) for 1 h, rinsed three times with PBS, then peroxidase-anti-peroxidase complex (1:100 dilution; Sternberger) for 1 h. The immunoreactive product was nickel-intensified (Benzing et al., 1995). Diaminobenzidine (DAB) (0.05% with 0.01% of H202) was used directly for development. ApoE KO mice were processed in parallel with normal mice.

Results

ApoE-like immunoreactivity was clearly present in the mouse olfactory nerve layer, where the large fascicles of olfactory nerve were often, but not always, outlined by immunoreactive product (figure 3, A and B). Cellular processes could be consistently

seen in the olfactory nerve and these appeared to be glial, although the type of glia was not ascertained. Glomeruli were weakly immunostained, but were clearly outlined by reaction product in the septae surrounding the glomeruli (figure 3, A and B). Immunostaining was in cellular processes of what appear to be glai although a neuronal source was not ruled out. Immunoreactivity in this periglomerular region was more intense than in the EPL. The EPL, although less intensely immunostained that the periglomerular region, did display lamination with the external part adjacent to glomeruli more immunoreactive that the internal part. The internal plexiform layer, just beneath the mitral cell layer, was obviously stained more intensely than the EPL or GCL. In addition, the core of the subependymal layer was intensely immunostained. A complete absence of staining in the apoE KO mice confirmed that we were visualizing apoE-like immunoreactivity.

Discussion

This study identifies apoE-like immunoreactivity surrounding olfactory nerves and glomeruli in normal olfactory bulbs in mice. Glia in this active region elaborate both trophic and inhibitory growth factors (Ramon-Cueto et al., 1995). Moreover, glia probably participate both in the recycling of membrane constituents of degenerating olfactory nerve fibers and the facilitation of axonal elongation and neuropil reorganization associated with regeneration. Therefore, apoE present in this region may be involved with either degeneration or regeneration of axons.

The distribution of olfactory bulb apoE is not spatially congruent with GAP43 which is a 25 kD protein that is associated with axonal growth cones and preterminal axons in the olfactory bulb (Ramakers et al., 1992; Verhaagen et al., 1989). Both the

glomerular zone and granule cell layer are rich in GAP43 immunoreactivity (Ramakers et al., 1992; Struble et al., 1998; Verhaagen et al., 1989), but apoE is primarily found in the glomerular zone with markedly less immunoreactivity present in the granule cell layer. This observation suggests that apoE is not simply related to axonal growth. The olfactory bulb might be an ideal structure for determining the role of apoE in regeneration or degeneration in light of the ability of the olfactory nerve to regenerate and the availability to reversibly lesion the nerve.

EXPERIMENT II: APOLIPOPROTEIN E IS UPREGULATED IN OLFACTORY BULB GLIA FOLLOWING PERIPHERAL RECEPTOR LESION IN MICE

Material and Methods

Two- to four-month-old C57BL/6J mice (Jackson, Bar Harbor, ME) were lesioned as previously described (Margolis et al., 1974; Rochel et al., 1980; Verhaagen et al., 1989). Briefly, a 25-gauge needle with a rounded tip, was inserted approximately 2 mm into one nostril, and 100 µl of 0.7 % TX-100 in saline, or 100 µl of saline alone was irrigated into a nostril of unanesthetized mice. This technique results in extensive bilateral damage to the olfactory epithelium with approximately 70-80% of the olfactory epithelium lesioned (Nathan and Struble, personal observation). This procedure spares the basal cells which subsequently divide and differentiate into new ORN (Margolis et al., 1974; Rochel et al., 1980; Verhaagen et al., 1990). Mice were sacrificed at days 0, 3, 7, 14, 28, 44 and 56 days post-treatment (n=3 mice treated with TX-100 at each time point and one mouse at each time point treated with saline alone).

Following the survival periods after TX-100- or saline-irrigation, mice were anesthetized with pentobarbital (80mg/kg), and perfused transcardially with cold saline and then with 4% paraformaldehyde in 0.1 M PBS, pH 7.4. Olfactory bulbs were removed and post-fixed in buffered fixative for two hours. Following the fixation, olfactory bulbs were cryoprotected overnight in 30% sucrose in 0.1 M phosphate buffer. After cryoprotection, the bulbs were frozen with dry ice and sections were cut on a cryostat at 30 µm. Sections were incubated in 5% dried milk in 0.1 M PBS for 1 h, then incubated for 48 h with anti-apoE polyclonal goat primary antisera (Calbiochem) at 1:16,000 dilution in PBS. The sections were then incubated in rabbit-anti-goat solution

(1:200 dilution; Cappel) for 1 h, rinsed three times with PBS, then peroxidase-anti-peroxidase complex (1:200 dilution; Sternberger) for 1 h. The immunoreactive product was nickel-intensified (Benzing et al., 1995). DAB (0.05% with 0.01% of H202) was used directly for development.

To determine the glial cell type expressing apoE, double label immunocytochemistry was performed on olfactory bulb sections obtained from mice sacrificed at three and seven days post-nerve lesion or saline treatment. This double labeling technique uses sequential reactions with antisera and reaction products of DAB and benzidine dihydrochloride (BDHC) (Levey et al., 1986). In our hands BDHC is not nearly so sensitive as DAB as a chromogen, however, we purposely selected chromogens that were easy to discriminate. DAB results in a diffuse brown product and BDHC presents a granular blue reaction product. Hence, we could identify colocalization on the basis of both color and appearance. Although probably less sensitive than other techniques, the granular deposition of BDHC allowed unequivocal identification of cell types expressing apoE. Sections were initially processed for demonstrating apoE with goat antisera at 1:8 000 dilution overnight followed by rabbit-anti-goat serum (1:200 dilution; Cappel) and then goat peroxidase-anti-peroxidase (1:200 dilution; Sternberger). DAB (0.05% with 0.01% of H202) was used directly for development. Some sections were removed and mounted directly on slides. The remainder of the sections were reacted overnight with either monoclonal anti- mouse F4/80 (1:400 dilution; Serotec), an antigen expressed by activated microglia, (Andersson et al., 1991) or monoclonal antibody to GFAP (1:4,000 dilution, Accurate). These sections were then developed with 0.01% BDHC. The following controls were included to assure specificity (Levey et al.,

1986). 1) Sections were immunoreacted for the initial antigen (apoE), developed with DAB then reacted with BDHC in the absence of the secondary antibody. 2) The initial immunohistochemical steps to visualize apoE were omitted, the sections were reacted with DAB, and then the sections were immunoreacted for the second antigen (either F4/80 or GFAP). Changing the order of antisera (i.e., reacting first for GFAP or F4/80) was not feasible because the BDHC technique was not sensitive enough to reliably identify apoE immunoreactive perikarya in unlesioned mice.

To determine the distribution of apoE immunostained cells, sections form at least two mice, at ages up to 14 days, were reacted with DAB as the chromogen. Plotting of apoE-immunostained cells in these sections was performed with Neurolucida, which permits operator selection of marked objects on a live microscopic image. The lamina were outlined at low power (2x objective) then each immunostained perikarya was marked at higher power (20x objective). Areas of the section and cell counts were used to obtain a rough estimate of cell density.

Results

Immunocytochemical studies of saline treated mice displayed the previously described distribution of apoE-like immunoreactivity (Struble et al., 1999). Most apoE immunoreactivity in saline treated mice was diffusely present in the neuropil, primarily surrounding glomeruli. Few apoE immunoreactive cell bodies were visible and most of these were found in the glomerular layer (figure 4). Three days following TX-100 lesion, the olfactory nerve showed intense immunostaining for apoE although the glomeruli displayed about the same intensity of immunostaining as in the control mice (figure 5). Seven days following lesion, apoE continued to show intense staining in the olfactory

nerve and also showed glomerular immunoreactivity (figure 6). At 14 days, apoE-like immunoreactivity increased in the glomeruli to bring intensity levels comparable to that of the external plexiform layer (figure 7). By 28 days post-lesion, the apoE immunoreactivity was similar the non-lesioned mice (figure 8).

Colocalization studies at three and seven days following lesion showed that apoE was expressed by both astroglia expressing GFAP and microglia expressing immunoreactivity identified by F4/80 (figure 9). The granular/crystalline BDHC reaction product of GFAP or F4/80 was commonly associated with depositions of the more uniform DAB reaction product representing apoE. These observations show that both astroglia and microglia are associated with apoE.

Plotting of apoE-immunoreactive cells generally supported these conclusions. Although only two sections were available for each time period, the pattern was virtually identical between the sections at each age. In saline treated mice the density of visible apoE immunoreactive cells was about 18/mm^2 and theses cells were primarily in the glomerular layer. At three days after lesion immunoreactive cell bodies could be seen throughout the olfactory bulb including the external plexiform and granule cell layer. In this case, the density of identifiable cells was 253/mm^2. The cells containing apoE-like immunoreactivity appeared to be glia. At seven days, the olfactory nerve had degenerated, and apoE immunoreactive somata were seen primarily in the periglomerular region and a rough density of 119/mm^2. By 14 days, overall immunostaining for apoE-like immunoreactivity appeared to be increased throughout the olfactory bulb and density in this section was 27/mm^2. Most of these immunoreactive perikarya were present in the glomerular layer. By 42-56 days, no differences in the numbers of visible cells could be

seen between the control and lesioned mice. At no time did we observe apoE-like immunoreactivity in unequivocally recognized neurons.

Discussion

Degeneration and regeneration in the olfactory bulb following olfactory nerve lesion in this study replicates previous reports in mice (Rochel et al., 1980; Verhaagen et al., 1990; Graziadei et al., 1983; Harding et al., 1977; Nadi et al., 1981; Verhaagen et al., 1989) and rats (Schwob et al., 1995; Ravi et al., 1997; Deamer et al., 1995; Genter et al., 1994). OMP in the olfactory bulb declines slightly at three days following olfactory epithelium lesion, and fully by seven days, representing the degeneration of the ORN terminals. Olfactory nerve terminals were still present in the glomeruli at three days and immunoreact for either amyloid precursor protein-like immunoreactivity (Struble et al., 1998) or OMP (Struble and Nathan, personal observation). Within several days, the olfactory epithelium begins to regenerate from stem cells (Schwob et al., 1995; Graziadei et al., 1979). Progeny of these stem cells differentiate into new ORN, and grow axons into olfactory bulb to re-innervate glomeruli between 14 to 21 days (Schwob et al., 1999). Consequently these studies provide a framework for analyzing apoE expression in the temporal sequence of events in the regeneration process.

ApoE elevation is correlated with olfactory nerve degeneration and regeneration. Immunocytochemical observations of intense apoE-like immunoreactivity on the nerve and an increased number of visible perikarya in the glomerular layer support this interpretation. Glomeruli were only weakly immunoreactive at 3 days, when fibers are still present in the glomeruli (Struble et al., 1998), but intensity had increased by seven days and apoE levels were still elevated at 21 days. This pattern of apoE expression is

basically the reverse of OMP expression in the bulb following olfactory epithelium lesion. As OMP levels recovered, apoE levels declined.

Plotting of apoE immunoreactive perikarya raise several interesting questions, although these studies were not designed to rigorously evaluate density. Increased numbers of immunoreactive perikarya are seen throughout the olfactory bulb and total cell number is 10-fold that in control tissue at three days. In contrast, total apoE (by western blot) is only 2 fold of saline treated levels (Nisar, 1999). Furthermore, whole bulb levels of apoE remain elevated for 14 to 21 days, but the plotting results show that the number of visible apoE-immunoreactive perikarya rapidly decline to essentially normal levels by 14 days.

Part of the explanation for this discrepancy may be in the difficulty of localization of apoE. In our hand, apoE immunoreactivity in the normal mice olfactory bulb is diffuse; visible apoE-immunoreactive perikarya were very rare. Procedures to increase the intensity of perikaryal staining also raised the "background" similarly, suggesting that apoE immunoreactivity is primarily present either in small processes or extracellularly. This discrepancy between the density of apoE immunoreactive cells and whole bulb immunoreactivity probably represents the localization of apoE in the neuropil. These data suggest that initially glia produce apoE (hence a perikaryal localization) but it is rapidly translocated to an extracellular compartment. Neurons apparently are not a major source for apoE production. The mitral neurons are found in single laminae in the bulb and it is highly unlikely that we would not have seen a labeled cell. This suggests that intraneuronal apoE concentration *in vivo* is below the threshold of detectability by

immunocytochemical techniques. Hence, neuronal translocation of apoE and associated lipids is comparatively small or brief.

Translocation apparently occurs in the PNS. Resident macrophages and monocyte-derived macrophages, at the site of injury, are reported to be the primary sources of the large quantities of apoE-containing lipoproteins (Boyles et al., 1985). These lipoprotein particles probably scavenge cholesterol released from degenerating myelin. Scavenged cholesterol was subsequently used in membrane biosynthesis by growth cones of sprouting axons by the low-density lipoprotein (LDL) receptor-mediated endocytosis (Mahley, 1988). Reactive gliosis in the CNS may by a central equivalent of macrophage activity in the PNS.

Reactive gliosis appears to parallel apoE expression. Several studies have reported intense reactive astrogliosis within three to seven days of nerve lesion both around olfactory glomeruli and also throughout the olfactory bulb (Schwob et al., 1999; Struble et al., 1999; Deamer et al., 1995; Anders et al., 1990). Furthermore astroglial markers are elevated in the olfactory bulb for at least 2 to 4 weeks following lesion. These data suggest that processes related to degeneration continue for several weeks following nerve lesion. A previous study suggested that apoE might be involved in regeneration because hippocampal elevation of apoE mRNA trail that of GFAP following perforant path lesion (Poirier et al., 1991). These data are not contradictory. If astroglia and microglia must be activated before they produce apoE, this temporal relationship is logical.

The double labeling immunocytochemical studies confirmed that both reactive astroglia and microglia produce amounts of apoE following a lesion. It is probably fairly

safe to further suggest that apoE is produced by activated microglia. *In vitro* studies have shown apoE production by microglia, but *in vivo* studies have been equivocal (Boyles et al., 1985; Nakai et al., 1996; Uchihara et al., 1995; Fujita et al., 1999). This difference may represent a detection problem perhaps related to the "activated" status of astroglia and microglia. I was unable to unequivocally colocalize apoE and the microglail marker in non-lesioned mice, although I did identify GFAP-apoE co-localization in the same mice. Only following olfactory nerve lesion, when whole bulb levels of apoE increased 1.5 to 2-fold was the level of apoE high enough to detect when I used combined labeling with DAB and BDHC. Nonetheless, it would seem logical that detection of apoE in microglia may require up-regulation of both microglial antigen (F4/80) that occurs after lesion (Anderssen et al., 1991) and cellular localization of apoE. Our data show that both microglia and astroglia produce apoE in the olfactory bulb but activation of glia might be required for co-localization.

A hypothesis of injury-induced apoE production may explain variable morphological effects observed in the absence of the apoE gene. *In vitro*, apoE, but only in combination with a source of lipids, facilitates process growth (Nathan et al., 1994). I propose that *in vivo* apoE is transiently increased to scavenge lipids released form degenerating processes, and then provides those lipids to regenerating processes. Much of this lipid is postulated to come from degenerating myelin sheaths (Mahley et al., 1996). In the intact central nervous system, inefficient recovery may not be apparent, or perhaps could be compensated for by other less efficient apolipoproteins (Terrisse et al., 1998).

A hypothesis of apoE participation in lipoprotein recycling is also compatible with previous observations of the effects of apoE alleles in both AD and recovery form head injury. The apoE4 allele not only predisposes to AD, but also substantially increases the risk of developing AD after head trauma (Mayeux et al., 1995). Moreover, patients with the apoE4 allele manifest a poorer outcome following traumatic brain injury (Jordan et al., 1997), and boxers with the E4 allele were at much greater risk to develop chronic neurologic deficits than those not having the E4 allele (Jordan et al., 1997). Recently, it was suggested that blood-flow (measured by fMRI) in individuals with apoE4 allele may be greater than apoE3 homozygotes while performing the same task (Bookheimer et al., 2000) suggesting some level of inefficiency in learning. If apoE is involved in efficient recycling or redistribution of lipoproteins during brain function or following injury, a lifetime of inefficient functioning may results in an accentuation of mild dysfunction and an increased risk for dementia.

EXPERIMENT III: DELAYED OLFACTORY NERVE REGENERATION IN APOE-DEFICIENT MICE.

Materials and Methods

Breeding pairs of homozygous apoE KO mice that have been back-crossed at least 10 times to the C57BL/6J wild-type mice (Jackson, Bar Harbor, ME). ApoE genotype of the litters were verified by PCR and confirmed by immunoblot using anti-apoE (1:1000; Calbiochem).

Two- to four-month-old apoE KO and wild type mice were lesioned as previously described (Margolis et al., 1974; Rochel et al., 1980; Verhaagen et al., 1990). Briefly, a 25-gauge needle with a rounded tip, was inserted approximately 2 mm into one nostril, and 100 µl of 0.7% TX-100 in saline or 100 µl of saline alone was irrigated into a nostril of unanesthetized mice. This technique results in extensive bilateral damage to the olfactory epithelium with approximately 70-80% of the adult olfactory epithelium lesioned (Nathan and Struble, personal observation). This procedure spares the basal cells, which subsequently divide and differentiate into new ORN (Margolis et al., 1974; Rochel et al., 1980; Verhaagen et al., 1990). Mice were sacrificed at days 0, 3, 7, 14, 21, 42 and 56 days post-treatment (n=3 animals for each time point and treatment).

Following the survival periods after TX-100 or saline irrigation, mice were anesthetized with pentobarbital (80 mg/kg), and perfused transcardially with cold saline and then with 4% paraformaldehyde in 0.1 M phosphate buffer. Olfactory bulbs were post-fixed in buffered fixative for two hours. Following the fixation, olfactory bulbs were cryoprotected overnight in 30% sucrose in 0.1 M phosphate buffer. After cryoprotection, the bulbs were frozen with dry ice and sections were cut on a cryostat at

30 μm. Sections were incubated in 5% dried milk in 0.1 M PBS for 1 h. Sections were then incubated for 48 h with anti-OMP polyclonal goat primary antisera (gift from Frank Margolis, Ph.D., University of Maryland) at 1:4,800 dilution in PBS. The sections were then incubated in rabbit-anti-goat solution (1:200 dilution; Cappel) for 1 h, rinsed three times with PBS, then goat peroxidase-anti-peroxidase complex (1:200 dilution; Sternberger) for 1 h. DAB (0.05% with 0.01% of H202) was used directly for development.

Results

OMP

Day 3 Saline Treated: The WT-SA and KO-SA mice both showed intense immunoreactivity in the olfactory nerves and the glomerular layer. No differences were seen between the two groups.

Day 3 TX-100 Treated: The WT-TX mice (figure 10) and KO-TX mice (figure 11) showed intense immunoreactivity in the olfactory nerves. The WT-TX mice showed moderate immunoreactivity in the glomerular layer while the KO-TX mice showed light staining in the glomerular layer.

Because the WT-SA and KO-SA mice both showed the typical immunoreactivity seen in untreated WT mice, we decided not to immunostain the WT-SA mice on days 7, 21 and 56.

Day 7 Saline Treated: The KO-SA mice showed intense immunoreactivity in the olfactory nerve and glomerular layer. This immunoreactivity was the same as the KO-SA and WT-SA mice further supporting our idea not to stain the WT-SA mice on days 7, 21 and 56.

Day 7 TX-100 Treated: The WT-TX mice showed intense immunoreactivity in the olfactory nerve and moderate immunoreactivity in the glomerular layer. The KO-TX mice showed light to moderate immunoreactivity in the olfactory nerve and glomerular layer.

Day 21 Saline Treated: The KO-SA mice showed dark olfactory nerve immunoreactivity and moderate, well organized glomerular immunoreactivity.

Day 21 TX-100 Treated: The WT-TX mice (figure 12) showed strong immunoreactivity in the olfactory nerve and moderate immunoreactivity in the glomerular layer. The glomeruli were decreased in number but appeared well organized. The KO-TX mice (figure 13) had moderate immunoreactivity in the olfactory nerve and glomerular layer; however, the glomeruli were unorganized.

Day 42 Saline Treated: All of the 42-day mice were overall lightly staining. The WT-SA and KO-SA showed light immunoreactivity in the olfactory nerve and glomerular layer.

Day 42 TX-100 Treated: The glomeruli in the WT-TX mice were not as numerous as in the saline treated mice; however the immunoreactivity was the same. The KO-TX mice showed light immunoreactivity in the olfactory nerve, similar to the other 42-day mice. The glomerular layer was also lightly stained, but the glomeruli were smaller compared to the other 42-day mice.

Because the 42-day WT-TX mice showed almost complete recovery from olfactory nerve lesion, no WT mice were stained on day 56.

Day 56 Saline Treated: All of the 56-day mice were overall lightly staining. The KO-SA mice showed a light immunoreactivity in the olfactory nerve and glomerular layer. In addition, the glomeruli were small and unorganized.

Day 56 TX-100 Treated: The KO-TX mice showed light staining in the olfactory nerve and glomerular layer. Similar the 56-day KO-SA mice, the glomeruli were unorganized. In addition, the glomeruli were sparse and incompletely stained.

Discussion

On days 3 and 7, there was a loss of OMP immunoreactivity. This represents degeneration of olfactory nerve after lesion. By day 21, there was an increase in OMP immunoreactivity in the WT-TX mice. This reactivity showed that some of the axons had already reached the glomerular layer. The KO-TX mice did not have the same immunoreactive pattern as the WT-TX mice. KO-TX mice did not show the same pattern of mature olfactory neurons in the glomerular layer, as seen by OMP immunoreactivity. By day 42 the WT-TX mice appeared to be almost fully recovered from the lesion. In comparison the KO-TX mice had not recovered. OMP immunoreactivity was still poor at 56-days in the KO-TX mice representing a lack of neurons reaching the glomerular layer and forming mature synapses. In conclusion, it appeared that the absence of an apoE gene greatly diminishes the ability of the olfactory nerve to regenerate following lesion.

EXPERIMENT IV: EFFECTS OF HUMAN APOLIPOPROTEIN E ISOFORMS ON OLFACTORY NERVE PLASTICITY IN MICE

Materials and Methods

Two- to four-month-old male WT, apoE KO and transgenic mice (gift from David Holtzman, M.D., Washington University) were used for this study. The transgenic mice were apoE KO mice that had either a human apoE3 gene or a human apoE4 gene inserted under a GFAP promoter (Sun et al., 1998). Mice were lesioned as previously described (Margolis et al., 1974; Rochel et al., 1980; Verhaagen et al., 1990). Briefly, a 25-gauge needle with a rounded tip, was inserted approximately 2 mm into one nostril, and 100 µl of 0.7 % TX-100 in saline, or 100 µl of saline alone was irrigated into a nostril of unanesthetized mice. This technique results in extensive bilateral damage to the olfactory epithelium with approximately 70-80% of the adult olfactory epithelium lesioned (Nathan and Struble, personal observation). This procedure spares the basal cells which subsequently divide and differentiate into new ORN (Margolis et al., 1974; Rochel et al., 1980; Verhaagen, et al., 1990).

Mice were sacrificed at 14 days post-treatment which is the midpoint of olfactory nerve regeneration (n=3 mice for each genotype and treatment). Mice were anesthetized with pentobarbital (80 mg/kg), and perfused transcardially with cold saline and then with 4% paraformaldehyde in 0.1 M phosphate buffer. Olfactory bulbs were post-fixed in buffered fixative for two hours. Following the fixation, olfactory bulbs were cryoprotected overnight in 30% sucrose in 0.1 M phosphate buffer. After cryoprotection, the bulbs were frozen with dry ice and sections were cut on a cryostat at 30 µm. Sections

were incubated in 5% dried milk in 0.1 M PBS for 1 h. Each mouse olfactory bulb was assessed for five antigens as follows:

1. Anti-GFAP monoclonal mouse antibody (1:4,000 dilution; Accurate) 48 h incubation
2. Anti-apoE polyclonal goat antibody (1:8,000 dilution; Calbiochem) 24 h incubation
3. Anti-F4/80 monoclonal mouse antibody (1:400 dilution; Serotec) 24 h incubation
4. Anti-OMP polyclonal goat antibody (1:4,800 dilution; gift from Frank Margolis, Ph.D., University of Maryland) 24 h incubation
5. Anti-Synaptophysin monoclonal mouse antibody (1:500 dilution; Boehringer Mannheim) 48 h incubation

To visualize GFAP and Synaptophysin, sections were incubated in goat-anti-mouse solution (1:200 dilution; Cappel) for 1 h, and for OMP and apoE, sections were incubated in rabbit-anti-goat solution (1:200 dilution; Cappel) for 1 h. All sections were rinsed three times with PBS, then treated with either mouse or goat peroxidase-anti-peroxidase complex (1:200 dilution; Sternberger) for 1 h. DAB (0.05% with 0.01% H2O2) was used directly for development. An avidin/biotin (Vectastain ABC kit, Vector) technique was used with DAB to visualize F4/80 binding.

Results

GFAP

Saline Treated: No differences were seen between the WT-SA (figure 14), apoE3-SA, apoE4-SA and apoE-KO-SA groups. In general, immunoreactivity was more intense in the glomeruli.

TX-100 Treated: The WT-TX (figure 15) and apoE-KO mice (figure 16) showed heavy immunoreactivity in the glomerular layer as well as a general increase in

immunoreactivity in the olfactory bulb. ApoE3-TX and apoE4-TX mice (figure 17) demonstrated immunoreactivity in the glomerular layer, but lacked the increased immunoreactivity in the external plexiform layer characteristic of the WT-TX and apoE-KO mice.

Summary: Following lesion GFAP-immunoreactivity in apoE3-TX and apoE4-TX mice did not appear to be increased throughout the bulb so much as it did in the WT-TX and apoE-KO mice. The absence of a normally regulated apoE gene affected expression of the GFAP gene. The failure to identify clear upregulation of immunoreactivity in the apoE3-TX and apoE4-TX mice may represent a ceiling effect or some interaction with the an abnormally regulated apoE gene.

ApoE

Saline Treated: The WT-SA mice (figure 18) showed diffuse immunoreactive pattern in the olfactory nerve and in the periglomerular layer, which, in turn showed more immunoreactivity than the glomerular layer. Only astroglial-like cell body staining in the glomerular layer was seen in the apoE3-SA and apoE3-SA mice (figure 19), and the core of the glomeruli were immunoreactive and darker than the rest of the bulb. There was no immunoreactivity in the KO-SA mice.

TX-100 Treated: The WT-TX mice (figure 20) showed increased apoE immunoreactivity in the periglomerular layer. ApoE4-TX mice and apoE3-TX mice (figure 21) showed no increase in immunoreactivity. There was no immunoreactivity in the apoE-KO-TX mice.

Summary: The levels of apoE are increased following lesion in the WT-TX mice; however, in the apoE3-TX and apoE4-TX groups apoE immunoreactivity is not increased following lesion.

F4/80

Saline Treated: No differences were seen between the apoE-KO-SA, apoE3-SA, apoE4-SA and WT-SA mice (figure 22). In general, there was moderate diffuse immunoreactivity in the glomerular layer and external plexiform layer with a lamination between these two layers.

TX-100 Treated: The WT-TX mice (23) and apoE-KO-TX mice (figure 24) showed increased immunoreactivity in the glomerular layer and external plexiform layer compared to the transgenic mice (figure 25).

Summary: The expression of either the apoE3 or apoE4 inserted gene apparently turns down or blocks activation of microglia.

OMP

Saline Treated: No differences were seen between the apoE-KO-SA , apoE3-SA, apoE4-SA and WT-SA mice (figure 26). In general, the olfactory nerve and glomerular layer showed intense immunoreactivity.

TX-100 Treated: Immunoreactivity in the WT-TX mice (figure 27) showed smaller glomeruli than in the SA treated mice. The apoE4-TX and apoE3-TX mice (figure 28) both showed less glomerular staining than the WT-TX mice. The KO-TX mice (figure 29) showed less staining in the glomeruli than the transgenic mice.

Summary: Recovery appeared to follow the order of WT-TX, apoE3-TX or apoE4-TX and apoE-KO-TX. Apparently the presence of either the apoE3 or apoE4 gene can partially compensate for the absence of the WT gene.

Synaptophysin

Saline Treated: No differences were seen between the apoE-KO-SA, apoE3-SA, apoE4-WT-SA mice (figure 30). In general, there is intense immunoreactivity in the glomerular layer and lighter immunoreactivity in the external plexiform layer.

TX-100 Treated: The WT-TX mice (figure 31) had a general attenuation of immunoreactivity and smaller glomeruli compared to SA treated mice. Immunoreactivity in the apoE3-TX and apoE4-TX mice (figure 32) showed smaller and lighter glomeruli than seen in the WT-TX mice. The KO-TX mice (figure 33) had decreased immunoreactivity especially in the glomerular layer than the other treatment groups.

Summary: Following lesion either apoE3 or apoE4 was adequate to produce some recovery of synaptophysin.

Discussion

The results of this study revealed that apoE deficiency in apoE gene KO mice leads to a delay in nerve recovery as compared to wild type mice. Expression of either human apoE3 or human apoE4 can partially compensate for the absence of the wild type gene. Nerve recovery appeared to follow the order: 1. wild type mice, 2. apoE3 transgenic equivalent to apoE4 transgenic, and 3. ApoE-KO mice.

In summary, the data suggest that apoE has a tremendous impact on neuronal plasticity. Furthermore, human apoE3 was found to be equivalent to apoE4 in supporting some degree of olfactory nerve repair in mice; however, neither was as good as the WT apoE gene, and both were better than apoE-KO mice.

It is possible that the difference between apoE3 and apoE4 is just not distinguishable at two weeks, but would be distinguishable at three or more weeks post-

lesion. It is possible that apoE3 has a favorable affect on olfactory nerve recovery, but at two weeks there are no differences. Therefore, the apoE3 gene mice might have complete recovery in six weeks post lesion and apoE4 gene mice might take a week or two longer for complete olfactory nerve recovery. ApoE4 may initially be able to handle the recovery, but at some point beyond two weeks, its ability to keep up the regenerative process may decrease.

Conclusions

1. ApoE immuoreactivity surrounds the olfactory nerve and the glomeruli. This suggests that apoE may be involved with degeneration and regeneration. The olfactory bulb might be an ideal structure for determining the role of apoE in degeneration and regeneration of nerves.
2. ApoE immuoreactivity increased in the olfactory nerve during degeneration following lesion and increased in the olfactory bulb during the regeneration process. ApoE is mostly produced by astrocytes, but also by microglia.
3. The absence of an apoE gene diminished the ability of the olfactory nerve to recover following lesion.
4. Transgenic apoE3 or apoE4 partially compensate for the absence of a wild type apoE gene, but neither was as good as the wild type gene.
5. These studies all suggest that apoE has a tremendous impact on olfactory nerve recovery following lesion.

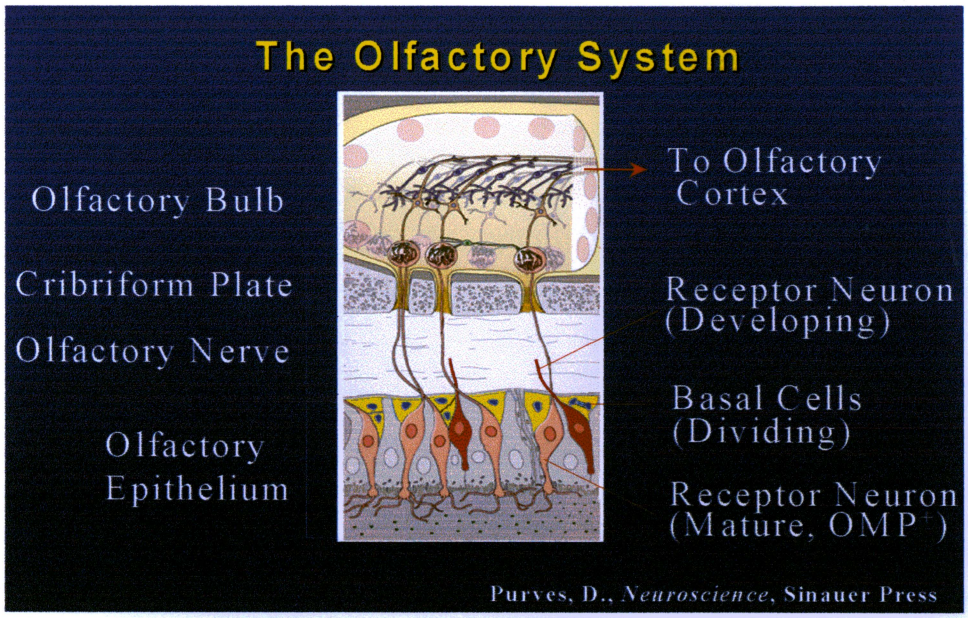

Figure 1. Olfactory epithelium and olfactory bulb.

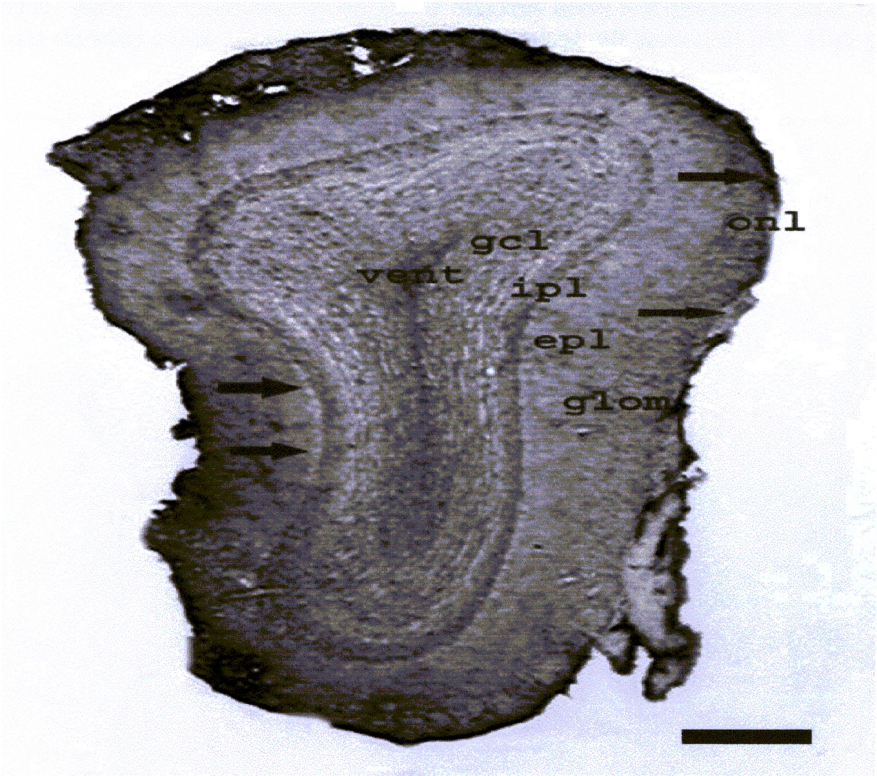

Figure 2. Lamina of the olfactory bulb. From outer to inner, olfactory nerve layer (onl), glomerular layer (glom), external plexiform layer (epl), mitral cell layer (arrows on left), internal plexiform layer (ipl), granule cell layer (gcl) and ventricular layer (vent).

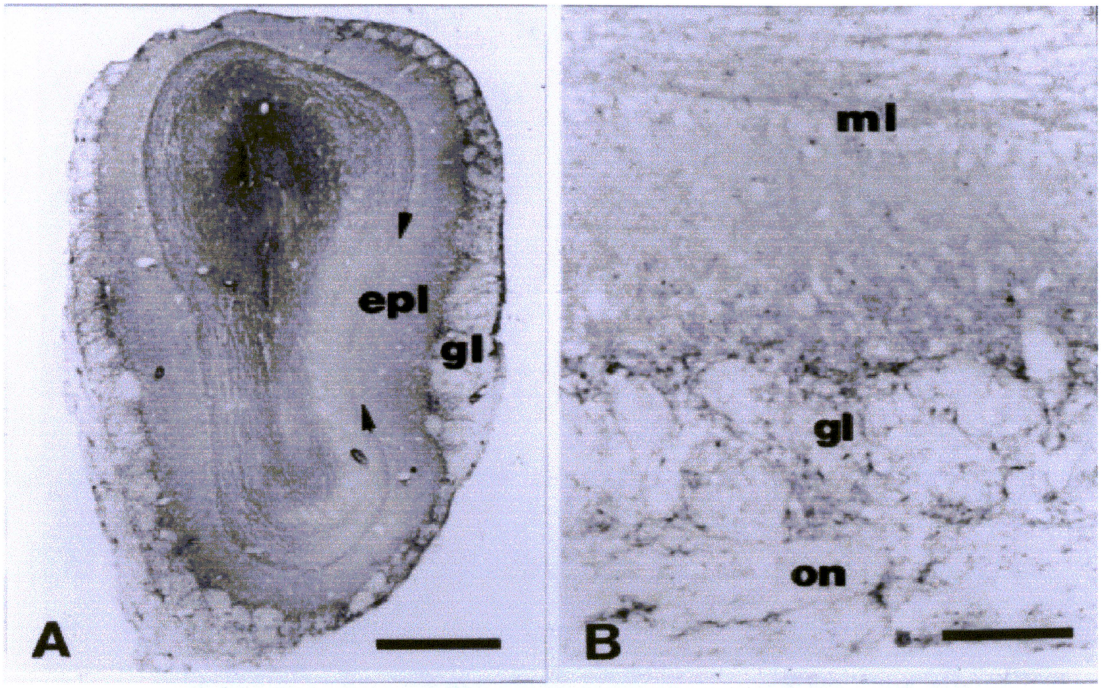

Figure 3 A and B. ApoE immunoreactivity present in olfactory nerve and glomerular layer. Low power view, arrowheads showing a lack of immunoreactivity in the mitral cell layer (ml) (A). High power view (B).

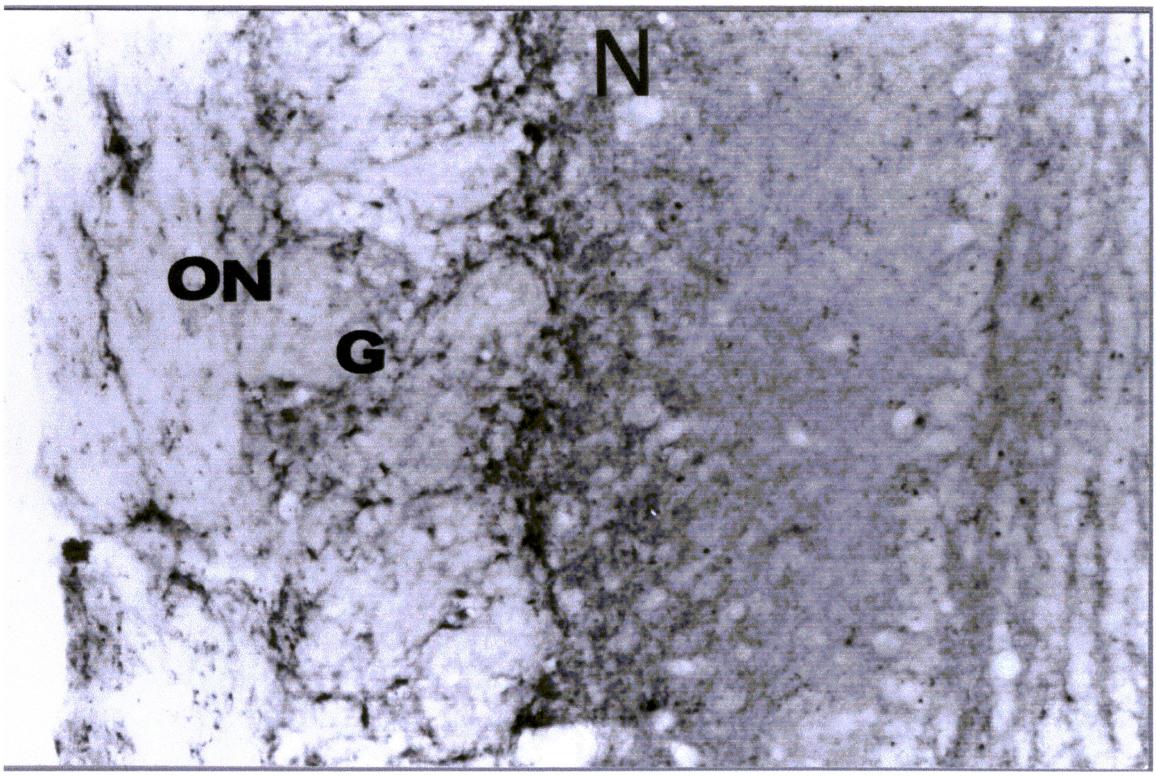

Figure 4. ApoE immunoreactivity in the olfactory nerve (ON) and glomerular layer (G) in saline treated (N).

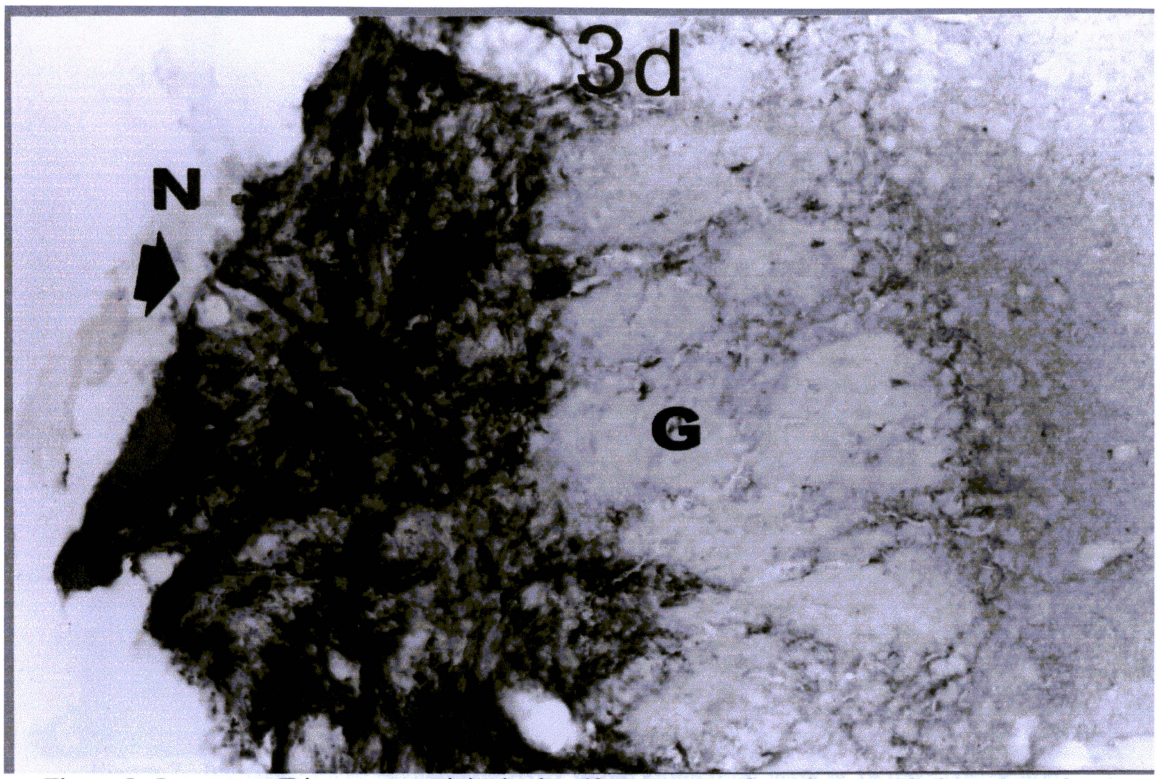

Figure 5. Intense apoE immunoreactivity in the olfactory nerve three days post-lesion. Arrowhead pointing to olfactory nerve.

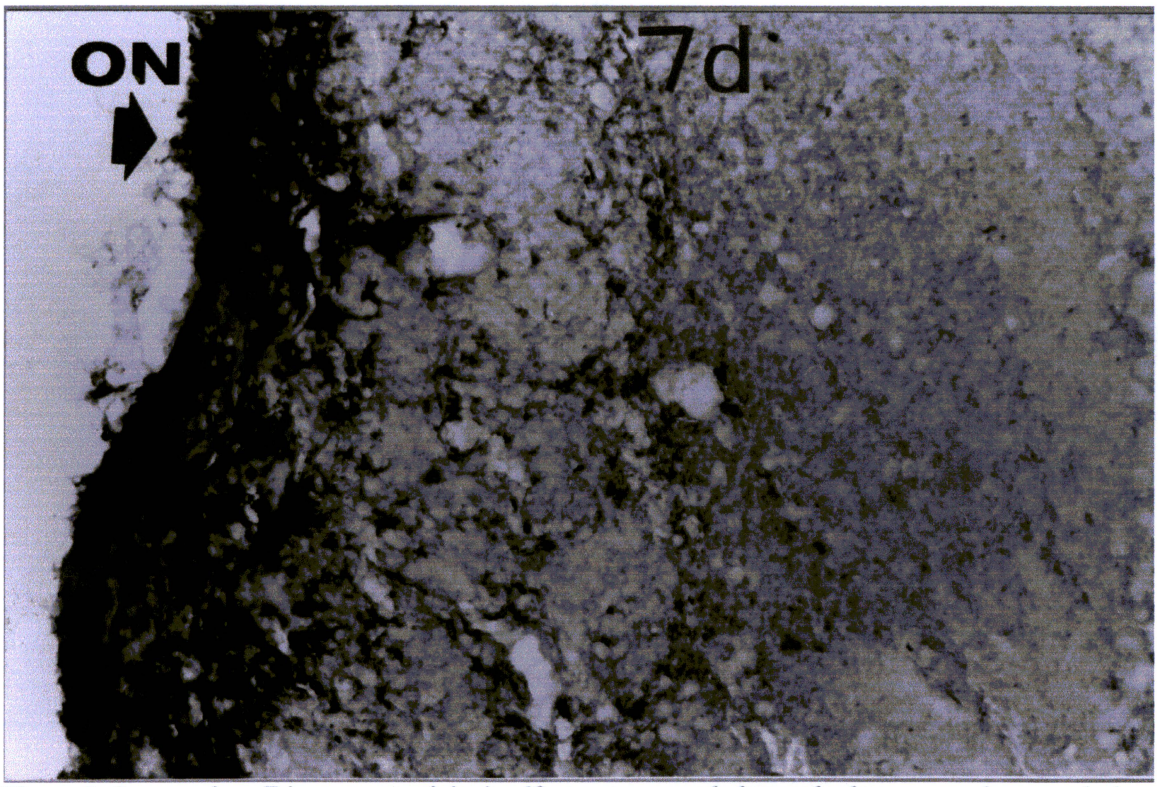

Figure 6. Increased apoE immunoreactivity in olfactory nerve and glomerular layer seven days post-lesion. Arrowhead pointing to olfactory nerve.

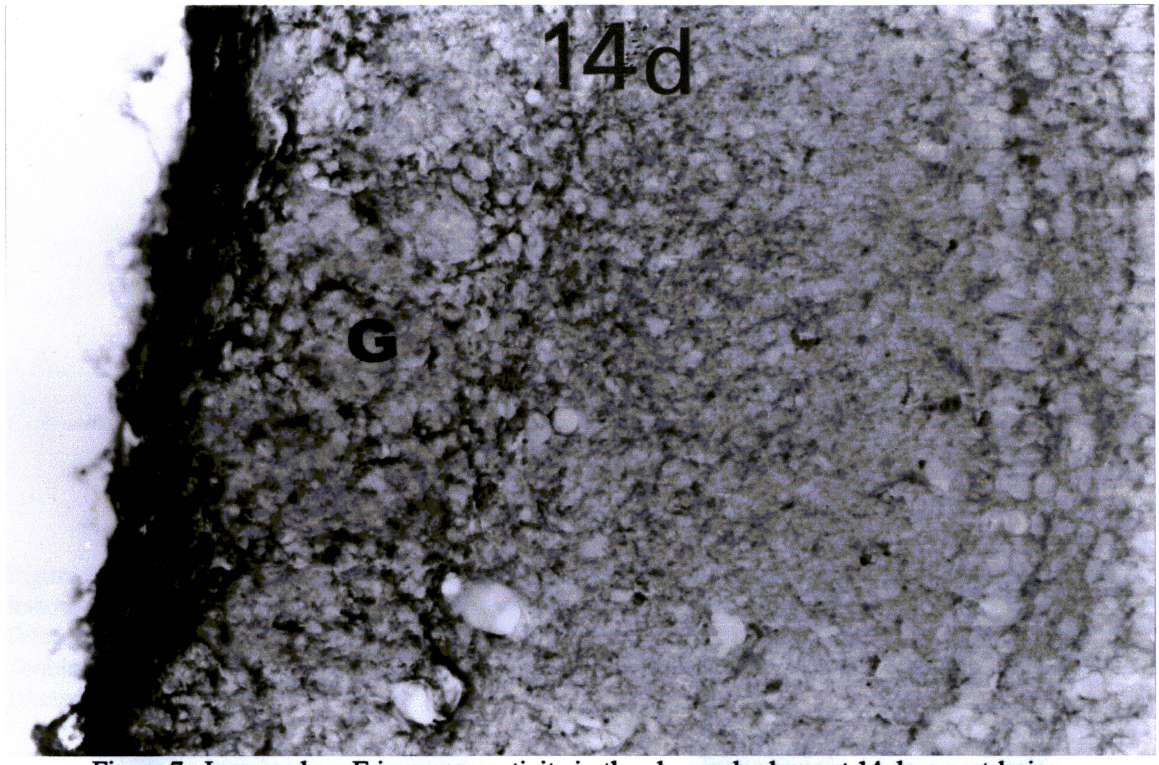

Figure 7. Increased apoE immunoreactivity in the glomerular layer at 14 days post-lesion.

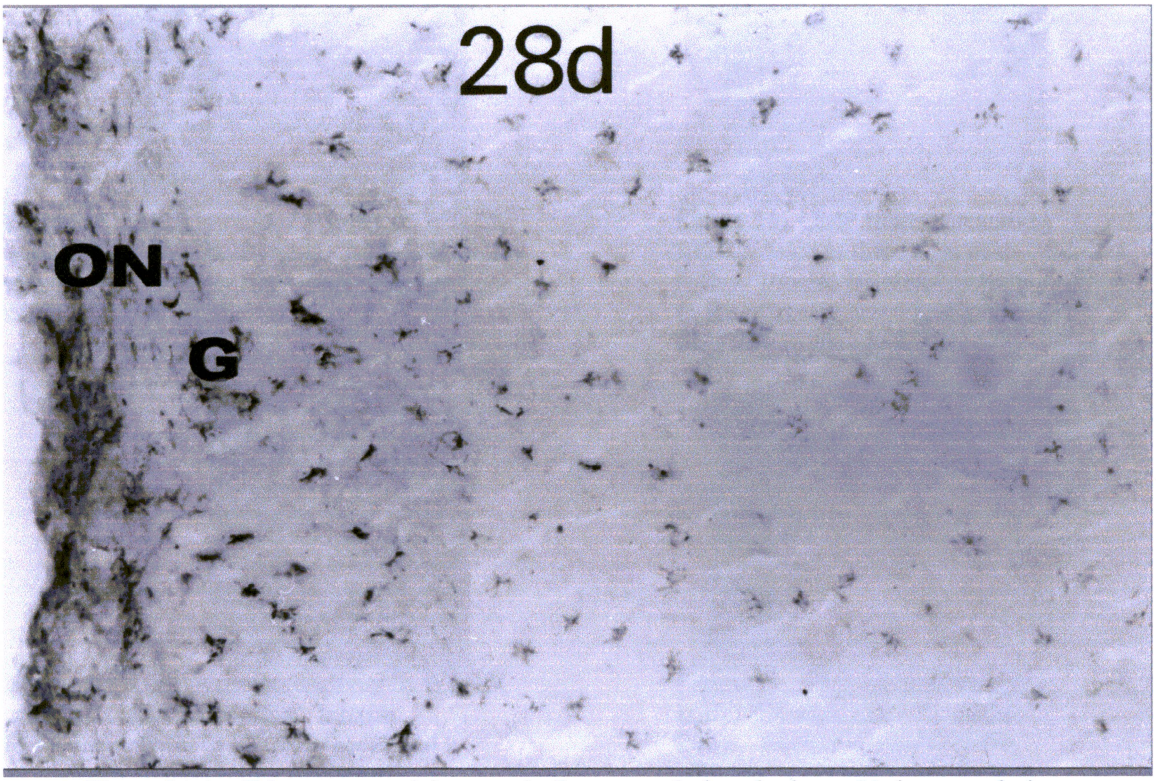

Figure 8. ApoE immunoreactivity was similar to non-lesioned mice at 21 days post-lesion.

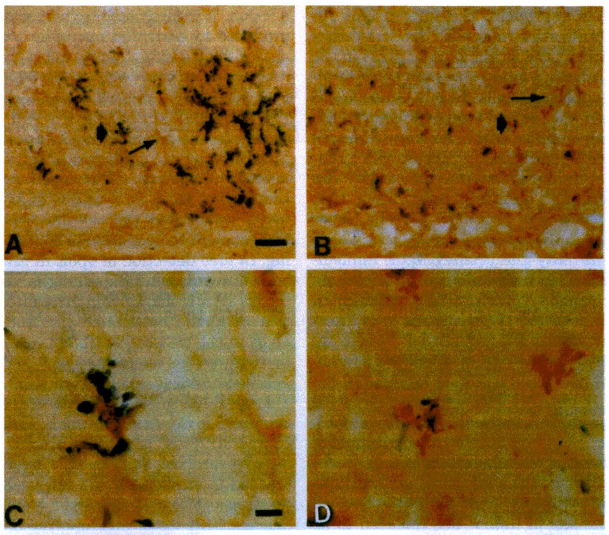

Figure 9. Double labeling of astroglia (A, C) and microglia (B, D) in the olfactory bulb at three days post-lesion. Low power views of the gomerular layer show double labeling with DAB for apoE and BDHC (blue granular) labeling for either GFAP (A) or F4/80 (B). The large arrowhead in A and B identify the double-labeled perikarya shown in C and D. The smaller arrows represent apoE-producing cells not labeled with the BDHC.

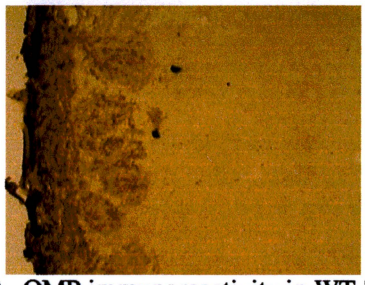

Figure 10. OMP immunoreactivity in WT-TX mice at three days post-lesion. Intense immuno-reactivity in olfactory nerve and moderate reactivity in the glomeruli.

Figure 11. OMP immunoreactivity KO-TX mice at three days post-lesion. Intense immunoreactivty In the olfactory nerve and moderate reactivity in the glomeruli.

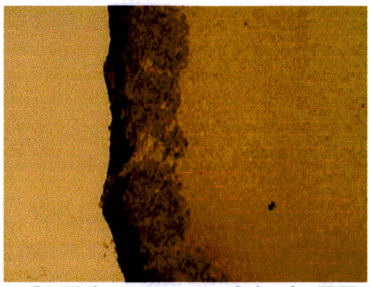

Figure 12. OMP immunoreactivity in WT-TX mice at 21 days post-lesion. Intense immunoreactivity in olfactory nerve and moderate reactivity in the glomeruli.

Figure 13. OMP immunoreactivty in KO-TX mice at 21 days post-lesion. Intense reactivity in the olfactory nerve with unorganized glomeruli reactivity.

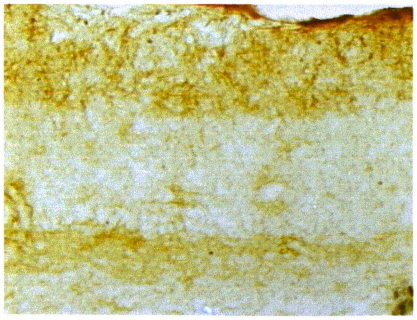

Figure 14. Wild-type, GFAP immuno-reactivity, saline treated. Intense reactivity in the glomerular layer. All saline groups were similar.

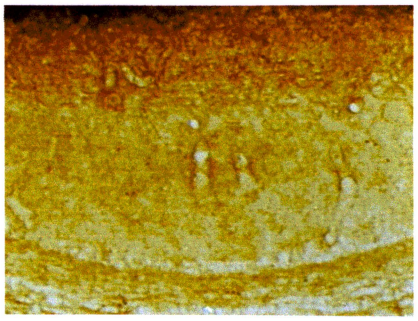

Figure 15. Wild-type, GFAP immunoreactivity, two weeks post-lesion. Intense immuno-reactivity in the glomerular layer and an overall increase in reactivity.

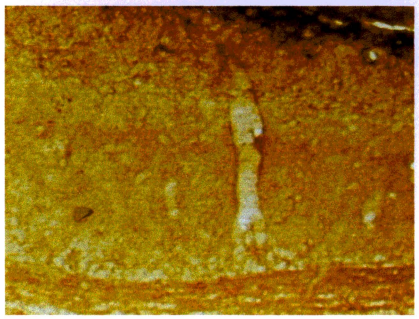

Figure 16. ApoE-KO, GFAP immuno-reactivity, two weeks post-lesion. Intense reactivity in glomerular layer and an overall increase in reactivity.

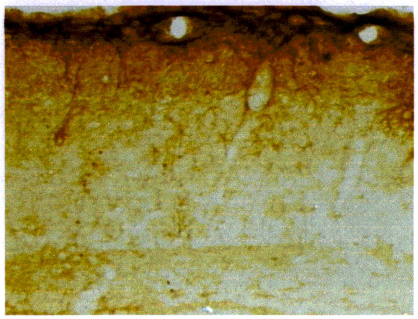

Figure 17. ApoE4, GFAP immunoreactivity two weeks post-lesion. Intense reactivity in the glomerular layer and an overall increase in reactivity.

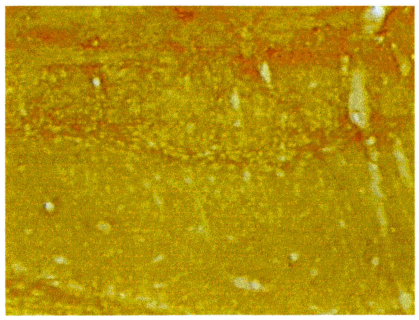

Figure 18. Wild-type, apoE immuno-reactivity, saline treated. Diffuse reactivity in the olfactory nerve and glomerular layer.

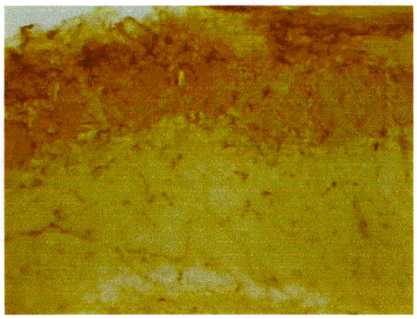

Figure 19. ApoE3, apoE immuno-reactivity, saline treated. Astroglial-like staining in the glomerular layer. ApoE4 was similar.

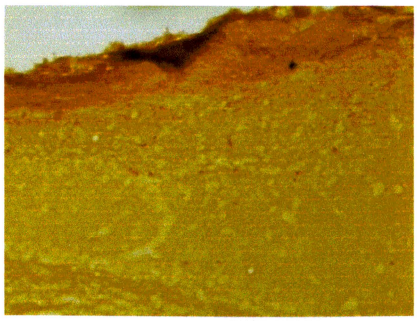

Figure 20. Wild-type, apoE immuno-reactivity, two weeks post-lesion. Reactivity increased in olfactory nerve and glomerular layer.

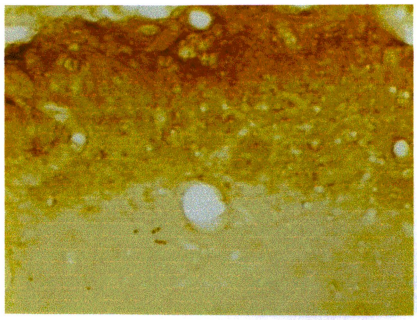

Figure 21. ApoE3, apoE immuno-reactivity, two weeks post-lesion. No increase in reactivity. ApoE4 was similar.

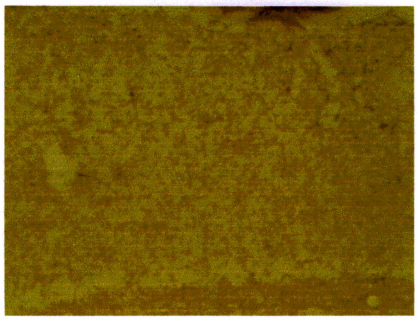

Figure 22. Wild-type, F4/80 immuno-reactivity, saline treated. Moderate reactivity in the glomerular layer and external plexiform layer. All saline groups were similar.

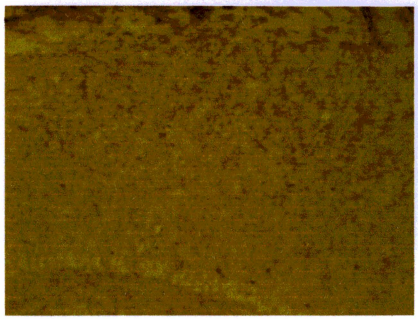

Figure 23. Wild-type, F4/80 immunoreactivity, two weeks post-lesioin. Increased immunoreactivity in the glomerular layer and external plexiform layer.

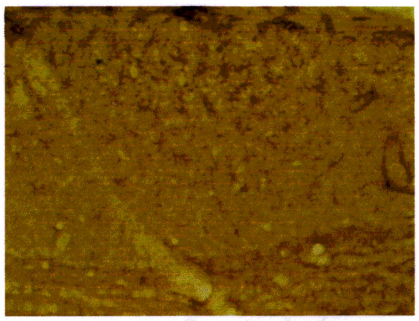

Figure 24. ApoE-KO, F4/80 immuno-reactivity, two weeks post-lesion. Increased immunoreactivity in the glomerular layer and external plexiform layer.

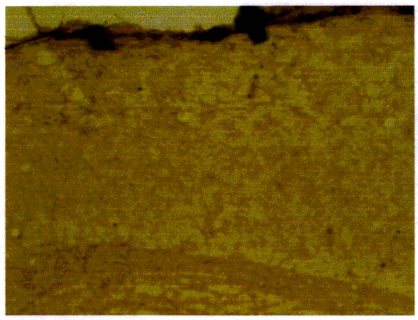

Figure 25. ApoE3, F4/80 immuno-reactivity, two weeks post lesion. No increase in reactivity. ApoE4 was similar.

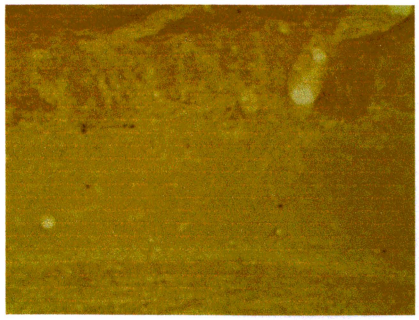

Figure 26. Wild-type, OMP immuno-reactivity, saline treated. Reactivity in the olfactory nerve and glomerular layer. All saline groups were similar.

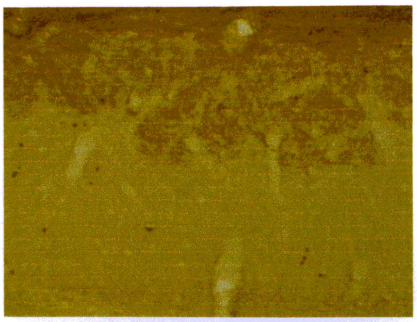

Figure 27. Wild-type, OMP immunoreactivity, two weeks post-lesion. Gomeruli are smaller compared to saline treated mice.

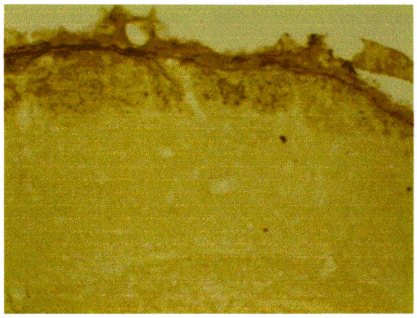

Figure 28. ApoE3, OMP immunoreactivity, two weeks post-lesion. Less glomerular reactivity than wild type mice. ApoE4 was similar.

Figure 29. ApoE-KO, OMP immunoreactivity, two weeks post-lesion. Less glomerular reactivity than the transgenic mice.

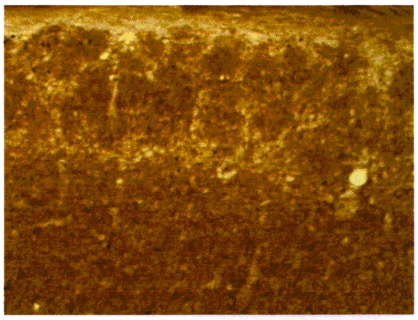

Figure 30. Wild-type, Synaptophysin immunoreactivity, saline treated. Intense reactivity in the glomerular layer and external plexiform layer. All saline groups were similar.

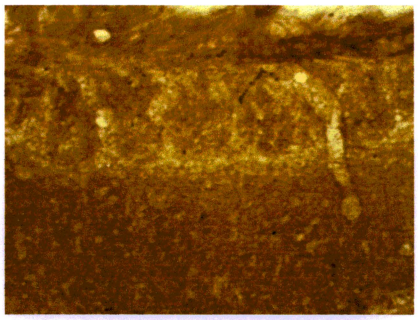

Figure 31. Wild-type, Synaptophysin immunoreactivity, two weeks post-lesion. Attenuation of reactivity and smaller glomeruli than saline treated groups.

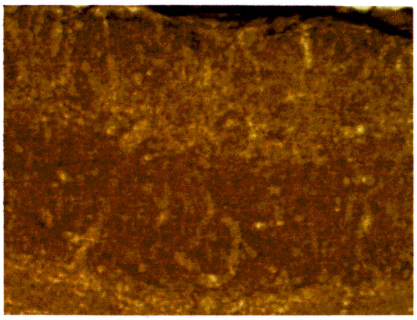

Figure 32. ApoE4, Synaptophysin immunoreactivity, two weeks post-lesion. Smaller and lighter glomeruli than wild-type mice. ApoE3 was similar.

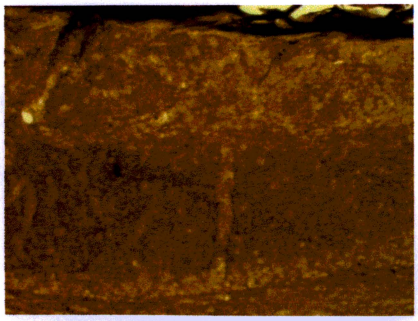

Figure 33. ApoE-KO, Synapto-physin immunoreactivity, two weeks post-lesion. Decreased reactivity in glomerular layer compared to the wild type and transgenic lesioned mice.

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Acknowledgements

- Britto P. Nathan, PhD, advisor and mentor
- Robert G. Struble, PhD, mentor
- Kipp Kruse, PhD, committee member
- Kip McGilliard, PhD, committee member
- American Federation for Aging Research, Glenn/AFAR Scholarship
- Western University of Health Sciences, Summer Research Fellowship