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Effects Of Apolipoprotein E On Olfactory Neuron Plasticity In Mice

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Effects Of Apolipoprotein E On Olfactory Neuron 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 Requirements For The Degree
Master of Science
In
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By
Rafia Nisar
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The Undersigned Faculty Committee Approves
The Thesis of Rafia Nisar:

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Department of Biological Sciences
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ABSTRACT

Previous studies have shown that apoE is upregulated in injured nerves, and that it may participate in nerve regeneration by recycling lipids from the degenerating myelin sheath to axonal growth cones. However, these studies fail to differentiate apoE function in degeneration and regeneration, because of temporal juxtaposition of degeneration and regeneration in these models. In this study, we characterized the apoE expression during olfactory nerve regeneration in mice, which occurs over an extended seven week period. Olfactory nerves were lesioned in 2-3-month-old mice by intransal irrigation of Triton X-100. Following lesioning the olfactory bulbs were collected at 0, 3, 7, 21, 42, and 56 days post-injury, and apoE levels in the bulbs were determined by immunoblot analysis. ApoE level peaked at 3 days-post lesion, reaching a concentration twice which is found in the normal olfactory bulb. The apoE level stayed elevated by approximately 1.5 times the normal level at 7 through 21 days after injury, and thereafter gradually returned to normal by 56 days. These data suggest that apoE functions in the central nervous system to promote efficient repair of neural structures following injury.

An extension of this observation is the hypothesis that if apoE is important in nerve repair, then nerve regrowth should be either incomplete or delayed in the absence of apoE. I tested this postulate by comparing olfactory nerve repair in apoE gene deficient/apoE gene knockout (apoE KO) with control mice. Olfactory nerve recovery was assessed by immunoblotting of GAP 43, a marker for juvenile olfactory neurons. Immunoblot analysis revealed that GAP 43 levels in control animals increased by approximately two times the normal level at 3 days post-lesion, and returned back to baseline at 7 days after injury. At 21 days post-lesion the GAP 43 level increased dramatically reaching a concentration 3 times which is found in the normal olfactory bulb. The GAP 43 level stayed elevated until 42 days, and thereafter gradually returned to normal by 56 days. Even though the GAP 43 time course in apoE KO mice followed a pattern similar to that observed in control animals, the major increase in GAP 43 was observed on 42 days post lesion. This is a two weeks delay from that observed in control animals, suggesting that the generation of new olfactory neurons is delayed by two weeks in apoE KO mice.

To confirm the GAP 43 data and examine the effects of apoE deficiency in the maturation of olfactory neurons, I examined the expression of the olfactory marker protein (OMP), a marker for adult olfactory neurons. Immunoblot analysis revealed that OMP levels in the bulb of control animals gradually declined following nerve lesioning, decreasing to approximately 50% of the normal level at 7days post lesion. Thereafter OMP levels sharply increased to about 80% of the normal level at 21 days post lesion, and then gradually increased to normal by 56 days. In apoE KO animals the OMP time course was different than that of control animals. First, OMP levels continued to decrease in apoE KO animals until 21 days post-lesion decreasing to

approximately 40% of normal OMP levels. Thereafter OMP levels increased slowly, reaching only 50% of normal level on 42 days post-lesion. The OMP levels gradually returned to normal after 56 days following injury in apoE KO animals, similar to that observed in control mice. Taken together the OMP data demonstrate that maturation of olfactory neurons is delayed by about two weeks in apoE KO mice as compared to control animals, and that it is consistent with the results from our GAP 43 studies.

The results from this study demonstrate that apoE has a tremendous impact on neurite outgrowth, and consequently has a significant effect on neuronal plasticity. The apoE is upregulated during periods predominated by degeneration of the olfactory nerve and at the early stages of regeneration. We hypothesize that apoE is upregulated to facilitate efficient repair of neural structures following injury. The association of apoE phenotype with dementing illnesses, like Alzheimer's disease, may represent a diminished ability to support a lifetime of nerve regeneration.

INTRODUCTION:

Apolipoprotein-E (apoE) is a 34-kDa protein component of lipoproteins that functions in the redistribution of cholesterol and other lipids by interacting with lipoprotein receptors on target cells (1-4). Receptor-lipoprotein binding initiates cellular uptake and degradation of the lipoproteins. The lipid is then available for use in the regulation of intracellular cholesterol metabolism. The gene for apoE is located on the long arm of chromosome 19 and is highly polymorphic. The primary structures of apoE from 10 different species have been determined (5). ApoE ranges in length from 279 to 310 residues, with a high degree of sequence conservation among species. There are three major isoforms of apoE that exist in humans-: apoE2, apoE3 and apoE4- that differ in amino acids at positions 112 and 158 (5-8). The most common isoform, apoE3, is secreted as a 299 amino acid protein with a cysteine at position 112 and arginine at position 158. Both positions contain cysteine in apoE2 and arginine in apoE4. Mice have one form of apoE which resembles apoE4 by having arginine at both positions 112 and 158 (5).

ApoE In The Nervous System:

Apolipoprotein-E is the major apolipoprotein in the brain and cerebrospinal fluid (CSF) (9-11). It is principally synthesized and secreted by glial cells, particularly astrocytes and microglia (9, 12, 13). The apoE containing lipoproteins in CSF have cholesterol, cholesteryl esters, phosphatidyl-choline and small amounts of sphingomyelin and phosphotidylethanolamine. It has been shown that apoE is the only lipoprotein in the CSF that can interact with lipoprotein receptors (10, 11).

Cells within the brain have been known to express four 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, and d) the glycoprotein (gp) 330. The LDL receptor and the LRP are expressed by neurons (10, 12). It has been reported that the VLDL receptor in the CNS is expressed in some human neurons, whereas gp330 is expressed by brain ependymal cells (4, 15-17). It has been demonstrated that human apoE-containing 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 (18). These studies suggest that 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.

ApoE And Peripheral Nerve Regeneration:

Peripheral nerve maintenance and repair have been suggested as possible roles of apoE in the PNS (1, 19- 22). In rats, the synthesis of apoE increases by 250- to 350- fold within 3 weeks post-injury of peripheral nerve (23, 24). It has also been reported that following peripheral lesioning, macrophages synthesize and release apoE which accumulates to 5% of total extracellular protein (25) and has been proposed to scavange cholestrol from the degenerating myelin, and recycle it to the growth cones of sprouting axons by LDL-receptor -mediated endocytosis for membrane biosynthesis (1, 26, 27). During regeneration of the peripheral nerve, the increased expression of apoE is negatively regulated by translational or post-translational control, but not by transcriptional control (28). It has been proposed that apoE might be involved in neurodegenerative processes by isoform specific effects on neurite extension and cytoskeletal stability (29, 30). In vitro studies have shown that addition of apoE3 to dorsal root ganglion neurons in culture stimulates neurite extension whereas apoE4 decreased outgrowth (31). These data suggest that apoE is important for peripheral nerve regeneration (1); however, its precise role in the CNS remains unknown.

ApoE And CNS Plasticity:

The functional importance of apoE in the CNS is even less clear. ApoE immunoreactivity has been reported in neurons of human AD brains (32, 33, 34) and in the rat brain after ischemia (35). It has been shown that expression of apoE increases following optic nerve injury in rats, but absolute levels do not (19) and apoE mRNA expression by astrocytes in the hippocampus increases after entorhinal cortex lesion (36). Neuronal expression of apoE mRNA has been detected in the human brain (36). It has been demonstrated that addition of apoE3 stimulates neurite extension in transformed murine neuroblastoma (Neuro-2A) cells whereas apoE4 inhibited neurite extension (18). Recent studies in apoE KO mice have shown that these mice display significant loss of synapses and marked disruption of the dendritic cytoskeleton with age and a reduced recovery following perforant pathway lesioning (38- 40). Synaptophysin (a marker for the presynaptic terminals), and microtubule-associated protein 2 (MAP-2, a dendritic marker) in the hippocampus and neocortex of apoE KO mice have been shown to decrease when compared to age-matched controls. However, other studies have not observed any significant morphological abnormalities in apoE KO mice (41). The reasons behind these discrepancies are not clear but differences in the strain and age of control and apoE KO mice used may have contributed to the inconsistant results. In contrast to the morphological studies, behavioral studies have consistently shown that apoE KO animals exhibit

spatial learning deficits (39, 42, 43). Infusion of recombinant apoE into the lateral ventricles of apoE KO mice reversed behavioral and morphological abnormalities (44). More studies in apoE KO mice have also suggested that apoE helps protect the brain against acute injury (45). These data suggest that apoE could exert critical effects within the CNS.

ApoE and Alzheimer's Disease:

The role of apoE as an important risk factor for AD has been suggested by several studies. The characteristic pathology of AD consists of both neuritic plaques and neurofibrillary tangles. The neuritic plaques are primarily extracellular and constitute classical amyloid deposits and often a neuritic component. The major protein in the plaque is the amyloid beta peptide (Ab), which arises by cleavage of the amyloid precursor protein (APP) (46). Studies have shown that apoE is associated with Ab deposits in neuritic plaques and in the angiopathy of cerebral vessels (47). In contrast to neuritic plaques, neurofibrillary tangles are intracellular and contain structures referred to as paired helical filaments (48). ApoE has also been co-localized in these filaments (49). The role of plaques and tangles in the etiology of AD is unclear.

There are three forms of AD: early-onset familial, late-onset familial and sporadic disease. Early-onset AD represents approximately 5% of patients, where as the late-onset AD accounts for the majority of the cases. Recent studies have demonstrated an association between the apoE4 allele and late onset familial AD (47). Studies have also shown a significantly higher apoE4 allele frequency in the late onset sporadic AD (50). It has been shown that the risk of having early onset AD and disease progression increases with an increased dosage of the apoE4 allele (50, 51,). The frequency of the apoE4 allele has been shown to be 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 (51). A recent report suggests that apoE4 also modulates the age of onset of Parkinson's disease. These findings strongly demonstrate a close link between apoE polymorphism and the development of neurodegenerative diseases ; however, the mechanism behind the pathological effects of the apoE remains unknown.

One possible mechanism of apoE involvement in AD may be on the process involved with neuronal plasticity, given that previous studies suggested that apoE may play an important role in peripheral nerve regeneration. However, the effects of apoE on the CNS remain unknown.

Aim Of The Study:

The primary objective of this study was to investigate the in vivo effects of apoE on axonal growth and synaptogenesis in the CNS. I hypothesized that, lipid redistribution is a critical element of neuronal remodeling and that the absence of apoE would disrupt plasticity. To test this hypothesis, olfactory nerves of apoE KO mice and wild-type littermate mice were used as a model system. The olfactory system was selected because lesioning techniques could be used to amplify the processes of tissue repair that normally occur in this system. To determine the time course of changes associated with degeneration and regeneration of the olfactory nerve, biochemical studies were used.

The first aim of this study is to determine the time course of expression of apoE in the olfactory bulb of mice following reversible olfactory nerve lesioning. The second aim is to characterize the recovery rate of olfactory nerves following nerve lesioning in apoE KO and wild-type mice. Recovery was assessed by the expression of marker proteins GAP43 and OMP. GAP 43 is specifically expressed in newly formed olfactory neurons (52) and OMP is expressed in mature olfactory neurons (53) if they form a functional synapse.

Basic Structure Of The Olfactory Bulb:

The anatomy of the olfactory system is relatively simple. The transduction of olfactory information occurs in the olfactory epithelium, the sheet of neurons and supporting cells lining the nasal turbinates. The epithelium includes several distinct cell types; the most important of these is the olfactory receptor neuron (ORN) which is a bipolar neuron. The apical process of this neuron contains microvilli, called olfactory cilia which possess membrane receptors. These receptors when contacted by substances in inspired air and dissolved in the overlying mucous, transduce odor information. This information is carried to the olfactory bulb by the axons of the ORN. These axons collect in bundles and form the olfactory nerve which passes through the skull and terminates in the olfactory bulb. Besides the ORN, the olfactory epithelium contains two other cell classes, basal and sustentacular cells. Basal cells are critical because they divide and form new olfactory neurons (54, 55). Sustentacular cells help to detoxify chemicals that come in contact with the epithelium.

The olfactory bulb is a part of the cerebral hemisphere. It is the first CNS relay for olfactory input. The bulb is organized in concentric laminae each with characteristic cell types and synaptic connectivity. The olfactory nerve layer is the most peripheral layer which is composed of the axons of the ORN, sheathing glia and microglia/macrophages. The olfactory receptor axons terminate in the glomerular layer, synapsing on the dendrites of mitral cells in large structures called glomeruli.

Internal to the glomeruli 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 the internal granule cell layer. The granular cell layer, lying below the mitral cell layer, is principally composed of small interneurons, called granule cells, whose dendrites ramify in the external plexiform layer. The olfactory bulb also contains two other interneurons, tufted cells in the external plexiform layer and periglomerular cells surrounding glomeruli.

Biochemical Denervation Of The Olfactory Bulb:

Numerous chemicals and physical transection are known to damage the olfactory nerve in mice (52- 64). Some chemicals, such as zinc sulphate, can induce degeneration of both the ORN and the basal cells and result in either a very prolong regeneration process or complete failure to regenerate. Reversible lesioning techniques spare the basal cells and allow regeneration of ORN and replacement of the olfactory epithelium. It has been shown that intranasal irrigation of TX in mice causes rapid retrograde degeneration of the ORN in the epithelium, with attendant orthograde degeneration of their synaptic terminals in the bulb (56, 57), while sparing the basal cells. These basal cells differentiate into mature ORN that restablishes functional connections in the bulb. The entire regeneration process occurs in approximately 2 months.

Olfactory System Changes Following Olfactory Nerve Lesion:

Previous studies have reported a reasonably good timeline for regeneration of the olfactory nerve following acute lesions of the receptor mucosa in mice (52, 53, 58- 60). Nerve degeneration occuring in the first week following lesioning of the mucosa, is evidenced by loss of olfactory marker protein (OMP) and silver stains for degeneration. Three days post lesion, basal cells start to proliferate peaking by about five weeks. New axons enter the olfactory nerve layer at one to two weeks; and at three weeks, new fibres are visible in the glomeruli. Therefore, within a relatively short time, fibres regenerate and reinnervate the olfactory bulb, although the epithelial cell division is not complete for five weeks.

Olfactory nerve lesioning causes visible shrinkage of the olfactory bulb. Wet weight decrease by about 30% at two weeks and by 40% at three to four weeks (57, 61, 62). Olfactory nerve-produced proteins within the bulb, such as OMP and growth associated protein 43 (GAP43) parallel olfactory nerve loss, decreasing by 60-80% (52, 53, 63).

It has been reported that following a reversible lesion in mice, olfactory nerve recovery starts at about two weeks, and consists of two distinct stages (52, 53). The first stage is characterized by the formation of a large population of immature olfactory neurons. These newly formed neurons express GAP43, a phosphoprotein related to neuronal growth and plasticity. During the second stage the newly formed neurons mature when their axons make contact with the olfactory bulb. This stage is characterized by a decrease in the expression of GAP43, and an increase in the expression of OMP, a protein present only in mature neurons (61). These data suggest that new axons from the epithelium enter the bulb at two to three weeks (55, 63).

The Olfactory System As A Model For Regeneration:

The adult olfactory system represents an ideal model for studying proteins involved in neuronal growth-related processes. First, the primary olfactory receptor neurons regenerate from stem cells throughout the life of the organism (65). The exceptional ability of the ORN to regenerate makes it an important neural system for studying the mechanisms operative in neuronal degeneration, and post-lesion plasticity. Second, the cytoarchitecture and connectivity of the bulb are relatively simple which permits reasonably simple interpretations of experimental manipulations. 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.

MATERIALS & METHODS

Nasal Irrigation

The wild type C57BL6J and apE KO mice on the same background used in this study were purchased from Jackson Laboratories. Two to three month old mice were lesioned as previously described (53, 56, 61). For each time point, an equal number of wild type (n = 8) and apoE KO mice (n = 8) were lesioned. Briefly, a 25 gauge needle 10 mm in length with a rounded tip, was inserted about 2 mm into one nostril of an unanesthetized mouse, and 100 ul of 0.7% (v/v) Triton-X100 (TX) (BP151-500, Fisher, St. Louis, MO) in 0.9% saline or 100 ul of 0.9% saline (control) was squirted into the nostril. The excess solution was drained from the nasal passages by gently shaking the mouse. This technique results in complete bilateral nerve lesion. Mice were sacrificed at 3,7,21,42 and 56 days post-treatment . This time course was selected to cover the range of degeneration and recovery as previously described (53).

Sacrifice Procedures

Following the appropriate survival periods after TX or saline irrigation, mice were anesthetized with intraperitonial injection of 100 ul of pentobarbital (80 mg/kg) . A needle was then inserted transcardially and animals were thoroughly perfused with phosphate buffered saline (pH 7.4) until the perfusate was free of blood. Following perfusion, brains were removed and olfactory bulbs collected and homogenized in ice cold 100 ul homobuffer (25 mM Tris HCl pH 7.6, 3 mM MgCl2, 100 mM NaCl, 0.28 mM PMSF and 0.25 mM EDTA). Twenty ul of 10% IGEPAL (1-3021 CA-630, Sigma, St. Louis, MO) was added to the homogenate and the samples incubated on ice for 30 minutes and centrifuged at 14,000 g for 10 minutes to remove the insoluble material. The supernatant was collected and stored at -20°C.

Protein Assay:

Protein assays were performed by the Lowry method as modified by Peterson (66). Briefly, 5 ul of homogenized olfactory bulb sample was diluted to 50 ul with double deionized water. Ten microliters of diluted protein samples (in triplicates) were precipitated in order to remove interfering substances. One hundred microliters of 0.15% Na-deoxycholate (DOC) (BP 349-100, Fisher, St. Louis, MO) was added to samples, vortexed and allowed to stand for 10 minutes at room temperature. One hundred microlitres of trichloroacetic acid (TCA) (A322-100, Fisher) were added

and the samples vortexed and centrifuged at 14,000 g for 25 minutes. The supernatant was discarded immediately after centrifugation and the pellet was saved for the assay step.

The pellet was resuspended in 400 ul of Reagent A (equal volume of dH2O, 0.8N NaOH, copper tartarate carbonate (CTC) and 10% SDS) vortexed and allowed to stand for 10 minutes at room temperature. To samples, 200 ul of Reagent B (1 part Folin-Ciocalteu's phenol reagent (F-9252, Sigma, St.Louis, MO) and 5 parts dH2O) was added, vortexed and incubated for 50 minutes at room temperature for color development. Following incubation, absorbance was recorded at 750 nm using a spectrophotometer. BSA-Bovine serum albumin (A-7511, fatty acid free, Sigma, St.Louis, MO) was used as standard sample for protein assay.

SDS-Polyacrylamide Gel Electrophoresis:

Proteins in the olfactory bulb extracts were resolved by SDS-PAGE as previously described (18). Briefly, 12% separating gel solution was prepared by mixing 1.67 ml dH2O, 1.5 M Tris-HCl (pH 8.8), 50 ul of 10% SDS (BP166-100, Fisher) and 2 ml of 30% acrylamide/bis (29.2 gm acrylamide (BP200-A, Fisher) and 0.8 gm bisacrylamide (BP200-B, Fisher). To accelerate the rate of polymerization, 25 ul of 10% ammonium per sulfate (BP200-C, Fisher) and 5 ul TEMED (N, N, N', N'- Tetramethylethylenediamine) (BP200-D, Fisher) was added to mixture and the solution was immediately poured into the assembled glass plates and allowed to polymerize for 45 minutes. To make the 4% stacking gel, 1.525 ml dH2O, 625 ul 0.5M Tris-HCl (pH 6.8), 25 ul of 10% SDS and 335 ul of 30% acrylamide/bis were mixed gently for 10 minutes. To the mixture, 12.5 ul of 10% ammonium per sulfate and 5 ul TEMED were added and it was poured into the gel cassettes. A comb was inserted into the stacking gel solution to create wells. The stacking gel was allowed to polymerize for 30 minutes at room temperature.

Eighty micrograms of supernatant was mixed with an equal volume of 2X Lammeli sample buffer (6.25 ml 4X Tris/SDS pH 6.8, 5 ml glycerol, 1 gm SDS, 0.5 ml 2 mercaptoethanol, bromophenol 13.25 ml dH2O) and heated at 100°C for 5 minutes. Samples were centrifuged for 5 minutes at 14,000 g and the supernatant collected. The gel cassettes were inserted into the buffer tank of an EC120 Mini gel vertical system (E-C Apparatus Corporation, St. Petersburg, FL) and the upper and lower buffer compartments filled with one liter of 1X running buffer pH 8.3 [250 ml of 5X running buffer (15 g Tris-base, 72 g glycine(BP381-1, Fisher), 5 g SDS and 750 ml dH2O]. The samples and 5 ul of kaleidoscope prestained standards (161-0324, Bio-Rad Laboratories, Hercules,

CA) were loaded in the wells. The safety cover was replaced in the gel system and the samples were run at 80 volts in the stacking gel and at 140 volts in the separating gel.

Protein Transfer:

Following gel electrophoresis, the gel cassettes were removed from the buffer tank and the plates separated. The stacking gel was removed with a razor blade and the separating gel placed in transfer buffer pH 8.3 (3.03 gm Tris-base, 14.4 gm glycine, 200 ml methanol and 800 ml dH2O) for 20 minutes on a shaker. The transfer membrane (Immobilon-P IPVH00010, Millipore) was first soaked in methanol for 5 seconds and then washed in dH2O for 5 minutes to remove the excess methanol. The gel was placed on presoaked filter paper in a gel holder and the transfer membrane was placed on top of the gel. The gel holder was placed in the buffer tank of trans-blot transfer cell and the entire chamber filled with transfer buffer. Proteins from the gel were transferred onto the membrane by passing 100 volts for an hour. The membrane was transferred to blotto (25 gm Carnation skim milk powder, 50 ul antifoam A, 5 ul 10% thimerosol, 500 ml borate buffer pH 7.4) and incubated overnight on a shaker at 4°C.

Western Immunoblotting:

For quantitation of apoE in the samples, the blots were incubated in monoclonal goat anti-human apoE antibody (1:1000 dilution in TBS/Tween) (178479, Calbiochem, San Diego, CA) for an hour on a shaker, then washed four times (10 minutes each) with wash solution pH 7.4 (8.75 gm NaCl, 20 ml 1 M Tris, 1 ml Tween 20 and 2 ml 10% NaN3). Blots were incubated in diluted alkaline phosphatase-conjugated donkey anti-goat IgG (1:2000 dilution in TBS/Tween) (705-055-093, Jackson Immuno Research Laboratories Inc, West Grove, PA) for an hour at room temperature on a shaker. Blots were then washed with wash solution five times (10 minutes each) and washed twice for 10 minutes each with 0.15 M Tris pH 8.8. Blots were processed in developer containing 5 ml 0.15 M Tris pH 8.8, 33 ul NBT (Nitro BT) (BP108-1, Fisher) and 16.5 ul BCIP (5-Bromo- 4 Chloro-3-Indolyl Phosphate P-Toluidine salt) (BP1610-100, Fisher) until bands appeared. The primary antibody recognized a single band around 35 kDa which is consistent with the published molecular weight of apoE (1).

For quantitation of GAP 43 and OMP, the blots were incubated in monoclonal mouse IgG2 against GAP 43 antibody (1: 700 dilution in TBS/Tween) (G9264, Sigma, St.Louis, MO) and in goat

anti-rat OMP (1:7000 dilution in TBS/Tween) (67, 68) respectively for an hour. The blots were washed with wash solution pH 7.4 four times (10 minutes each). Blots were incubated for GAP 43 in secondary alkaline phosphatase-- conjugated goat anti-mouse IgG (1:2000 dilution in TBS/Tween) (115-055-062, Jackson Immuno Research Laboratories Inc, West Grove, PA) for an hour. Blots for OMP were incubated in alkaline phosphatase--conjugated donkey anti-goat IgG (1:2000 dilution in TBS/Tween) (705-055-093, Jackson Immuno Research Laboratories, Inc, West Grove, PA) for an hour. Blots were washed with wash solution pH 7.4 for five times (10 minutes each) and twice with 0.15 M Tris pH 8.8 (10 minutes each). Blots were processed in developer until bands appeared. The primary antibody recognized a 46 kDa band for GAP 43. A single band around 19 kDa was recognized for OMP by the primary antibody (69). All experiments were repeated at least three times to assure reproducibility of the results. Bands were quantified by densitometery (Scion Image). As an internal control, the blots also contained an olfactory bulb extract from unlesioned animals whose level of apoE, GAP 43 or OMP was arbitrarily given a value of one, and all values from different time points of the TX- or saline irrigated animals were expressed relative to the unlesioned mouse values.

RESULTS

Time Course Of ApoE Expression Following Olfactory Nerve Lesioning In Mice

In this study, olfactory nerve lesioning was performed by intranasal irrigation of Triton X-100 (TX) in saline. Following lesioning, olfactory bulbs were collected at 3, 7, 14, 21, 42 and 56 days. The time course of apoE expression in the olfactory bulb of wild-type mice was examined using immunoblotting techniques. ApoE level peaked at 3 days post-lesioning reaching a concentration twice of that found in the normal olfactory bulb. ApoE levels stayed elevated by approximately 1.5 times the normal level at 7 through 21 days after injury, and thereafter gradually returned to normal by 56 days (Fig. 1 & 2) (Table 1). Immunoblots for saline irrigated animals showed no significant difference in apoE levels at different time periods (Fig. 1 & 2) (Table 1).

Impact Of ApoE Deficiency On Olfactory Nerve Regeneration In ApoE KO Mice

Based on the data from this study on apoE expression during nerve regeneration, it was hypothesized that if apoE is important in nerve repair, then nerve regrowth should be either incomplete or delayed in the absence of apoE. This postulate was tested by comparing olfactory nerve repair in apoE KO with control mice. Olfactory nerve recovery was assessed by immunoblotting of GAP 43, a marker protein for juvenile olfactory neurons. Immunoblot analysis revealed that GAP 43 level in control animals increased by approximately two times the normal level at 3 day post-lesion, and returned back to baseline at 7 days after injury. At 21 days post –lesioning GAP 43 levels increased dramatically reaching a concentration 3 times which is found in the normal olfactory bulb. GAP 43 levels stayed elevated until 42 days, and thereafter gradually returned to normal by 56 days (Fig. 4 & 5) (Table 4).

Even though the GAP 43 time course in apoE KO mice followed a pattern similar to that observed in control animals, there were two significant differences. First, the major increase in GAP 43 was observed on 42 days post- lesioning, which is a two week delay from that observed in control animals, suggesting that the generation of new olfactory neurons is delayed by two weeks in apoE KO mice. Second, the maximum increase of GAP 43 in apoE KO mice was only two- fold as compared to a three- fold rise in control mice. In sum, the data from GAP 43 experiments demonstrate that olfactory nerve regeneration is delayed by approximately two weeks in apoE KO mice as compared to control animals (Fig. 4 & 5) (Table 7).

To confirm the GAP 43 data and examine the effects of apoE deficiency in the maturation of olfactory neurons, the expression of olfactory marker protein (OMP) was examined. Immunoblot analysis revealed that OMP levels in the bulb of control animals gradually declined following nerve lesioning, decreasing to approximately 50% of the normal level at 7 days post- lesioning. Thereafter, OMP levels sharply increased to about 80% of the normal level at 21 days post- lesioning, and then gradually increased to normal by 56 days (Fig. 6 & 8) (Table 11). These results are consistent with previously published results in rats. In apoE KO animals the OMP time course was different than that of control animals. First, OMP levels continued to decrease in apoE KO animals until 21 days post- lesioning decreasing to approximately 40% of normal OMP levels. Thereafter, OMP levels increased slowly reaching only 50% of normal levels on 42 days post- lesioning. OMP levels gradually returned to normal on 56 days following injury in apoE KO animals similar to that observed in control mice (Fig.7 & 8) (Table 14). Taken together the OMP data demonstrate that maturation of olfactory neurons is delayed by two weeks in apoE KO mice as compared to control animals, and that it is consistent with the results from our GAP 43 studies.

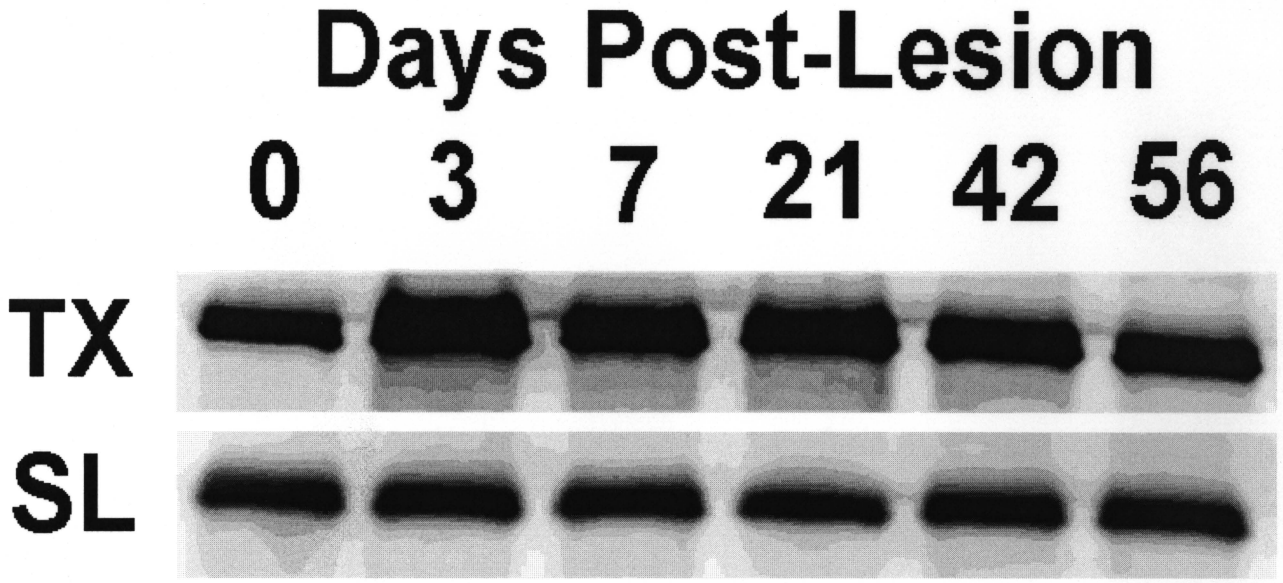

Figure 1. Time Course of Apo E expression following olfactory nerve lesion.

Table 1. Mean comparison of Apo E levels in saline and TX irrigated mice following olfactory nerve lesion.

Saline

	0 Days	3 Days	7 Days	21 Days	42 Days	56 Days
Exp 1	1.00	1.00	0.97	0.88	0.97	0.97
Exp 2	0.90	1.20	1.30	1.30	0.88	0.88
Exp 3	1.20	1.10	1.10	1.20	1.10	1.00
Mean	1.03	1.10	1.12	1.12	0.98	0.95
Std Dev	0.15	0.10	0.17	0.22	0.11	0.06
SEM	0.09	0.06	0.09	0.12	0.06	0.04

Triton

	0 Days	3 Days	7 Days	21 Days	42 Days	56 Days
Exp 1	1.03	2.26	1.66	1.76	1.60	1.40
Exp 2	0.96	1.70	1.40	1.60	1.20	0.70
Exp 3	0.70	2.10	1.70	1.40	1.00	1.00
Mean	0.89	2.02	1.58	1.58	1.26	1.03
Std Dev	0.17	0.28	0.16	0.18	0.30	0.35
SEM	0.10	0.16	0.09	0.10	0.17	0.20

Table 2. Two way ANOVA for ApoE levels between Saline and TX treated control mice.

	0d	3d	7d	21d	42d	56d
Pooled Variance	0.027	0.046	0.027	0.040	0.053	0.063
Hypothesized Mean Difference	0	0	0	0	0	0
Df	4	4	4	4	4	4
t stat	1.027	-5.219	-3.451	-2.805	-1.510	-0.404
P(T<=t) one-tail	0.182	0.003	0.013	0.024	0.103	0.353
t Critical one-tail	2.132	2.132	2.132	2.132	2.132	2.132
P(T<=t) two-tail	0.364	0.006	0.026	0.048	0.205	0.706
t Critical two-tail	2.776	2.776	2.776	2.776	2.776	2.776

Table 3. Two way ANOVA for ApoE levels between TX treated control mice.

	0d & 3d	3d & 7d	7d & 21d	21d & 42d	42d & 56d
Pooled Variance	0.057	0.549	0.026	0.063	0.108
Hypothesized Mean Difference	0	0	0	0	0
df	4	4	4	4	4
t stat	-5.777	2.266	0	1.562	0.868
P(T<=t) one-tail	0.002	0.043	0.5	0.097	0.217
t Critical one-tail	2.132	2.132	2.132	2.132	2.132
P(T<=t) two-tail	0.004	0.086	1.000	0.193	0.434
t Critical two-tail	2.776	2.776	2.776	2.776	2.776

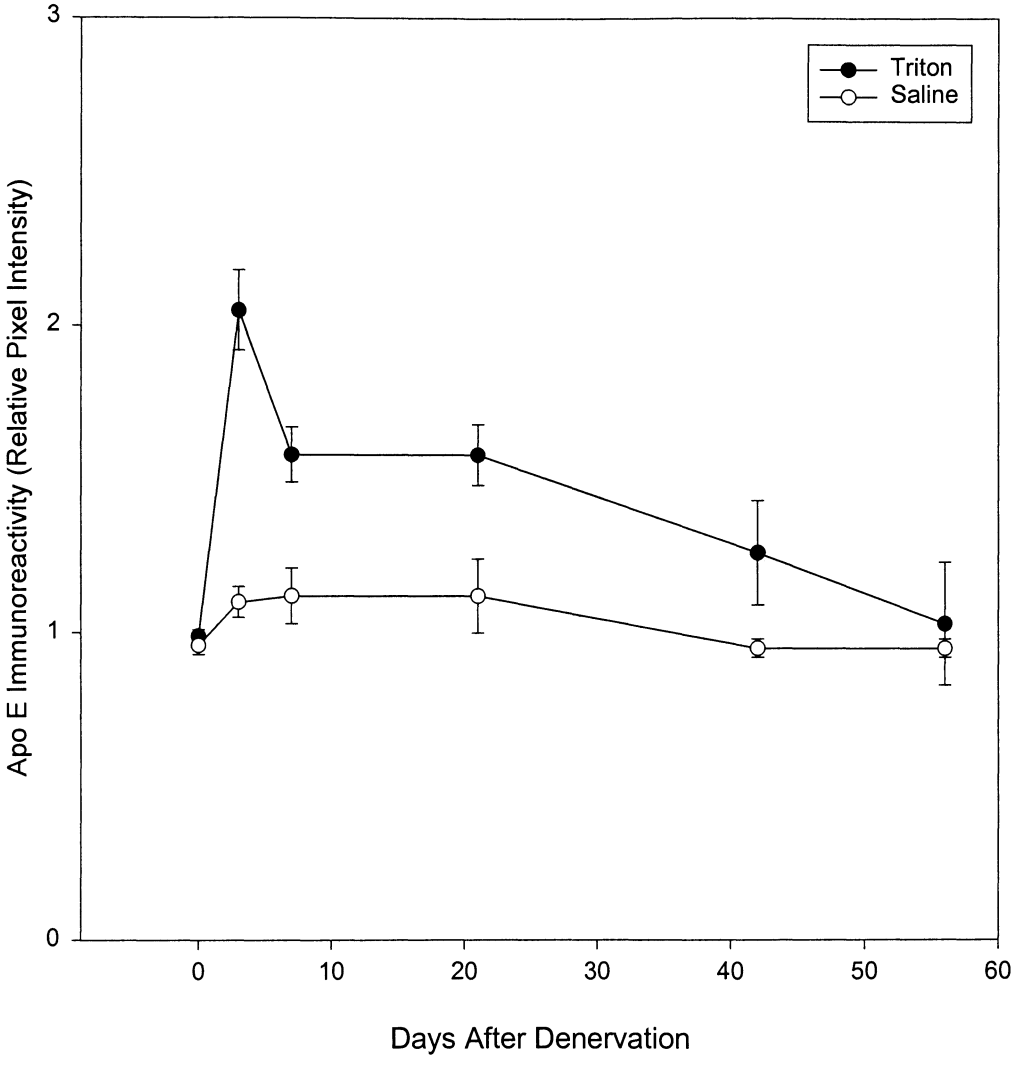

Figure 2. Comparison of Apo E levels following olfactory nerve lesion.

Figure 3. Time course of GAP 43 expression in wild type mice following olfactory nerve lesion.

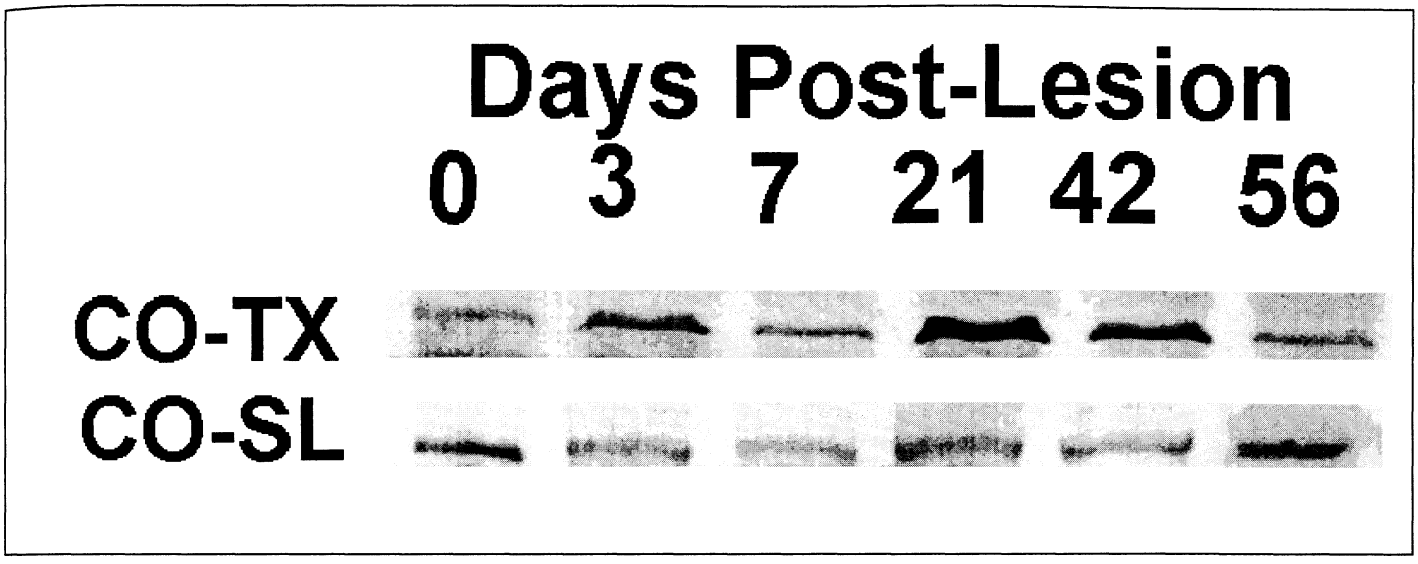

Table 4. Mean comparison of GAP 43 levels in Saline and TX irrigated wild type mice.

Saline

	0 Days	3 Days	7 Days	21 Days	42 Days	56 Days
Exp 1	1.40	1.60	1.30	1.40	1.60	1.40
Exp 2	1.50	1.00	1.20	1.50	1.40	1.90
Exp 3	1.50	1.30	1.40	1.80	1.10	1.60
Mean	1.47	1.30	1.30	1.57	1.37	1.63
Std Dev	0.06	0.30	0.10	0.21	0.25	0.25
SEM	0.03	0.17	0.06	0.12	0.15	0.15

Triton

	0 Days	3 Days	7 Days	21 Days	42 Days	56 Days
Exp 1	1.00	2.10	1.40	2.50	2.80	1.30
Exp 2	1.00	1.60	1.30	2.70	2.70	1.40
Exp 3	0.90	1.90	1.10	3.40	2.00	0.90
Mean	0.96	1.87	1.27	2.87	2.50	1.20
Std Dev	0.07	0.25	0.15	0.47	0.44	0.26
SEM	0.04	0.15	0.09	0.27	0.25	0.15

Table 5. Two way ANOVA for GAP 43 levels between Saline and TX treated Wild type mice.

	0d	3d	7d	21d	42d	56d
Pooled Variance	0.003	0.076	0.017	0.133	0.136	1.633
Hypothesized Mean Difference	0	0	0	0	0	0
Df	4	4	4	4	4	4
t stat	10.606	-2.506	0.316	-4.360	0.126	2.055
P(T<=t) one-tail	0.000	0.033	0.384	0.006	0.008	0.054
t Critical one-tail	2.132	2.132	2.132	2.132	2.132	2.132
P(T<=t) two-tail	0.000	0.066	0.767	0.012	0.017	0.109
t Critical two-tail	2.776	2.776	2.776	2.776	2.776	2.776

Table 6. Two way ANOVA for GAP 43 levels between TX treated Wild type mice.

	0d & 3d	3d & 7d	7d & 21d	21d & 42d	42d & 56d
Pooled Variance	0.033	0.043	0.123	0.206	0.130
Hypothesized Mean Difference	0	0	0	0	0
df	4	4	4	4	4
t stat	-6.037	3.530	-5.580	0.988	4.416
P(T<=t) one-tail	0.002	0.012	0.002	0.189	0.005
t Critical one-tail	2.132	2.132	2.132	2.132	2.132
P(T<=t) two-tail	0.003	0.024	0.005	0.379	0.011
t Critical two-tail	2.776	2.776	2.776	2.776	2.776

Figure 4. Time course of GAP 43 expression in Apo E KO mice following Olfactory nerve lesion.

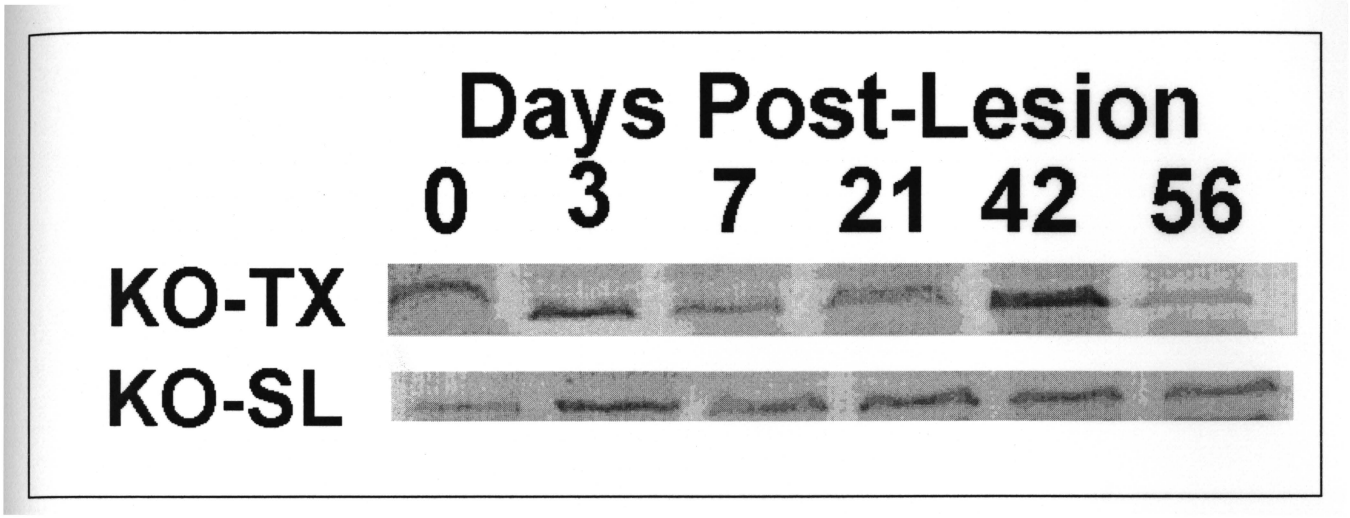

Table 7. Mean comparison of GAP 43 levels in saline and TX irrigated Apo E KO mice.

Saline

	0 Days	3 Days	7 Days	21 Days	42 Days	56 Days
Exp 1	1.00	1.50	1.30	1.20	1.30	0.90
Exp 2	1.00	1.30	1.10	0.90	0.90	1.30
Exp 3	0.90	1.60	1.50	1.50	1.10	1.00
Mean	0.96	1.47	1.30	1.20	1.10	1.07
Std Dev	0.07	0.15	0.20	0.30	0.20	0.21
SEM	0.04	0.09	0.12	0.17	0.12	0.12

Triton

	0 Days	3 Days	7 Days	21 Days	42 Days	56 Days
Exp 1	1.10	1.60	1.60	1.70	2.30	1.10
Exp 2	0.90	1.70	1.20	1.50	2.00	1.10
Exp 3	1.10	1.90	1.00	1.50	1.80	1.00
Mean	1.03	1.73	1.27	1.57	2.03	1.07
Std Dev	0.12	0.15	0.31	0.12	0.25	0.06
SEM	0.07	0.09	0.18	0.07	0.15	0.03

Table 8. Two way ANOVA for GAP 43 levels between Saline and TX treated apoE KO mice.

	0d	3d	7d	21d	42d	56d
Pooled Variance	0.008	0.023	0.066	0.051	0.051	0.023
Hypothesized Mean Difference	0	0	0	0	0	0
Df	4	4	4	4	4	4
t stat	-0.894	-2.138	0.158	-1.976	-5.029	0.000
P(T<=t) one-tail	0.210	0.049	0.441	0.060	0.003	0.500
t Critical one-tail	2.132	2.132	2.132	2.132	2.132	2.132
P(T<=t) two-tail	0.421	0.099	0.882	0.119	0.007	1.000
t Critical two-tail	2.776	2.776	2.776	2.776	2.776	2.776

Table 9. Two way ANOVA for GAP 43 levels between TX treated apoE KO mice.

	0d & 3d	3d & 7d	7d & 21 d	21d & 42d	42d & 56d
Pooled Variance	0.018	0.058	0.053	0.038	0.033
Hypothesized Mean Difference	0	0	0	0	0
Df	4	4	4	4	4
t stat	-6.331	2.366	-1.591	-2.919	6.484
P(T<=t) one-tail	0.001	0.038	0.093	0.021	0.001
t Critical one-tail	2.132	2.132	2.132	2.132	2.132
P(T<=t) two-tail	0.003	0.077	0.187	0.043	0.003
t Critical two-tail	2.776	2.776	2.776	2.776	2.776

Figure 5. Comparison of GAP 43 levels between wild type and Apo E KO mice following olfactory nerve lesion.

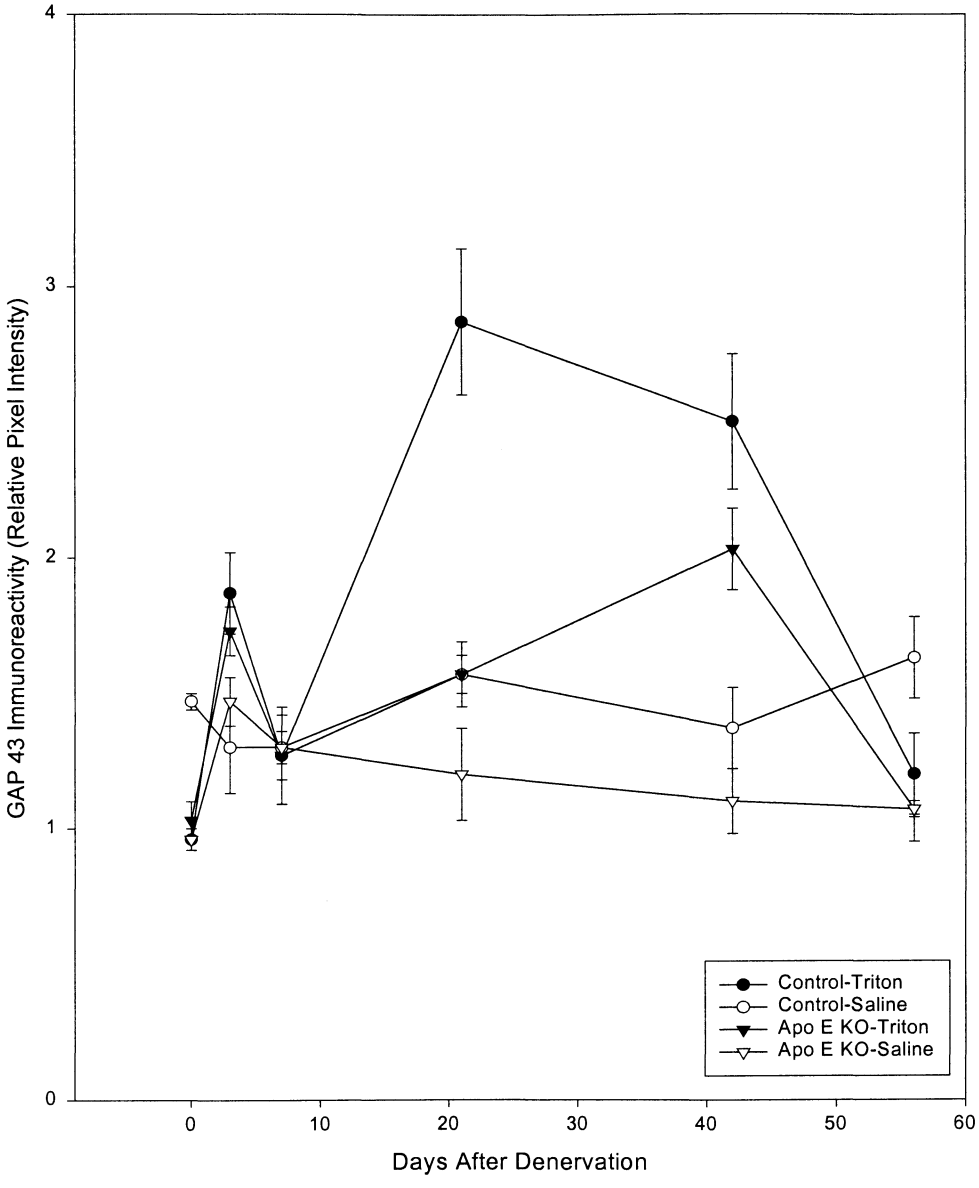

Table 10. Two way ANOVA for GAP 43 levels between TX treated Wild type and apoE KO mice.

	0d	3d	7d	21d	42d	56d
Pooled Variance	0.008	0.043	0.025	0.118	0.126	0.036
Hypothesized Mean Difference	0	0	0	0	0	0
df	4	4	4	4	4	4
t stat	-0.894	0.784	0.000	4.628	1.606	0.852
P(T<=t) one-tail	0.211	0.238	0.500	0.005	0.091	0.221
t Critical one-tail	2.132	2.132	2.132	2.132	2.132	2.132
P(T<=t) two-tail	0.421	0.476	1.000	.001	0.183	0.441
t Critical two-tail	2.776	2.776	2.776	2.776	2.776	2.776

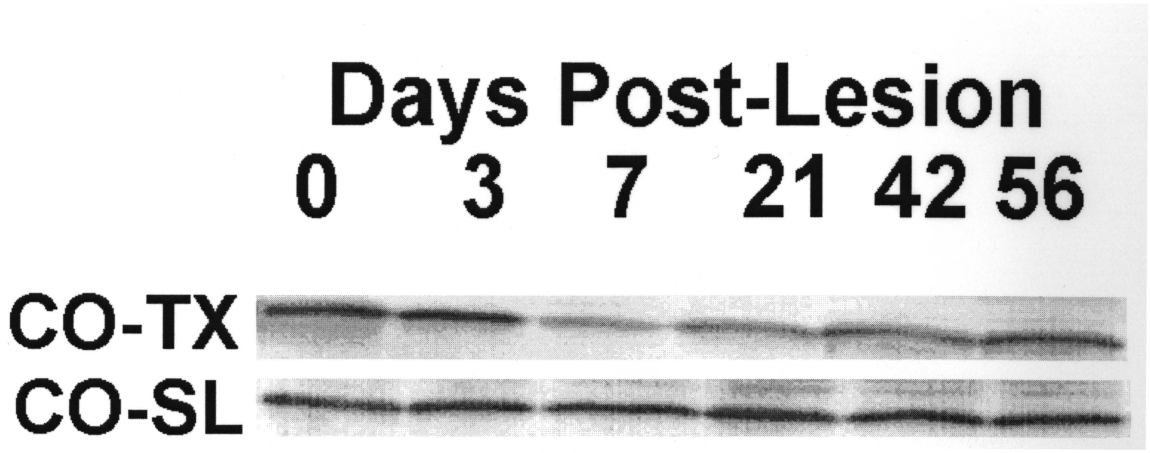

Figure 6. Time course of OMP expression in wild type mice following olfactory nerve lesion.

Table 11. Mean comparison of OMP levels in saline and TX irrigated wild type mice.

Saline

	0 Days	3 Days	7 Days	21 Days	42 Days	56 Days
Exp 1	1.00	1.00	0.90	1.00	1.00	1.10
Exp 2	0.90	1.10	1.20	1.10	1.20	0.80
Exp 3	1.10	1.10	1.00	1.20	1.10	1.00
Mean	1.00	1.07	1.03	1.10	1.10	0.97
Std Dev	0.10	0.06	0.15	0.10	0.10	0.15
SEM	0.06	0.03	0.09	0.06	0.06	0.09

Triton

	0 Days	3 Days	7 Days	21 Days	42 Days	56 Days
Exp 1	1.00	0.80	0.70	0.80	0.80	0.90
Exp 2	1.00	0.80	0.50	0.90	0.90	1.00
Exp 3	0.70	0.70	0.40	0.60	0.80	0.90
Mean	0.90	0.77	0.52	0.77	0.83	0.93
Std Dev	0.17	0.06	0.13	0.15	0.06	0.06
SEM	0.10	0.03	0.08	0.09	0.03	0.03

Table 12. Two way ANOVA for OMP levels between Saline and TX treated Wild type mice.

	0d	3d	7d	21d	42d	56d
Pooled Variance	0.020	0.003	0.023	0.017	0.007	0.013
Hypothesized Mean Difference	0	0	0	0	0	0
Df	4	4	4	4	4	4
t stat	0.866	6.364	4.009	3.162	4	0.353
P(T<=t) one-tail	0.217	0.001	0.008	0.017	0.008	0.370
t Critical one-tail	2.132	2.132	2.132	2.132	2.132	2.132
P(T<=t) two-tail	0.435	0.003	0.016	0.034	0.016	0.741
t Critical two-tail	2.776	2.776	2.776	2.776	2.776	2.776

Table 13. Two way ANOVA for OMP levels between TX treated Wild type mice.

	0d & 3d	3d & 7d	7d & 21d	21d & 42d	42d & 56d
Pooled Variance	0.016	0.013	0.023	0.013	0.003
Hypothesized Mean Difference	0	0	0	0	0
Df	4	4	4	4	4
t stat	1.265	2.475	-1.870	-0.707	-2.121
P(T<=t) one-tail	0.137	0.0342	0.067	0.259	0.050
t Critical one-tail	2.132	2.132	2.132	2.132	2.132
P(T<=t) two-tail	0.274	0.068	0.134	0.518	0.101
t Critical two-tail	2.776	2.776	2.776	2.776	2.776

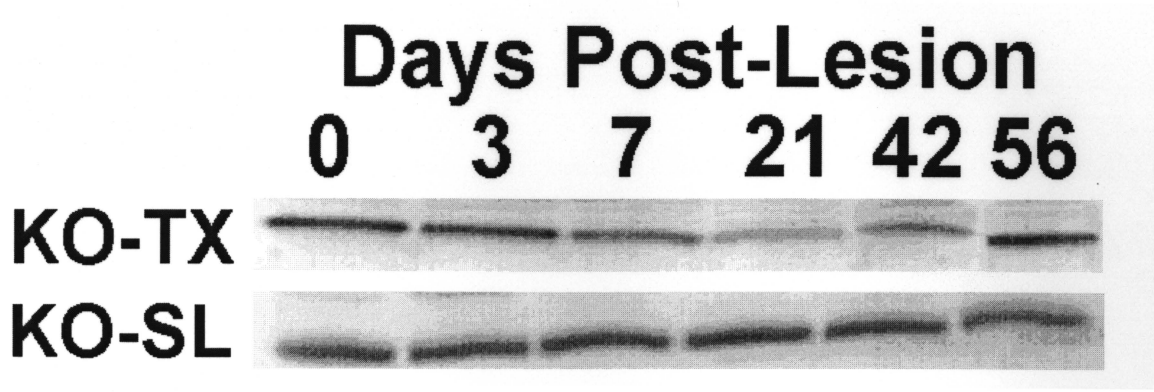

Figure 7. Time course of OMP Expression in Apo E KO mice following olfactory nerve lesion.

Table 14. Mean comparison of OMP levels in saline and TX irrigated Apo E KO mice.

Saline

	0 Days	3 Days	7 Days	21 Days	42 Days	56 Days
Exp 1	1.00	1.00	0.90	1.10	1.00	0.70
Exp 2	0.80	1.10	1.00	0.90	0.90	0.90
Exp 3	1.10	1.00	1.10	1.10	1.10	1.10
Mean	0.97	1.03	1.00	1.03	1.00	0.90
Std Dev	0.15	0.06	0.10	0.12	0.10	0.20
SEM	0.09	0.03	0.06	0.07	0.06	0.12

Triton

	0 Days	3 Days	7 Days	21 Days	42 Days	56 Days
Exp 1	1.00	0.80	0.70	0.50	0.60	0.70
Exp 2	1.00	0.80	0.50	0.40	0.40	0.90
Exp 3	0.90	1.00	0.40	0.30	0.70	0.90
Mean	0.95	0.87	0.53	0.40	0.57	0.83
Std Dev	0.05	0.12	0.15	0.10	0.15	0.12
SEM	0.03	0.07	0.09	0.06	0.09	0.07

Table 15. Two way ANOVA for OMP levels between Saline and TX treated apoE KO mice.

	0d	3d	7d	21d	42d	56d
Pooled Variance	0.013	0.008	0.016	0.011	0.016	0.026
Hypothesized Mean Difference	0	0	0	0	0	0
Df	4	4	4	4	4	4
t stat	1.185	2.236	4.427	7.181	4.110	0.500
P(T<=t) one-tail	0.500	0.044	0.006	0.001	0.007	0.321
t Critical one-tail	2.132	2.132	2.132	2.132	2.132	2.132
P(T<=t) two-tail	1.000	0.089	0.011	0.002	0.015	0.643
t Critical two-tail	2.776	2.776	2.776	2.776	2.776	2.776

Table 16. Two way ANOVA for OMP levels between TX treated apoE KO mice.

	0d & 3d	3d & 7d	7d & 21d	21d & 42d	42d & 56d
Pooled Variance	0.008	0.018	0.016	0.016	0.018
Hypothesized Mean Difference	0	0	0	0	0
Df	4	4	4	4	4
t stat	1.341	3.015	1.264	-1.581	-2.412
P(T<=t) one-tail	0.125	0.019	0.137	0.094	0.036
t Critical one-tail	2.132	2.132	2.132	2.132	2.132
P(T<=t) two-tail	0.251	0.039	0.274	0.189	0.073
T Critical two-tail	2.776	2.776	2.776	2.776	2.776

Figure 8. Comparison of OMP levels between wild type and Apo E KO mice following olfactory nerve lesion.

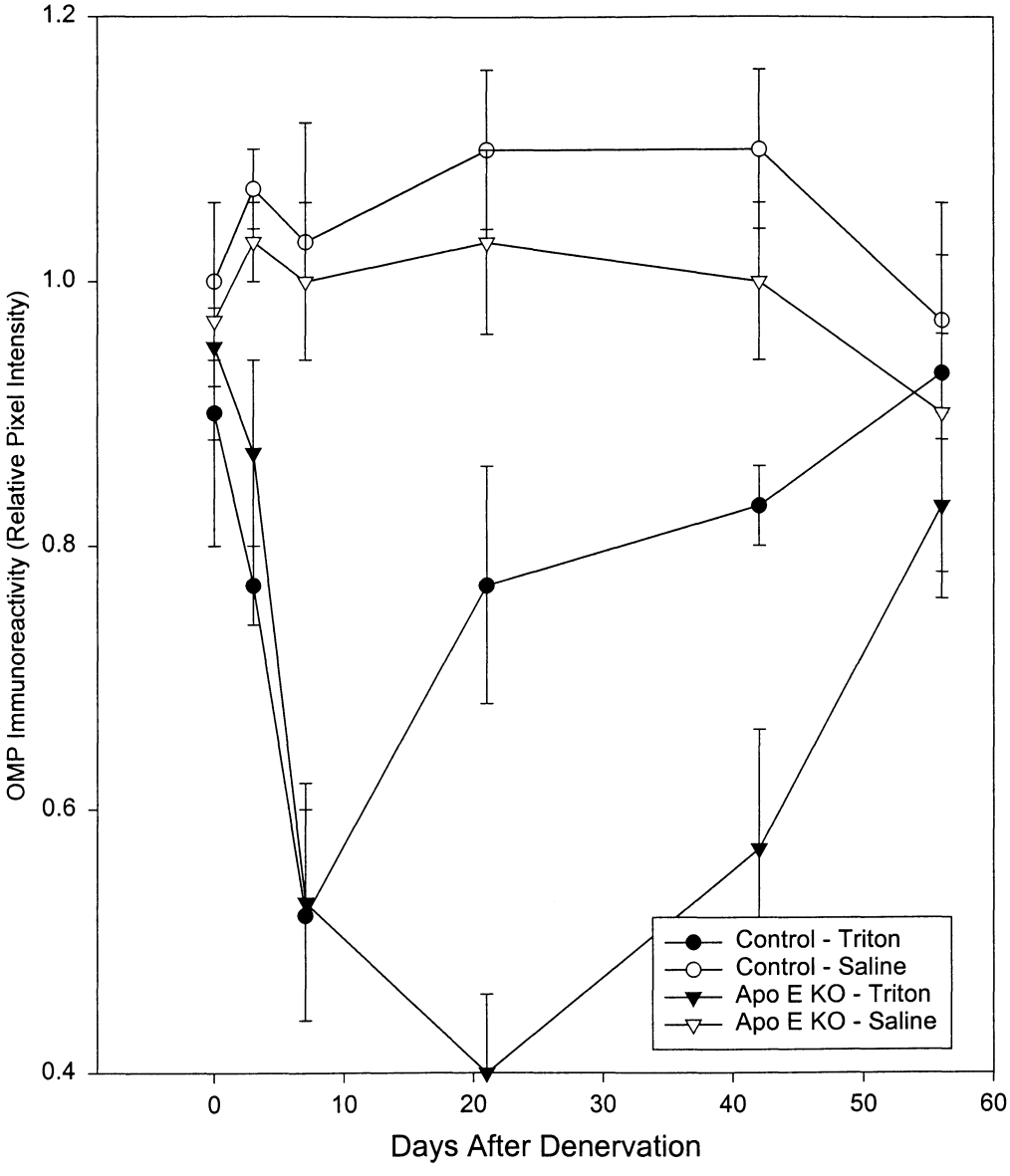

Table 17. Two way ANOVA for OMP levels between TX treated Wild type and apoE KO mice.

	0d	3d	7d	21d	42d	56d
Pooled Variance	0.016	0.008	0.023	0.016	0.013	0.008
Hypothesized Mean Difference	0	0	0	0	0	0
df	4	4	4	4	4	4
t stat	-0.632	-1.341	0.000	3.478	2.828	1.341
P(T<=t) one-tail	0.280	0.125	0.500	0.012	0.023	0.125
t Critical one-tail	2.132	2.132	2.132	2.132	2.132	2.132
P(T<=t) two-tail	0.561	0.250	1.000	0.025	0.047	0.250
t Critical two-tail	2.776	2.776	2.776	2.776	2.776	2.776

DISCUSSION

Apolipoprotein E is unique among apolipoproteins in that it has a special relevance to the nervous system. The role of apoE in neurobiology has been suggested by a series of observations made over the past several years. First, apoE messenger RNA is abundant in the brain and is synthesized and secreted primarily by astrocytes. Second, apoE-containing lipoproteins are found in the cerebrospinal fluid and appear to play a major role in lipid transport in the CNS. Third, apoE, together with a source cholesterol, promotes marked neurite extension in cultured dorsal root ganglion cells. Fourth, apoE levels increase dramatically after peripheral nerve injury. Recently, apoE4 has been implicated in the pathogenesis of AD, but the mechanism by which apoE is linked to the disease is unknown. The aim of this study was to understand the role of apoE in the CNS during nerve regeneration.

To test my hypothesis, I used olfactory nerves of mice as the model system. The ability to cause central degradation by peripheral manipulation makes the primary olfactory pathway a very attractive neural system to study. This model system has many distinct advantages. First, this pathway is quite suited to regeneration studies since the perikarya lie outside the central nervous system and are fairly accessible in the nasal epithelium while the synaptic endings are within the cranium itself, in the olfactory bulb. Second, the olfactory bulb has a simple organization with discrete input and output neurons and it is the only region in the CNS in which total degeneration of the primary sensory nerves can be experimentally induced after which reinnervation results in reconstitution of the system (63, 70, 71, 72, 73). Third, the olfactory receptor cells have a unique property among the neurons of the central nervous system in that they are capable of regenerating and reinnervating the olfactory bulb following chemical or mechanical lesion of their axons (63, 71, 74). Fourth, the olfactory epithelium contains a population of immature, precursor cells which are capable of differentiating into functional neurons following the death of mature neurons (75, 76), and this process continues even in the absence of any exogenous insult. Experimental manipulation of the olfactory nerve by chemical lesioning is a useful technique in determining the functions of apoE in a well-defined neural system. For this purpose, I used olfactory nerves of apoE KO mice that are specifically and completely deficient in the expression of apoE. These mice provide a useful system to test directly whether apoE is an essential component of nerve regeneration. Intranasal irrigation with zinc sulfate or vinblastine sulfate causes severe damage to the olfactory epithelium, and prevents any neural regeneration from occurring (62, 63, 77). I used 0.7% Triton X-100 for intranasal irrigation to perform olfactory nerve lesioning in mice. It has been shown that intranasal irrigation with Triton X-100 (0.5%) destroyed mature olfactory receptor neurons, but allowed regeneration to occur.

Morphological studies have shown an extensive loss of the olfactory epithelium from the turbinates, the accumulation of epithelial debris in the airways and absence of a ciliary layer from the mucosa following TX lesion in mice (78). TX treatment has been shown to severely damage the olfactory epithelium both in wild-type and in apoE KO mice. After one week post-lesioning, the mean damaged olfactory mucosa has been shown to be similar in wild-type and apoE KO mice (89% of total olfactory mucosa in wild-type and 90% in apoE KO mice).

Previous studies have indicated a possible role for apoE in the plasticity of the CNS (79, 80, 81), suggesting that apoE might be critical in maintaining the integrity of the CNS during aging and after lesions (27, 82). This study substantiates previous investigations which have indicated a role for apoE in the CNS response to injury (27, 36, 83). In initial experiments, I examined the temporal expression of apoE levels following olfactory nerve injury. The results indicate that there is a significant increase in apoE levels at 3 day post-lesioning which gradually declines to normal levels by 56 day. In the central nervous system, apoE is primarily synthesized and secreted by astrocytes and macrophages (9, 81, 84). In normal nerve tissue, small amounts of apoE are produced primarily by resident macrophages and LDL receptors are expressed on many nerve cells, indicating that apoE is involved in cholesterol transport within the nervous system at all times and its synthesis is increased after injury. It may be that injury and repair only modulate the activity of this system.

Cholesterol liberated from degenerating axons serves as a local source for the subsequent reactive synaptogenesis that takes place in this system (85). Studies on cholesterol fed macrophages have shown that free cholesterol increases mRNA levels and results in increased apoE biosynthesis and secretion (86). During first week post-lesion, astrocytes engulf the degenerated synaptic terminals and preterminal axons which generate a large store of cholesterol and lipids readily available for the synthesis of membrane components for new synapses, and may act as a trigger to induce the up-regulation of apoE. As a result of increased apoE expression and in the presence of active synaptogenesis and terminal proliferation, there is a decrease in intracellular cholesterol synthesis (27).

Alterations in the cellular localization of apoE can occur following brain injury. There is evidence that apoE is increased in astrocytes following transient cerebral ischemic injury and acute subdural hematoma in rat and are taken up into neurons which are destined to die (35, 87). Following injury, apoE transports cholesterol and lipids to cells for synaptic remodeling and repair. An increase in apoE immunoreactivity in neurons following the initial ischemic insult indicates a rapid protective response by supplying cholesterol to neurons for repair (88). Receptors for apoE are located in neuronal cell bodies and dendrites (89), suggesting that apoE could be incorporated into neurons

through this mechanism. Furthermore, apoE can be internalized by neurons (20) and may serve as a major cholesterol carrier to the neurons (80). As axons regenerate, their advancing tips express high levels of LDL receptors which have been postulated to participate in uptake of apoE containing lipoproteins (90). Cholesterol rich apoE containing lipoproteins bind with high affinity to these LDL receptors (1, 91, 92). The presence of apoE and expression of LDL receptors at the elongating tips of axons provide a mechanism for cholesterol transfer among macrophages and regenerating axons. It has been shown that nonesterified cholesterol is released, esterified, and transported by apoE to neurons undergoing regeneration where it is taken up through the LDL receptor pathway and it is used as a precursor for the synthesis of new synaptic terminals (27). The presence of this cholesterol transport system to the damaged neurons involving apoE, coupled with conservation of cholesterol within the degenerating nerve, suggests that cholesterol is important for regeneration to keep up with the massive membrane biogenesis that occurs in the regenerating nerve.

The relative rate of apoE biosynthesis and secretion has been shown to decrease to normal values in crushed peripheral nerves 56 days after injury (93). A decline in apoE secretion and accumulation of apoE containing lipoprotein particles within the regenerating nerve are concurrent with the depletion and the unloading of cholesterol stores from macrophages. As axonal growth proceeds, the apoE levels reduce and reach normal levels once axons make their contact with their target sites in the CNS. ApoE has also been shown to be present around the glomeruli and the olfactory nerve in both mouse and human (78). These studies and our results suggest that apoE is involved in cholesterol transport and associated with the continuous degeneration and regeneration processes that occur in the CNS.

The significance of apoE uptake into neurons and increase in extracellular apoE may have important implications regarding human pathology particularly in relation to Alzheimer's disease. It has been demonstrated that intraneuronal apoE occurs in response to injury (87). A similar mechanism may occur in Alzheimer brain such that following a compromise in cellular homeostasis apoE is released and taken up into neurons. The apoE present in neurons may interact with microtubule associated proteins such as tau, and depending on the apoE isoform, ultimately lead to neurofibrillary tangle formation.

ApoE knockout mice offer a unique opportunity to study, in vivo, the role of apoE in the CNS. ApoE KO mice have been shown to have decreased resistance to focal cerebral ischemia when compared with wild-type animals (94). Following middle cerebral artery occlusion, apoE KO mice had large infarcts and high mortality rates as compared to controls (94). Reinnervation of the molecular layer of dentate gyrus after interruption of the perforant pathway is

abnormal (82). Severe motor and behavioral deficits after close head injury and reduced brain cholinergic activities and impaired cognition have also been reported (41, 45). ApoE KO mice display an age-dependent disruption of the synaptic and dendritic organization of the neocortex and hippocampus when compared to controls (38). Infusion of apoE into the lateral ventricles of apoE KO mice has been shown to correct the synapto-dendritic pathology, indicating that these alterations are reversible (40). These observations suggest that apoE plays a possible role in synaptic remodeling and maintaining the integrity of the CNS during aging and after injury (27, 36).

To further investigate the importance of apoE in the CNS, I used olfactory nerves of apoE KO mice compared to wild-type mice to determine the rate of axonal growth, synaptogenesis and olfactory neuron maturation in the absence of apoE. For this purpose, GAP 43 and OMP were used as marker proteins. It was found that olfactory nerve regeneration occurred in apoE KO mice, but the rate of axonal growth and maturation of olfactory neurons is delayed in these mice by two weeks compared to controls. These results are consistent with previous reports that nerve regeneration in the peripheral nervous system in apoE KO mice is normal, and that apoE is not essential for nerve repair (95). It has been reported that HMG-CoA reductase, a rate limiting enzyme in cholesterol synthesis, decreased mRNA and activity levels to the same degree in the crushed nerves of wild-type and apoE KO mice (95, 96). These studies suggest that regenerating nerves of apoE KO mice do not synthesize large quantities of cholesterol, and therefore the cholesterol present in the nerve before injury is conserved and reutilized in the regenerating nerves of these animals. This apparent recycling of lipids in apoE KO mice suggest that other apolipoproteins in the regenerating nerve may be capable of functionally substituting for apoE. There is a ~500 fold increase in the concentration of apolipoprotein D, as well as 15-25 fold increase in concentrations of apolipoprotein A-I and apolipoprotein A-IV in the crushed nerves of wild-type animals (79). Apolipoprotein D is expressed by fibroblast-like cells and glial cells in the CNS (97), and a rapid and sustained local increase in apoD mRNA and apoD immunoreactive protein has been observed following entorhinal cortex lesion in the rat hippocampus (98). It appears that any of the substituting apolipoproteins can fill the role of apoE as an acceptor for cholesterol and ligand for the LDL receptor, and in the normal transfer of cholesterol to the regenerating nerve, thus ensuring lipid transfer within CNS for the maintenance of normal function.

The rate of nerve regeneration depends in part upon the efficiency of lipid transport from the local environment to the elongating axon. The presence of axonal degeneration products in wild-type animals peaks within the first week following the lesion, and products are then cleared from the

denervated zone by glial cells by the second week (99, 100). ApoE expression has been shown to increase in astrocytes within the first post-lesion week supporting a role for apoE in these events (36). Persistence of degenerating products in the hippocampus of apoE KO mice has been reported in the injured brain. Therefore, it has been suggested that apoE is involved in the clearance of neuronal degeneration products in the injured brain, perhaps via its ability to transport cholesterol and other lipids released from damaged neuronal membranes. The delay observed in axonal extension and olfactory neuron maturation in apoE KO mice in this study could be explained due to inefficient clearance of cholesterol-laden neurodegeneration products, and a delay in their transport to new membrane components and growing axons.

The aim of this study is to understand the role of apoE in the CNS during nerve regeneration. The results show that apoE expression is upregulated following olfactory nerve injury and stay elevated for a long time, which suggests it is involved not only in degeneration process but also during nerve regeneration. Consistent with the hypothesis that lack of apoE would disrupt the CNS plasticity, our results show that nerve regeneration is delayed in apoE KO mice following olfactory nerve injury. It suggests that may be in the absence of apoE, some other protein is taking over apoE's function, but it is not as efficient as apoE in lipid transport. Similar to what has been shown in mice, if apoE3, the most common isoform of apoE is present in humans, may help facilitate nerve repair in the aging brain. However, if apoE4, the mutant form is present, then it may not fulfil apoE's function in nerve repair processes, and therefore will lead to AD. Thus, it is concluded that apoE plays a pivotal role in nerve repair following injury, and apoE deficiency in leads to delayed nerve repair.

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