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## The distribution of apolipoprotein E in mouse olfactory epithelium

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### Abstract

Previous studies from our laboratory suggest that apolipoprotein (apoE), a lipid transporting protein, facilitates olfactory nerve regeneration. We have shown that apoE is enriched in the olfactory nerve and around the glomeruli of the olfactory bulb (OB). The studies reported herein were undertaken to identify possible sources of apoE in the olfactory epithelium (OE). Immunoblotting results revealed apoE expression in the OE of wild-type (WT) mice, but not in apoE deficient/knockout (KO) mice. Immunohistochemical studies revealed that the perikarya and processes of sustentacular (Sus) cells expressed apoE-like immunoreactivity. Minimal neuronal apoE immunostaining was seen, although apoE was observed in the interstitial spaces between olfactory receptor neurons (ORN). Substantial apoE-like immunoreactivity was localized to the endfeet and terminal process of Sus cells surrounding the basal cells. Double labeling immunocytochemical studies confirmed that the cell bodies and endfeet of Sus cells expressed high levels of apoE. The endothelial cells of blood vessels were intensely stained for apoE in the lamina propria. Cells forming Bowman's gland also immunostained for apoE. The apoE staining in the nerve fascicles was less intense, but was uniformly distributed throughout the core of the nerve bundles. Heavily stained cells, probably ensheathing glia, surrounded the nerve fascicles. These results revealed that apoE is expressed in the adult OE and lamina propria at strategic locations where it could facilitate the differentiation, maturation and axonal growth of the ORN, perhaps by recycling lipids from degenerating ORN for use by growing axons.

### Keywords

apoE; olfactory; glia; glial proteins; knockout mice

### 1. Introduction

Apolipoprotein E (apoE) is a component of lipoproteins that functions in the redistribution of cholesterol and other lipids (Kowal et al., 1990; Mahley, 1988; Takahashi et al., 1992; Willnow et al., 1996). It is primarily synthesized in the liver, but is also expressed in significant amounts in the nervous system. Previous studies have shown apoE mRNA and protein increase at the site of neural regeneration and that apoE containing lipoproteins stimulate neurite outgrowth in a variety of neuronal cultures (Bellosta et al., 1995; Holtzman et al., 1995; Ignatius et al.,

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1987; LeBlanc and Poduslo, 1990; Nathan et al., 1994; Nathan et al., 1995; Nathan et al., 2001; Nathan et al., 2002; Nathan et al., 2004; Snipes et al., 1986; Teter et al., 1999; White et al., 2001). We have previously shown that apoE surrounds glomeruli of the olfactory bulb (OB) (Struble et al., 1999). Subsequent studies found that olfactory nerve regeneration and morphological recovery of the OB were significantly delayed in mice lacking apoE/knockout (KO) compared to wild-type (WT) mice following OE lesioning (Nathan et al., 2005). The olfactory nerve undergoes constant regeneration and axonal growth throughout the life of the organism (Graziadei and Graziadei, 1979; Graziadei and Monti Graziadei, 1985). One hypothesized function of apoE in the nervous system is efficient recycling of lipids from degenerating neural tissue to growth cones of regenerating neurons to support neuroplasticity (Beffert et al., 1998; Mahley, 1988; Struble et al., 2006).

The current study was carried out to localize apoE in the adult mouse OE to complement our previous studies of apoE in the OB. Because published studies have indicated a local effect of apoE on axonal growth (Hayashi et al., 2004), we predicted that apoE should be present in the OE. We identified apoE expression in the OE of WT mice, but not in KO mice. Intense apoE immunoreactivity was present in the cell body of sustentacular (Sus) cells. Little or no neuronal staining was seen although apoE was observed in the interstitial spaces between olfactory receptor neurons (ORN). A high level of apoE immunoreactivity was observed in the basal cell layer, where apoE expression was primarily localized to the endfeet and terminal process of Sus cells surrounding the basal cells. In addition apoE was seen in olfactory nerve fascicles. A variety of cell types in the lamina propria also expressed high levels of apoE.

## 2. Results

Immunoblotting techniques confirmed apoE expression in mouse OE (Figure 1) and OB tissues from adult WT mice. Immunoblotting revealed similar bands at about 35 kDa in both the OE and OB extracts from WT mice. The absence of the apoE band in both OE and OB extracts from KO mice confirmed the specificity of the antibody used in this study. Actin immunoblotting was used as a loading standard.

Immunohistochemistry extended the immunoblotting studies by localizing apoE in the OE and its underlying lamina propria. The results revealed that apoE immunoreactivity was present in most of the layers of OE and the lamina propria. Intense apoE immunoreactivity was observed in the olfactory epithelial surface and in cytoplasmic processes of the Sus cells extending to the epithelial surface (Figure 2). Immunoreactive processes also traversed between the ORN. Intense apoE immunoreactivity was clearly present in the basal cell region of the OE. At high magnification it was clear that the immunoreactivity was predominantly localized in the cytoplasm of the Sus cells (Figure 3A), and in knob like structures which appear to be Sus cells endfeet surrounding basal cells (Figure 3B). ApoE staining in the respiratory epithelium was considerably lower than that in the OE (Figure 2C) and the change was striking in contrast to the OE. A complete absence of apoE-like immunoreactivity in the KO mice confirmed that we were visualizing apoE immunoreactivity (Figure 2D).

Double-labeling immunohistochemical studies were performed to concurrently examine apoE expression with SUS-4 (a marker for sustentacular cells), and GBC-1 (marker for GBC). The results revealed that apoE immunoreactivity consistently colocalized with SUS-4 in the perikarya of Sus cells (Figure 4A). Double labeling studies with apoE and GBC-1 also revealed similar colocalization of apoE and GBC, except that some apoE-only positive processes were not in spatial register with the GBC (Figure 4B). Our impression of the spatial mismatch is that the apoE was in endfeet surrounding the Sus cells. These endfeet abutted the GBCs. However, in the absence of electron microscopy we cannot exclude the possibility that some immunoreactive endfeet-like profiles represent GBC.

In the lamina propria, the endothelial cells of blood vessels were intensely stained for apoE (Figure 5). Cells forming Bowman's gland also stained for apoE. The apoE staining pattern in the nerve fascicles was composed of intensely immunostained cells surrounding, uniformly distributed less intensely immunostained apoE throughout the core of the nerve bundles. The distribution and morphology of these intensely stained cells suggested they were ensheathing glia.

### 3. Discussion

The results from this study demonstrate that the lipid transporting protein apoE is expressed throughout the OE and its underlying lamina propria. In the OE, Sus cells expressed significant levels of apoE in their perikarya and villar processes, located in the surface of OE, and in their end feet, which were in close proximity to basal cells lying above the basal lamina. ApoE was only present at low levels surrounding the ORN. In the lamina propria, apoE was expressed at high levels in the endothelial cells of the blood vessels. Furthermore, ensheathing cells were intensely stained whereas the olfactory nerve fascicles showed faint staining of apoE. Occasionally we observed apoE expressing macrophage-like cells surrounding the olfactory fascicles. In addition, cells forming the Bowman's gland were also stained for apoE.

Our study did not completely replicate a previous study of apoE expression in the OE of elderly humans (Yamagishi et al., 1998). They reported intense apoE staining in the blood vessels and diffuse staining in the olfactory nerve fascicles, which is consistent with our data. However, they did not report any detectable level of Sus staining. The reason for these discrepant findings between our data from young mice and these data from elderly humans could have several explanations. The absence of Sus staining for apoE might be due to age-related structural and physiological alterations (Loo et al., 1996). Sus cells in aged rats are swollen and contain abundant eosinophilic material. Additionally, a linear decline in CSF apoE levels has been shown during aging in humans and apoE expression in the OE may similarly decrease as animals age (Fukuyama et al., 2000). Alternatively, the absence of apoE in human Sus cells may represent post-mortem changes. It is possible that maintenance of Sus apoE is metabolically maintained. With death there is a general release of apoE to the neuropil and an artifactual absence in Sus cells.

The dichotomy between olfactory and respiratory epithelia suggests a disproportionate importance of apoE to ORN replacement and regeneration compared to routine cellular replacement in respiratory epithelium. Sus and ensheathing glia derived apoE could be critically involved in recycling of membrane components liberated from the senescing ORN population and recycling it to support basal cell division and differentiation and axonal elongation of the newly differentiated ORN. We propose that Sus-derived apoE facilitates the various functions attributed to Sus and ensheathing glia (Barnett, 2004; Dahl et al., 1982; Getchell et al., 1984; Ramon-Cueto and Avila, 1998).

Disposal of cellular debris in a variety of peripheral tissue is primarily carried out by macrophages. And a previous study has shown that apoE promotes clearance of cellular debris at the site of neural injury (Fagan et al., 1998). ApoE appears to be vital to phagocytotic function of macrophages (Grainger et al., 2004). Thus one potential role of apoE produced by Sus in a normal/uninjured OE is to support the continuous disposal and recycling of cellular debris as a result of the constant turnover of the receptor neurons in the OE.

A hypothesis of apoE function in lipid recycling in the OE is also compatible with published studies that examined the impact of apoE deficiency on the olfactory system in apoE KO mice. We reported a striking qualitative and quantitative increase of apoE in the olfactory nerve and bulb concurrent with olfactory nerve degeneration (Nathan et al., 2001). In addition, we found

that olfactory nerve regeneration and recovery in the OB following OE lesion were significantly delayed in mice lacking apoE (Nathan et al., 2005). Reappearance of OMP in the OB following reversible lesion of OE in normal mice occurred between 7 and 21 days post lesion, reaching statistically normal levels by 42 days. In contrast, in KO mice, OMP remained indistinguishable from baseline for 42 days, but then recovered by 56 days. Although the exact mechanism for this delay is open to question, it is tempting to propose that the absence of apoE production by the Sus cells and ensheathing glia in the OE impairs re-innervation of the OB by disruption of the lipid recycling process. The absence of apoE in Sus cells may disrupt regenerative processes of basal cells. ApoE secreted by Sus cell endfeet, found in close proximity to basal cells of OE, may support efficient transport of lipids to facilitate basal cell division and differentiation to mature neurons. Absence of apoE in ensheathing glia may slow the growth of newly generated axons. This hypothesis was supported by compartmental culture paradigms which showed that apoE facilitated neurite growth, but only when added at the site of the growth cone (Hayashi et al., 2004). Hence, delayed regeneration in apoE KO mice could represent both slower regeneration of ORN and slower axonal growth. Our previous data tend to support that possibility.

It is important to note that olfactory function shows early severe deficits in various chronic neurological disorders where apoE genotype is a major risk factor (Ansari and Johnson, 1975; Hawkes, 2006; Talamo et al., 1989). We propose that rather than a disease-specific effect of apoE on the course of the disease, dysfunctional apoE would delay repair and underlie abnormalities of olfactory function found in chronic neurological diseases. The olfactory system, with its unique ability to continuously regenerate, may be an ideal structure for determining the role of apoE in nervous system during normal and pathological condition.

## 4. Materials and Methods

### 4.1. Animals

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

### 4.2. Tissue preparation

For immunoblotting analysis, mice were anesthetized with sodium pentobarbital (80 mg/kg), and perfused transcardially with 0.9% saline until the perfusate was clear. OB and OE from the turbinates were dissected, washed with ice cold 0.1M phosphate buffered saline (PBS, pH=7.4), and processed for immunoblotting as described below. For fluorescence immunohistochemistry, mice were anesthetized with sodium pentobarbital (80 mg/kg), transcardially perfused with cold saline (0.9% NaCl), followed by 4% paraformaldehyde in 0.1M PBS. Olfactory turbinates were removed and cryoprotected overnight in 30% sucrose in 0.1 M PBS. After cryoprotection, the turbinates were frozen with dry ice and sections were cut on a cryostat at 18  $\mu$ m, and air dried for 2 h at room temperature.

### 4.3. Immunoblotting

Quantitative immunoblotting for OB and OE levels of apoE was performed as described (Bellosta et al., 1995; Nathan et al., 1995). Briefly, OB and OE were homogenized in 100  $\mu$ l of ice-cold TMN buffer (25 mM Tris-HCl [pH 7.6] 3 mM MgCl<sub>2</sub>, 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride). The homogenate was lysed by adding 20 % Triton X-100, 10 % deoxycholate, and 10 % SDS on ice for 5 min. The homogenate was then centrifuged (10,000 X g) for 2 min, and 20  $\mu$ g of supernatant was mixed with an equal volume of 2X Laemmli sample buffer and heated at 100 °C for 5 min. The proteins in the supernatant were resolved by 10–

20% gradient SDS-PAGE and transferred on to Immobilon PDVF membrane (Fisher, St. Louis, MO) using a wet trans-blot transfer cell (Bio-Rad, Hercules, CA) following the manufacturer's procedure. Blots were incubated with goat anti-apoE (Calbiochem, San Diego, CA) at 1:1,000 dilution in T-TBS (0.1 M Tris, 0.15 M NaCl, 0.1% Tween-20) for 30 min on a shaker at room temperature. Following incubation with anti-apoE, the blots were washed six times (5 min each) in T-TBS, and incubated with HRP conjugated donkey anti-goat secondary (Chemicon, Temecula, CA) at 1:1250 dilution in T-TBS for 30 min at room temperature. The blots were washed six times (5 min each) in T-TBS and immunoreactivity was visualized with Pico Chemiluminescence kit (Pierce, Rockford, IL). To confirm antibody specificity, immunoblots were performed with a 30-fold excess of normal goat serum in place of the primary antisera.

For actin immunoblotting, the apoE blots were soaked for 1h at room temperature in stripping buffer (Pierce, Rockford, IL), washed in TBST [20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20, 0.1 g BSA, pH 7.5], and incubated for 1h at room temperature with mouse anti-actin (Developmental Studies Hybridoma Bank, The University of Iowa, Iowa City, IA) at 1:10,000 dilution in TBST. Blots were washed with TBST, and incubated for 1h at room temperature in HRP conjugated goat anti-mouse IgG (Chemicon, Temecula, CA) at 1:10,000 dilution in TBST. Blots were washed in TBST and bands visualized as previously described for apoE. All experiments were repeated at least three times to assure reproducibility of the results.

#### 4.4. Immunohistochemical Analysis

OE sections on slides were rinsed in 0.1 M PBS, and permeabilized with 0.2 % Triton X-100 in PBS for 30 minutes at room temperature. The slides were rinsed once with PBS and treated with 70, 90, 100, 90, and 70% ethanol for two minutes each (Jang et al., 2003). Non specific immunoreactivity was attenuated by incubation in 2.25% gelatin in 0.1 M PBS for 1 h, followed by overnight incubation at 4 °C with primary antisera solution (see Table 1 for source and concentration used). The sections were washed three times in PBS, and incubated for 1 hour at room temperature with secondary antibody solution as listed in Table 1. The sections were washed three times in PBS, mounted in Vectashield (Vector labs, Burlingame, CA). Double labeling immunohistochemistry was performed similarly to single staining, except using cocktails of primary and secondary antisera at concentration indicated in Table 1. For apoE staining, KO mice were processed in parallel with WT mice. Specificity was determined by incubation with normal serum in place of the primary antisera which resulted in no staining.

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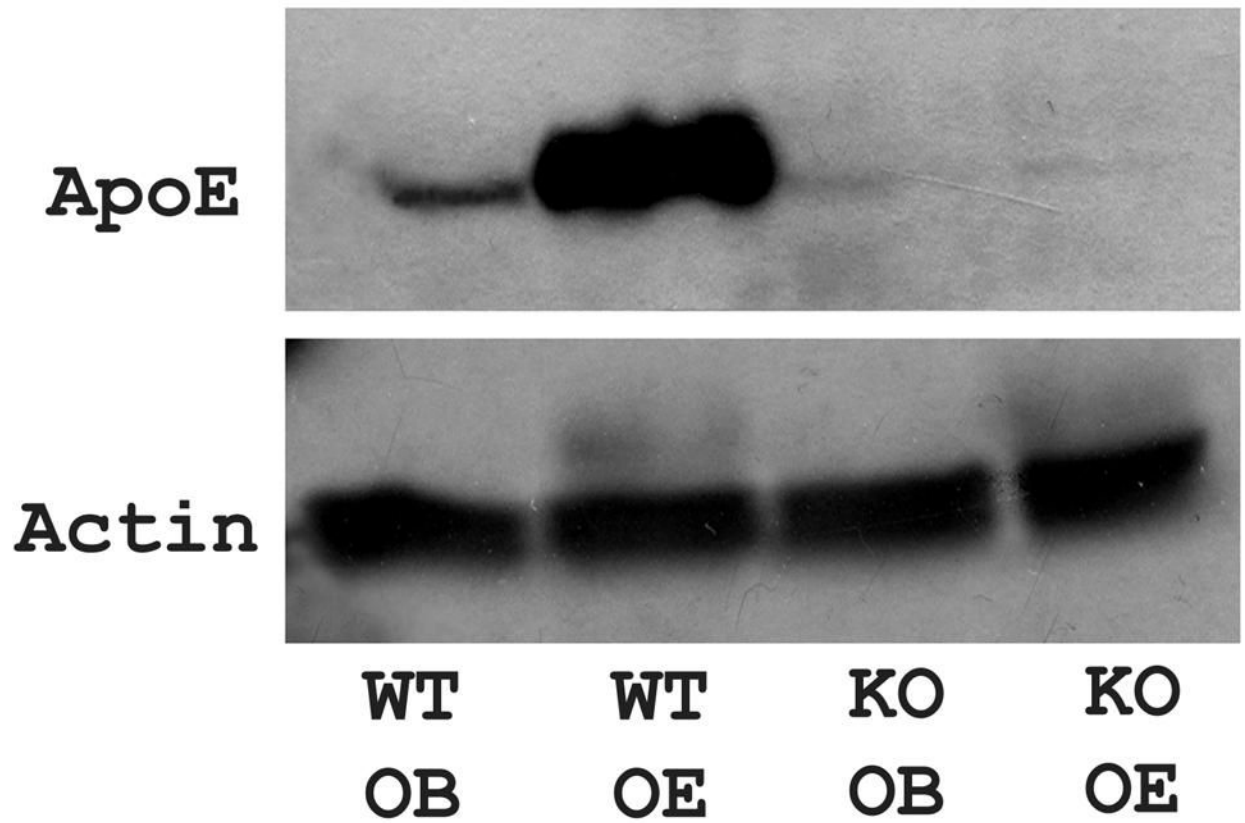
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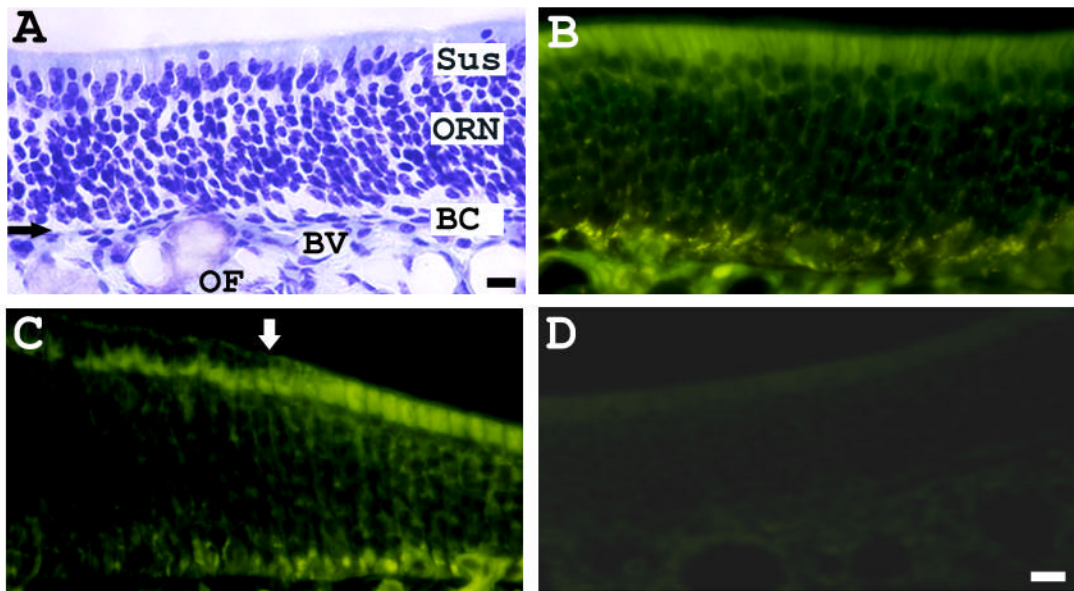
## Abbreviations

<b>apoE</b>	apolipoprotein E
<b>OB</b>	olfactory bulb
<b>OE</b>	olfactory epithelium
<b>ORN</b>	olfactory receptor neuron
<b>GBC</b>	globose basal cells
<b>Sus</b>	Sustentacular cells

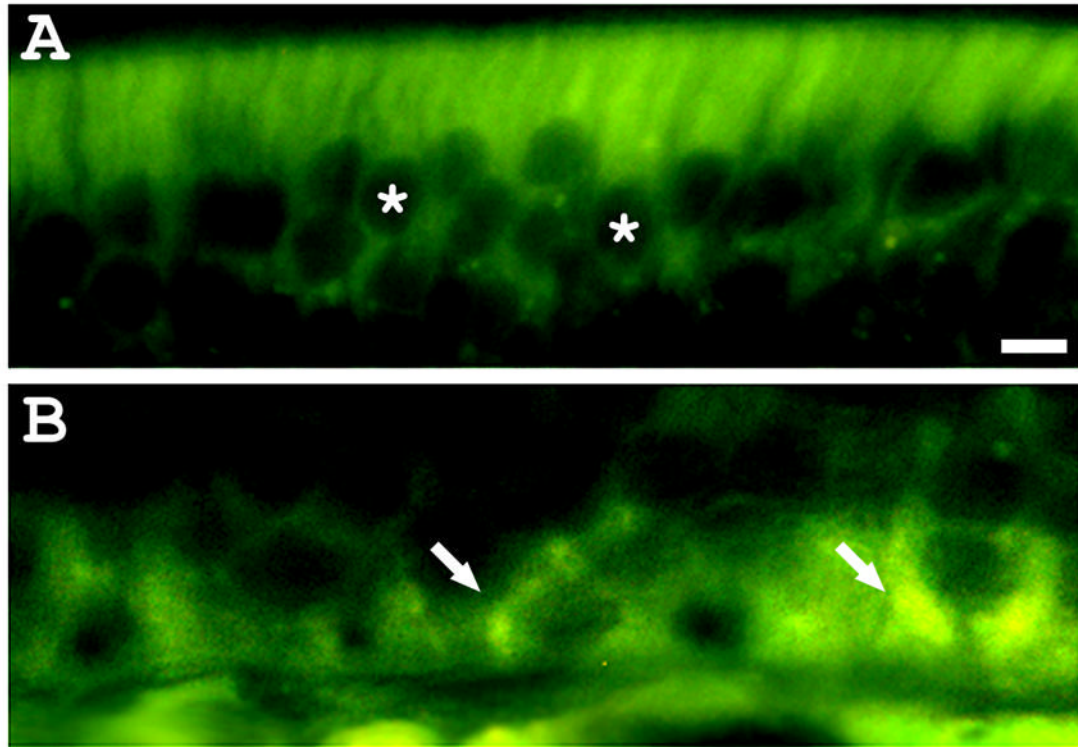




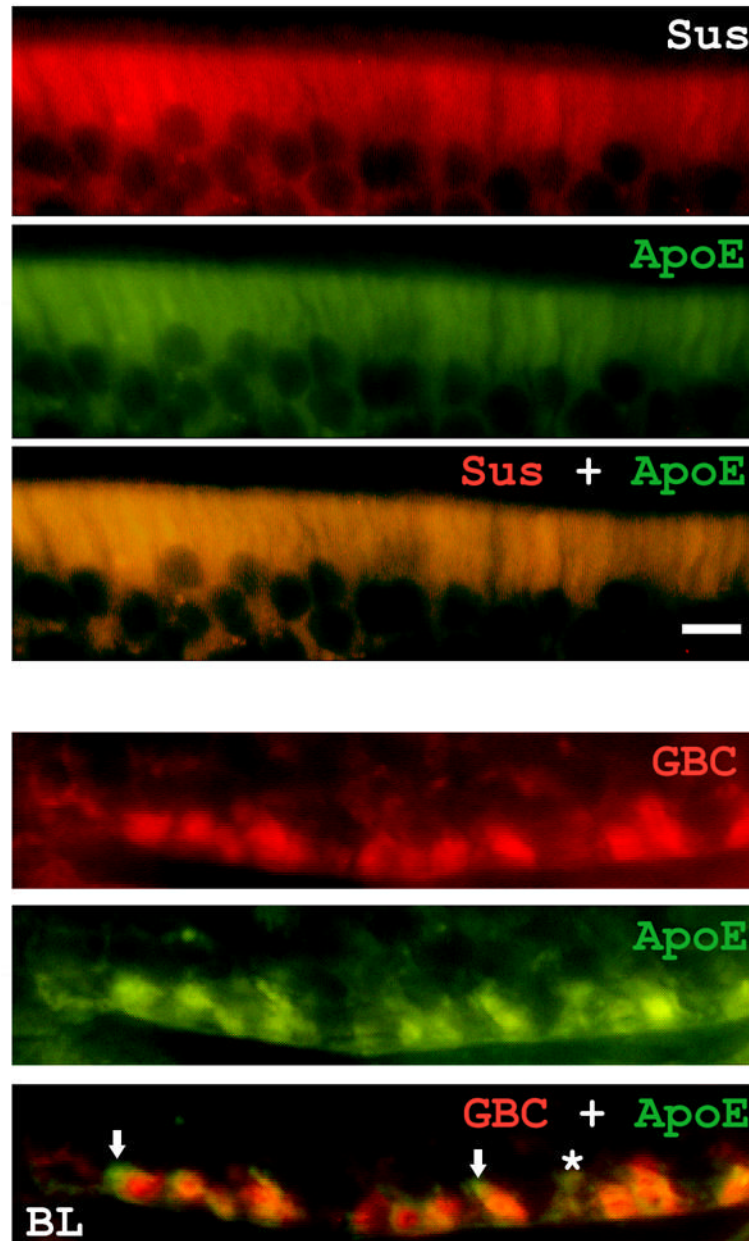
**Fig. 1.** Immunoblot analysis of OB and OE extracts from WT and KO mice. Twenty micrograms of tissue extracts were immunoblotted with polyclonal apoE antibody. A single 35 kDa band was detected in OB and OE extracts from WT, but not from KO mice. Actin was used as internal standard for protein loading.



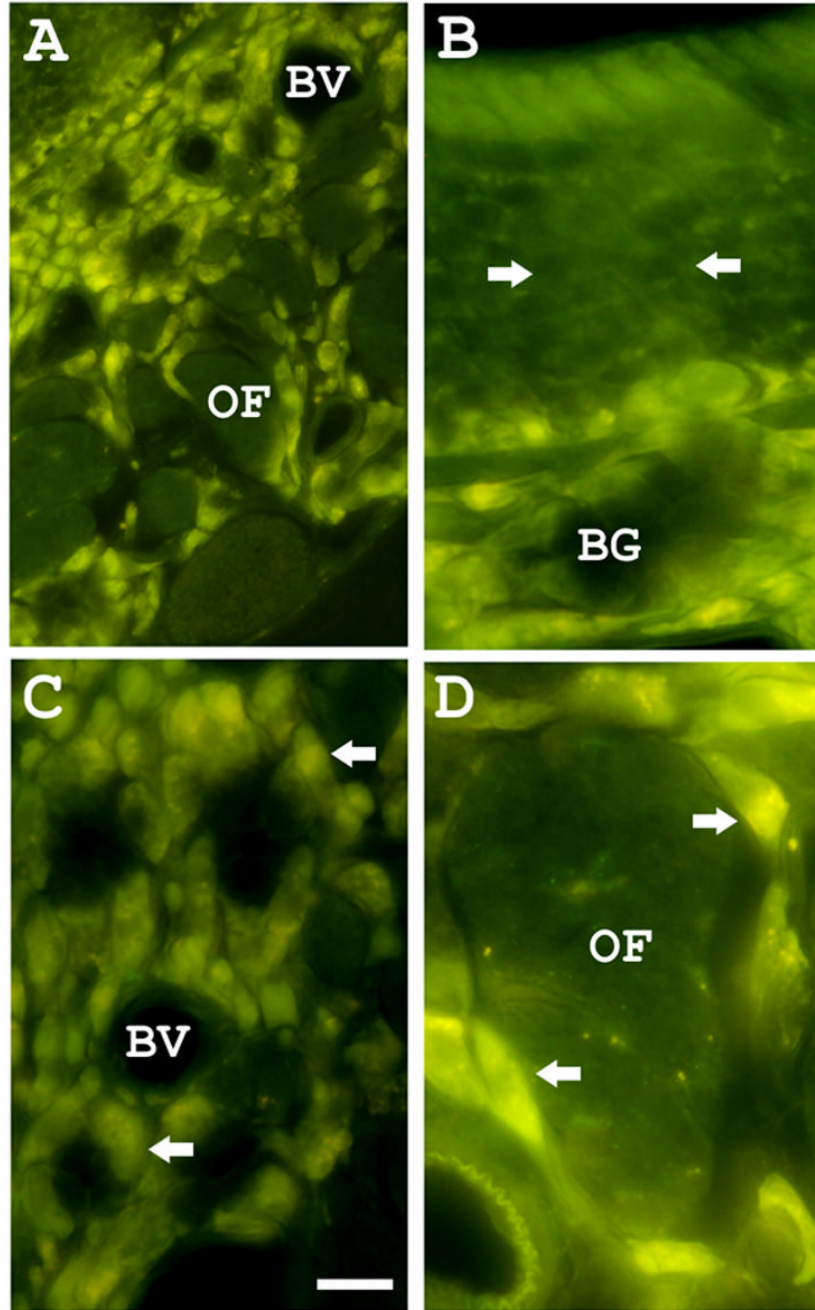
**Fig. 2.** ApoE expression in adult mouse olfactory epithelium. **(A)** Cresyl-violet stained section of olfactory epithelium and the underlying lamina propria. Sus, sustentacular cells, ORN, olfactory receptor neuron, BC, basal cell zone, BV, blood vessel, OF olfactory fascicle. Arrow indicates basal lamina. **(B)** ApoE immunoreactivity in WT mice was intense in the perikarya and end feet of sustentacular cells. **(C)** Low level of apoE expression in the respiratory epithelium of WT mice. ApoE immunoreactivity in the respiratory epithelium was highly reduced. The transition zone of the olfactory (OE) and respiratory (RE) is marked by an arrow. **(D)** Absence of apoE staining in the olfactory epithelium of KO mice. Scale bars = 10  $\mu$ m in A and 15  $\mu$ m in D (also for B and C).



**Fig. 3.** Perikarya and end feet of Sus cells express apoE. **(A)** Higher magnification photographs show apoE immunoreactivity in the cytoplasm, but not the nucleus of Sus cells (asterisk). **(B)** Endfeet of Sus cells (arrows) surrounding GBC intensely stain for apoE. Scale bars = 10  $\mu\text{m}$  in A (also for B).



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



**Fig. 5.**

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

**Table 1**

List of primary and secondary antibodies used in this study

<b>Antibodies</b>	<b>Host</b>	<b>Source</b>	<b>Dilution</b>
ApoE	Goat	Calbiochem, San Diego, CA	1:500
Sus-4	Mouse	Gift of Dr. Schwob	1:50
GBC-2	Mouse	Gift of Dr. Schwob	1:50
FITC-Anti goat	Donkey	Jackson, West Grove, PA	1:1000
TR-Anti mouse	Donkey	Jackson, West Grove, PA	1:1000