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Impact of apoE deficiency during synaptic remodeling in the mouse olfactory bulb

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Abstract

In this study we examined the role of apoE on the rate of synaptic recovery in the olfactory bulb (OB) following olfactory epithelium (OE) lesioning in mice. We used both immunoblotting and immunohistochemical techniques to compare the density of OB synaptophysin (Syn, a synaptic marker) in apoE-gene deficient/knockout (KO) mice and wild-type (WT) mice following OE lesion. We found that the whole bulb concentrations of Syn, measured by immunoblotting, declined sharply following injury in both WT and KO mice during the degenerative phase (3–7 days). After this initial decline, the Syn concentration gradually increased to normal levels by 56 days in WT mice. In contrast, Syn concentration in KO mice did not recover by day 56 when Syn density in WT was essentially normal. Glomerular Syn density, measured by immunohistochemistry, found a lower density in KO mice at all time points post lesion. This lower concentration of whole bulb Syn parallels the slower recovery of glomerular area in KO mice. The data indicate that apoE deficiency in KO mice is associated with a delayed recovery of the glomerular area and a slower recovery in Syn concentration in the OB.

Keywords

apoE; synaptophysin; olfactory bulb; glia; olfactory nerve; glial proteins; transgenic mice

Apolipoprotein E (apoE), a lipid transporting protein, is widely expressed in the primary olfactory pathway [6,12,13,23,25]. Previous studies from our laboratories and others have shown apoE expression in the olfactory nerve and around the glomeruli in the OB of adult mice [12,23]. ApoE levels in the OB were two fold higher than normal immediately following OE lesion, and remained elevated over a 3-week period when axons from the newly differentiated olfactory receptor neurons (ORN) grew to reestablish the synaptic connections with cells in the glomeruli[13]. The precise function of apoE in the olfactory system during normal and injury-induced remodeling is unclear; however, studies suggest a role for apoE in the synaptogenesis of the CNS [2,8,20,27].

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Approximately, fifty percent of all synapses in the glomerulus of the olfactory bulb (OB) are contributed by axon terminals of the ORN residing in the olfactory epithelium (OE) [4,26]. The cell bodies of the ORN can be easily lesioned. Following a lesion they regenerate an axon to reinnervate the glomeruli and form new synapses. Hence, the olfactory system provides a simple model to quantify synaptic regeneration. We used this model system to evaluate the role of apoE in synaptic regeneration of the OB by examining the time course of synaptophysin (Syn, a synaptic marker) recovery in the OB of wild-type (WT) and apoE knockout (KO) mice following OE lesioning. We found that synaptogenesis was delayed in KO mice.

Breeding pairs of homozygous KO mice that had been backcrossed at least 10 generations to the C57BL/6 strain and C57BL/6 WT controls were purchased (Jackson Laboratory, Bar Harbor, ME). ApoE genotype of the litters were verified by PCR and confirmed by immunoblotting using anti-apoE (1:1000; Calbiochem, San Diego, CA) as described [9].

Two- to four-month-old male KO and WT mice were lesioned as previously described [7,18, 24]. Briefly, a 25-gauge needle with a rounded tip, was inserted about 2 mm into the nostril, and 50 µl of 0.7% (v/v) Triton-X100 (TX) (Sigma, St. Louis, MO) in saline, or 50 µl of saline alone (SL, control) was infused into a nostril of mice. The excess solution was drained from the nasal passages by gently shaking the mice. The procedure was repeated on the other nostril. This technique results in extensive bilateral damage to the OE with approximately 70–80% of the adult OE lesioned [13]. This procedure, however, spares the basal cells, which subsequently divide and differentiate into new receptor neurons [7,18,24].

Mice were sacrificed on days 0, 3, 7, 21, 42, and 56 days post-nasal irrigation (n = 6 mice for each time point), and OB prepared for either immunoblotting or immunocytochemistry of Syn. Briefly, for immunoblotting analysis mice were perfused transcardially with 0.9% saline until the perfusate was free of blood. OB were dissected, washed with ice cold 0.1M phosphate buffered saline (PBS, pH=7.4), and homogenized in 100 µl of ice-cold TMN buffer (25 mM Tris-HCl [pH 7.6] 3 mM MgCl2, 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride) (Xu et al., 1996). The homogenate was lysed by adding 20 % Triton X-100, 10 % deoxycholate, and 10 % SDS on ice for 5 min, and centrifuged (10,000 X g) for 2 min, and 20 µg proteins in the supernatant were resolved by 10–20% gradient SDS-PAGE and immunoblotted with rabbit anti-human Syn (Dako, Carpinteria, CA) at 1:1,000 dilution in T-TBS (0.1 M Tris, 0.15 M NaCl, 0.1% Tween-20) for 60 min on a shaker at room temperature. Following incubation with anti-Syn, the blots were washed in T-TBS, and incubated with HRP conjugated donkey anti-rabbit secondary (Chemicon, Temecula, CA) at 1:1250 dilution in T-TBS for 30 min at room temperature. The blots were washed 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 non-immune rabbit serum in place of the primary antisera.

For actin immunoblotting (loading standard), the Syn 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) at1:10,000 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 1:1250 dilution in T-TBS for 30 min at room temperature. The blots were washed 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 non-immune rabbit serum in place of the primary antisera.

For immunohistochemistry, the mice were deeply anesthetized with pentobarbital (80 mg/kg, ip) and perfused transcardially with PBS followed by freshly prepared 4% paraformaldehyde.
(Sigma, St. Louis, MO) in PBS. The OB were post-fixed for two hours, cryoprotected overnight in 30% sucrose (Fisher, Hanover Park, IL) in 0.1M PBS at 4°C and then frozen with dry ice and stored at −80°C. The SL- and TX-treated WT and KO mice were available at days 0, 3, 7, 14, 21, 42 and 56. OB from groups of three mice per time point were cut at 12 µm thick on a Reichert-Jung 2800 Frigocut E cryostat. Serial sections were thaw mounted onto slides pre-subbed with 2% gelatin (ACROS Organics, New Jersey) and 0.5% Chrome Alum (Sigma, St. Louis, MO). After air drying the slides, they were rinsed in 0.1M PBS and dried at 37°C in an oven. Sections were stored at 4°C until needed.

Sections were immunoreacted with anti-Syn or with 4% non-immune rabbit serum (control). Briefly, sections were permeabilized with 0.02% Triton X-100 in 0.1M PBS for 30 min followed by a 5 minute rinse in 0.1M PBS. Non specific immunoreactivity was attenuated by incubation in 4% normal goat serum (Vector, Burlingame, CA) in 0.1 M PBS for 1 hour, followed by a 5 minute rinse in 0.1M PBS. The tissues were incubated with rabbit anti-Syn (Cell Marque, Hot Springs, AZ) at 1:2000 dilution in the blocking solution for 24 hours at 4°C. The sections were washed thrice in PBS, and incubated for 1 hour at room temperature with goat anti-rabbit secondary antibody labeled with fluorescein isothiocyanate (FITC) (Jackson ImmunoResearch, West Grove, PA) at 1:200 dilution in PBS. The sections were washed thrice in PBS, mounted in Vectashield (Vector, Burlingame, CA). Saline and TX-irrigated mice from WT and KO were stained and photographed on the same day to ensure comparison of staining density.

Quantification of glomerular area and Syn immunoreactivity in the glomeruli was performed as described [5,16]. Briefly, microscopic lighting was adjusted such that the difference between pure black and 100% transmission was 256 gray levels. Images from the areas of ten contiguous glomeruli on the medial side of the OB at about the midpoint of the rostro-caudal extent and midway between the ventral and dorsal side of the OB glomerular layer were captured using a digital camera. Images were also taken from the subependymal zone, which showed background staining, and therefore were used as a measure of nonspecific immunoreactivity. Optical density analysis of the images was performed with image analysis software (Scion Image, Frederick, MD). Measurements within a 40 square pixel were made from the core of ten contiguous glomeruli and the subependymal zone. The corrected OD was obtained by subtracting the OD measurements of the subependymal zone from the OD of the glomeruli. Adjacent sections, stained with combined cresyl violet were available for unequivocal identification of olfactory nerves and glomeruli for comparison to the immunoreacted slides. Statistical analysis was performed using Systat© analysis of variance software. Significance was considered at p<0.05 level. Post-hoc testing was performed with a Bonferroni-corrected t-test for selected contrasts.

Statistical analysis of the whole bulb concentration of Syn, measured by immunoblot, revealed a significant interaction between day after lesion and genotype (F_{6,28}=13.84; p<0.001). Syn levels declined sharply between 3 and 7 days post lesioning in both WT and KO mice. Following this precipitous decline, OB Syn levels in WT mice steadily increased to about 80% of the normal levels by 56 days post lesion. Concentration was not significantly different between genotypes at 0 or 3 days post lesion. In KO mice, a trend for increase in Syn was observed on 21 days post lesion, which however did not sustain in the later time points post lesioning. Apart from this initial raise, Syn density did not increase in KO mice and was significantly less than that of WT mice on day 56.

Immunohistochemical studies were performed to confirm the immunoblot data and to more specifically examine Syn levels in the glomerular layer. When measuring Syn density in random glomerular field we found a significant effect of genotype only (F_{1,32}=26.58; p<0.001). Regardless of day (F_{7,32}=1.829; p=0.116) or the interaction between treatment and time after...
lesion, \( F_{7,32}=1.082; p=0.397 \) the KO mice had a lower intensity of Syn immunoreactivity than did WT mice. In essence, the overall major effect of genotype was represented by a lower general Syn optical density. A slight, but non-significant day effect was found, but it was equally present in both WT and KO mice.

We then analyzed the Syn density in relationship to glomerular area. We found a significant interaction of day post lesion and glomerular area \( F_{7,32}=3.62; p<0.006 \). As shown previously, glomerular area was slower to recover in KO mice than WT mice and recovery in KO mice occurred between 42 and 56 days post lesion while the WT mice were essentially normal by 42 days[14]. We multiplied this glomerular area by Syn density, which is roughly analogous to measuring total number or size of glomerular synapses, and found a significant main effect of genotype \( F_{1,32}=25.27; p<0.001 \) and day \( F_{7,32}=21.10; p<0.001 \). We found no significant interaction \( F_{7,32}=1.95; NS \) between day post lesion and genotype. In essence, the total amount of Syn per glomerulus in KO was about 25% less than the WT regardless of the lesion effect.

Results from the present study demonstrate that apoE significantly modulates recovery of whole bulb Syn concentration following a reversible OE lesion. After a seven day decline following lesion, whole bulb Syn concentration in WT mice recovered reaching statistically normal levels by 56 days post lesion. In contrast, Syn recovery in the KO mice did not reach comparability even by 56 days. This parallels our previous paper showing slower recovery in olfactory marker protein (OMP, mature ORN marker) and glomerular area in KO mice when compared to WT mice[14]. Hence, in the absence of apoE, synaptic recovery in whole bulb samples is substantially delayed compared to WT mice.

The analysis of Syn optical density in glomeruli showed a response that was more difficult to interpret but of substantial interest for understanding the role of apoE. We anticipated that Syn density in a glomerulus would decline as the presynaptic ORN degenerated. Although, not reaching significance \( p<0.11 \), we found that both WT and KO mice showed a minimal decline followed by recovery. However, we did find, regardless of treatment, lower Syn density in the KO compared to the WT mice. The lower glomerular Syn density in KO mice could represent less Syn per synapse but equal numbers of synapses or fewer presynaptic endings in a glomerulus of KO mice and glial processes may be “over represented” in KO glomeruli. Together, these data strongly suggest that apoE is involved in processes governing synaptogenesis in the CNS comparable to previous reports [2,8,20,27]. In vitro studies have demonstrated that cholesterol laden apoE is a glial-derived factor that promotes synapse development in neuronal cultures [10].

The more interesting aspect is that we did not find an interaction between glomerular Syn and day. The implication of these data is that the decline and recovery of Syn concentration we found in whole bulb included areas of the OB that were not receiving direct ORN input. This finding indicates transsynaptic degeneration and delayed recovery as previously reported in the rat olfactory bulb [21].

Our data seem to be best explained by positing a) less Syn in KO mice than in WT mice, and b) a slower regeneration in the KO mice. The significance of delay in Syn post injury in apoE KO mice may explain the association of human apoE4 isoform to several chronic neurological diseases [3,17]. We have proposed that the increased risk for these diseases in individuals with dysfunctional apoE isoforms may represent, not a direct effect on the disease, but a slowing of repair and regenerative processes in these individuals [22]. Moreover, apoE KO mice start with a lower synaptic density. Hence, repair is slower and a threshold of synaptic loss that reaches clinical expression may occur earlier in those with apoE4. Hence, expression of the disease occurs earlier. Data showing olfactory deficits in those with apoE4 may represent poorer repair processed in the constantly regenerating olfactory nerve [1,11,15,19]. Hence, rather than
directly relating to some disease process, declines in olfaction with aging may represent slower repair and regeneration.

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References

Fig. 1. Immunoblotting of Syn in the OB following OE lesioning in WT and KO mice
(A) OB homogenates (20 µg) collected at the indicated times following nasal irrigation of
Triton were immunoblotted using a polyclonal antibody to apoE as described under Materials
and Methods. Actin was used as a loading standard. (B) The relative changes (optical density,
OD) in the Syn concentration (Mean +/- SE). All data points were generated by densitometric
scans of immunoblots in three separate experiments.
Fig. 2. Syn immunoreactivity in the glomerular layer of OB following OE lesioning

(A) WT mice are shown on the left and KO are shown on the right. No obvious differences are apparent in saline-treated mice (A, B) and in 3 days post lesion (C, D). However, at 14 and 42 days post lesion, Syn is recovering in WT (E, G), whereas little or no Syn is seen in KO (F, H). By 56 days, in WT mice (I) Syn immunoreactivity has almost reached to normal levels seen in saline-treated WT mice. In contrast, in KO mice (J) Syn immunoreactivity is still lighter, and quite distinct from the saline-treated KO mice. The scale bar in J = 50 µm. (B)

Densitometric quantification of the changes in the Syn immunoreactivity in the glomeruli as a function of days post Triton lesion. In WT mice, Syn recovery occurred between day 21 and 42, whereas in KO mice it occurred between day 42 and 56 and was not complete by this latter date. Note that, the KO mice showed consistently lower levels of Syn than the WT mice. (C)

Glomerular area is shown in this graph as a function of days following lesion. A sharp decline in glomerular area is evident at 3 days post injury in both WT and KO mice. In WT mice, the area recovers to normal level by 42 days, whereas in KO mice it is delayed to 56 days.