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Effects Of Apolipoprotein E, Sex, And Estrogen On The Neuroplasticity Of Olfactory Receptor Neurons In Mice Following Olfactory Bulbectomy

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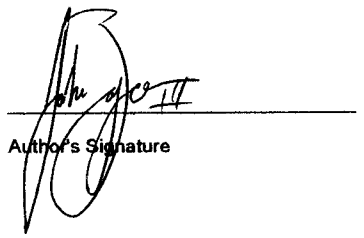

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The Effects of Apolipoprotein E, Sex, and Estrogen on the Neuroplasticity of Olfactory Receptor Neurons in Mice following Olfactory Bulbectomy

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John P. Boyce III

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Abstract

Apolipoprotein E (apoE) is well studied for its role in cholesterol transportation and metabolizing lipoproteins.ApoE is found in the olfactory system especially in the olfactory nerve and glomeruli of the olfactory bulb. Studies from our laboratory indicate that apoE is a required component for timely regeneration and olfactory receptor neuron maturation. The mechanism for which apoE contributes to neuron function and developing late onset Alzheimer's diseaseis still being studied. A mutant form of apoE (apoE 4) is believed to be a prominent genetic risk factor for this neurodegenerative disease. Over sixty percent of Alzheimer's disease patients in the United Sates are women. This revelation has concentrated more focus on the loss of estrogen as a potential risk factor. To better understand how apoE and estrogen may contribute to Alzheimer's disease, this study utilizes apoE knockout mice and estradiol treatment.

Using the olfactory system as a model, the right olfactory bulb was removed from all treatments of wild type (WT) and apoE knockout (KO) mice. Retrograde olfactory receptor neuron death and regeneration was observed between males and females of both genotypes in the olfactory epithelium. The results show massive olfactory receptor neuron death within days of olfactory bulb ablation in male mice compared to their female counter part. When estrogen was given at above the physiological dose, the duration of neuroprotection increased. However, when no estrogen was present olfactory receptor neurons died faster. Overall, mice treated with estrogen exhibited a more rapid rate of regeneration regardless of the presence of apoE.

Animals without apoE illustrated similar results to those with apoE but the average data values were slightly lower for apoE knockout mice than WTmice, illustrating that apoE is required for proper olfactory epithelium maintenance. These results were fairly consistent

throughout the data; however data for estradiol treated animals varied among genotypes. To measure the rate of olfactory receptor neuron death olfactory epithelium thickness was measured at 3, 7 and 21 days following olfactory bulbectomy. To observe the rate of death of mature olfactory receptor neurons olfactory marker protein (OMP) immunohistochemistry was performed. To gauge the rate of globose basal (stem) cell division bromodeoxyuridine (BrdU) immunohistochemistry was performed. By removing the olfactory bulb, the target for olfactory receptor neuron which served as the signal transduction, the olfactory epithelium fails toreturn to its original thickness suggesting olfactory receptor neuron maturity is dependent on proper target connectivity.

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Introduction

1.1 Alzheimer's disease: progression of research

Like many neurological illnesses, Alzheimer's disease (AD) is examined exponentially as more information surfaces regarding causes and treatments. The year was 1901 when Auguste Deter, at the age of 51, was admitted witha type of progressive dementia. The diagnosis was pre-senile dementia. Auguste D.would become the first reported case of Alzheimer's disease when Alois Alzheimer examined her brain in 1906 after she passed away. Using histology along with Nissl and Bielschowskystaining techniques, Alzheimer became arguably the first neuropathologist to observe amyloid plaques and neurofibrillary tanglesin a patient with early onset Alzheimer's disease (Graeber et al., 1999).

1.2 Apolipoprotein E and receptors

In 1993, one isoform of apolipoprotein E arose as a primary genetic risk factor for late onset Alzheimer's disease. With approximately 299 amino acid residues, apolipoprotein E is a 34KDa polymorphic protein subgroup of a class of lipoproteins responsible for redistributing lipids such as cholesterol (Boyle et al., 1989; Poirier et al., 1993). Intracellular lipid metabolism by way of phagocytosis is induced by lipoprotein-receptor interactions (Herz et al., 1988;Herz et al., 2001;Mahley et al., 1988; Takahashi et al., 1992; Yamagishi et al., 1998; Weisgraber et al., 1994). This is a general mechanism for lipid metabolism which varies depending on the locale and physiology of an organism.

The liver, known for its ability to process fatty substances, is where apoE was first found to be synthesized.Further investigation demonstrated high levels of apoE in the brain and the only apolipoprotein type in cerebral spinal fluid (CSF) which interacts with lipoprotein receptors

(Borghini et al., 1995; Pitas et al., 1997a; Pitas et al., 1997b; Herz et al., 1988; Bellosta et al., 1995). ApoE containing lipoproteins are believed to antagonize five different types of receptors in neurons: low-density (LDL) receptors, LDL-receptor related protein (LRP), very low-density lipoprotein (VLDL) receptor, apoE receptor 2(apoER2) and glycoprotein-330 (gp-330). Neurons mostly express LDL and LRP receptors and increase binding affinity and internalization of apoE containing lipids in vitro (Bellosta et al., 1995; Willnow et al., 1992). In order to fully comprehend how apoE may contribute to AD, a fundamental understanding of its mechanisms in the central nervous system along with its peripheral constituents is necessary.

1.3ApoE in the CNS and PNS

Understanding the role of apoE in peripheral nervous system (PNS) and central nervous system (CNS) with regard to maintenance and repair is a focal point for Alzheimer's disease research. Closer examination of areas where neuronal cell regeneration is increased, levels of both the mRNA of apoE and the protein itself increase. Also apoE producing lipoproteins, when applied to cell culture, accelerate neurite outgrowth (Bellosta et al., 1995;Holtzman et al., 1995; Ignatius et al., 1987; Nathan et al., 1994; Nathan et al., 2000; White et al., 2001; Nathan et al., 2001; Teter et al., 2002). LDL receptor and LRP receptor facilitate the binding and internalization of apoE-containing lipoproteins in vitro (Bellosta et al,. 1995). In rat models, apoE increases 250 to 350 fold following peripheral nerve insult (Holtzman et al., 1995; Ignatius et al., 1986; Snipes et al., 1986). Optic nerve and spinal cord injury also propagates a dramatic increase of apoE (Ignatius et al., 1986; Messmer et al., 1996; Snipes et al 1986). All indications support the idea that apoE recycles lipids from damaged tissue (Goodrum et al., 1995). However, there is morphological inconsistency with apoE expression of uninjured models.

ApoE deficient mice (apoE knock out/KO) have fewer synapses, vacoulized/swollen dendrites, and reduced recovery following insult (Masliah et al,. 1995; Masliah et al., 1996; Masliah et al., 1997). Conversely, other studies found no morphological differences between wild type mice and apoE KO mice (Gandy et al., 1995; Anderson et al.,1998). This discrepancy is understandable because apoE expression is tissue, genomic, and model dependent. Also when dealing with the olfactory system, olfactory receptor neurons (ORNs) die from natural insult increasing the likelihood of observing variation among members of similar uninjured models. Even in this project, uninjured epithelium has more variation then its injured counterpart. More consensuses can be found in behavioral modifications due to lack of apoE then in a morphological sense.

Animals lacking apoE have deficits in spatial learning (Masliah et al., 1996 Gordon et al., 1996). Applying apoE into lateral ventricles of apoE deficient mice reversed both morphological and behavioral abnormalities (Masliah et al., 1996). ApoE has also been found to prevent/protect against acute brain injuries (Chen et al., 1997). One study found that apoE is not necessary for the maintenance of the basal forebrain, rather, required for repair following injury (Fagan et al., 1998).Alterations in apoE levels appear to cause a variety of issues exemplifying the need for proper quantity of apoE in the CNS. Considering the alterations both behaviorally and possibly morphologically in apoE KO mice, understandably, this protein and its isoform apoE 4 (ε4) has been the center of Alzheimer's disease research.

1.4Biochemistry and physiology of apoE

The primary structure of apoE from 10 different species has been identified (Weisgraber, 1994). In humans this protein may exist in three different isoforms: apoE 2, apoE 3, and apoE 4.

They are produced by three alleles (ε2,ε3,ε4) ata single gene locus on chromosome 19 (Herz et al., 1988). These isoforms have similar primary conformations. However they vary at amino acid residue 112 and 158 (Menzel et al., 1984; Hallman 1991; Davingnon, 1988 and Raffai 2001). The most common isoform and least likely to cause complications is apoE 3. ApoE 3 has a cysteine at amino acid 112 and an arginine at 158. In apoE 2 both residues are cysteine and in apoE 4 both residues are arginine. This can be caused by a single point mutation at the first of threecodons for AA residue 112 in apoE 3. Cyteine can be either UGU or UGC: if there is a point mutation at the first codon and uracil in mRNA is replaced with adenine then UGC becomes AGC which codes for arginine or vice versa. This genetic alteration is called single nucleotide polymorphism (SNP) (Holtzman et al 2012).ApoE levels increase in the brain of patients with Creutfeldt-Jacob disease, Down's syndrome, and Alzheimer's disease (AD) (Namba et al., 1991). One study suggests that apoE may cause Parkinson's disease (Zareparsi et al., 2002). This single amino acid substitution leads to dramatic alterations in the biological properties of apoE, including its binding affinity for its lipoprotein receptors.

As previously stated, the apoE 4 allele results in the progression of late-onset Alzheimer's disease (Corder et al., 1993; Roses, 1997; Strittmatter., 1996; Schmechel et al., 1993). To understand how apoE is a risk factor for Alzheimer's disease, characteristics of AD must first bereasoned. Apoe4 genotypic individuals also have increased risk for dementia related symptoms follow traumatic brain injury (TBI) (Ignatius et al., 1987; Mayeux et al., 1995;Teasdale et al.,1997). Rehabilitation following a stroke and TBI is diminished in apoE 4 individuals which is evident in sports with increased risk for such trauma (Albertset al., 1995;Friedman et al., 1999; Jordan et al., 1997; Kutner et al., 2000).The prognosis of AD is a series of senile (neuritic) plaques, amyloid angiopathy, and neurofibrillary tangles. Individuals with apoE 4 alleles have

the risk of progressive late-onset AD noted by an increase inneurotic plaques and neurofibrillary tangles (Corder et al., 1993;Roses et al., 1997;Strittmatter et al., 1996; Schmechel et al., 1993; Ohm et al., 1999).Neuritic plaques characterized as: focal, spherical collections of dilated, tortuous, gallayas positive, dystrophic neurites. These neuritic plaques contain a clear corona surrounding an amyloid core. The components of this amyloid core is beta-amyloid, a peptide of approximately 40 to 43 amino acid residues derived from amyloid precursor protein (APP). Neurofibrillary tangles are intracellular anomalies that containpaired helical filaments along with numerous straight filaments with similar composition. These helical and straight filaments are remnants of tau that have beenhyprephosphorylated (Contran et al., 1999). To understand thoroughly how apoE and its isoforms contribute to AD, it is important to understand how apoE interacts with its receptors.

As previouslystated, apoE is the major ligand of two prominent receptors in CNS neurons: LRP and LDLR. LRP may be more related to AD since itcan bind to both APP andbeta-amyloid. Some possible mechanisms for which apoE receptors may contribute to AD are: inflammation control, cholesterol metabolism, neurogenesis and the generation/trafficking of amyloid precursor protein (APP) as well as beta amyloid (Aβ). Aβ42 (the long form) deposition in the brain is one major prognosis of AD and cerebral amyloid angiopathy (CAA) however, Aβ aggregation occurs naturally and its clearance involves apoE and apoE receptors (Holtzman et al., 2012; Bales et al 2009).

The physiological (lipidated) form of apoE along with its lipid free form interacts with Aβ. Altering the lipidization state of apoE can modify Aβ fibrillization. The adenosine triphosphate (ATP) binding cassette A1 (ABCA1) lipidates apoE and increasing ABCA1 levels decreases the deposition of amyloid(Wahrle et al., 2008). It is not believed that an increase of

apoE increases Aβ formation, Rather it is involved in its transport and clearance prior to its disposition. Isoforms of apoE alter the rate of Aβ clearance with an increased duration of clearance by apoE 4 and a more rapid clearance by apoE 2. This coincides with the level of fibrillization observed between the isoforms: apoE4 has greater fibrillization than apoE3 which has more fibrillization than apoE 2. These findings appear to still be up for debate with some conflicting evidence suggesting that apoE inhibits fibrillization altogether (Holtzman et al., 2012).

LRP1 is the best characterized receptor that directly protects against dendritic and neuronal loss and is weakly associated with AD. LRP1 are expressed more in neurons then in glia and binds to Aβ directly or indirectly via ligands like apoE. A decrease in LRP1 in brain capillaries may contribute to impair Aβ clearance. The loss of LRP1 in adipocytes and fibroblasts increases GSK3, a known catalyst for tau phosphorylation. LRP1 interacts with either long or short forms of APP extracellularly, endocytosis occurs quicker when LRP1 is APP bound increasing its processing to Aβ.Any increase of APP endocytosis and dispersal in endosomes increases Aβ production (Holtzman et al., 2012).

Impaired Aβ clearance may cause late onset AD. LRP1 (LDLR and VLDLR) are found in endothelial cells and smooth muscle cells at the blood brain barrier and its decreased expression in brain capillaries may contribute to lower Aβ clearance. Once apoE is bound to Aβ, apoE loses its lipid binding function in vitro. The overlying C-terminal of Aβ along with the lipid binding region of apoE is believed to cause apoE-Aβ binding complex. Aβ binding to apoE4 redirects its clearance from LRP1 to VLDLR causing internalization of apoE4-Aβ complexes at the blood brain barrier in a more leisurely manner than LRP1. Most internalized AB is delivered to lysomes or transcytosed into the plasma by the blood brain barrier. Both VLDLR and LRP1

are responsible for clearing Aβ-apoE2 and Aβ-apoE3. LRP1 may also bind directly to Aβ (Holtzman et al., 2012).

ApoE receptors contain short cytoplasmic tails which have no functional domain. These receptors can act as co-receptors by way of an extracellular domain and interact with other receptors such as NMDAR. NMDA and AMPA receptors interact with a common ligand of apoE receptors Apoer2 and VLDLR: Reelin. Apoer2 and VLDLR do not require association with other proteins to transmit an intracellular signal; however, both have a high affinity for Reelin. Reelin signaling alters the SRC family tyrosine kinase initiating events such as activating PI3K and Kinase B (AKT) which inhibits GS3K (well-documented for phosphorylating MAP tau) and causes tyrosine phosphorylation of NMDAR subunits allowing cation influx (Beffert et al., 2002; Beffert et al., 2004Jossin et al., 2007). A loss of Reelin may cause tau hyperphosphorylation but Reelin has no proven effects on P35: another protein involved in tau phosphorylation. Long term potentiation appears to be increased because of the association of Reelin, Apoer2/VLDLR with NMDAR (Weeber et al., 2002). If Aβ levels are at low to intermediate levels, Reelin can decrease Aβ synaptic suppression; however, intracellular compartmentalization of apoE4 can inhibit the properties Reelin has with regards to neuronal homeostasis of Aβ (Holtzman et al., 2012). Receptor coupling with NMDAR that increases its activity warrants a more in-depth understanding of calcium's role in synaptic plasticity.

1.5 Calcium regulation in neurons: channels

One electrophysiological goal of an action potential(AP) is to increase the intracellular concentration of calcium and drive signal relay between neurons. Of all the major ions (sodium, potassium, and chloride) calcium has the lowest concentration in the interstitial fluid and also the

lowest concentration inside of the neurons, yet, using the Nernst equation; it has the highest equilibrium potential: 127.73, compared to the second largest, sodium (Pelligrino et al., 1998; Ohm et al., 1999). Gene expression, neurotransmission, synaptic plasticity, and even neurite outgrowth are all affected by an increase of calcium concentration. Channels for this cation are seen in the dendrite, soma, and axon of neurons. The resting membrane potential of a typical neuron is anywhere between -70 to -75mV and an action potential is initiated when the intercellular threshold is reached (-50 to -55mV). Therefore, in a neuron, any electrochemical activities between resting potential and membrane potential may be considered a graded potential.

By way of olfactory bulbectomy and ion overstimulation a relationship between caspase 8 signaling and apoptosis has been found which involves calcium. Through OBX the initial cascade event that drives apoptosis is improbable to pinpoint, but by altering calcium channels via over-excitation, the initial caspase events can be isolated while maintaining synaptic connections. Like glutamate, NMDA excites NMDA receptors and increased NMDA concentration may cause apoptosis by vastly increasing calcium ion concentration at the axon terminal. One calcium receptor, p75, when missing causes mal-formation of glomeruli in the OB (Tisay et al., 2001). Limited caspase activation was observed in p75 KO mice post NMDA lesioning (Carson et al., 2005) which is further evidence that calcium regulation of ORNs may reduce apoptosis. This is important because intracellular calcium ion concentration must be constantly regulated and some neuron populations depend on neurotrophins for this. Retrograde apoptosis is a prominent mechanism for cell death in the olfactory epithelium following removal of the olfactory bulb (OB). Since retrograde apoptosis may be activated by a large increase or decrease of intracellular calcium ion concentration, it becomes apparent how neuron without a

target might die. Since retrograde apoptosis occurs from the axon terminal to the soma, mitochondria homeostasis is prominent cause of cell death in the OE following olfactory bulbectomy.

1.6 Cell death and mitochondria homeostasis

Retrograde cell death is failure of mitochondrial homeostasis which is dependent on calcium and oxy radical regulation. Mitochondria are intracellular powerhouses that use reactant oxygen species (ROS) like hydrogen and oxygen along with calcium to drive ATP synthesis using the enzyme ATPase and an influx of free hydrogen ions from the cytosol. The resting membrane potential of a neuron is about -70 mV and the mitochondrial membrane potential ψm between -150mV to -200mV: mitochondria are hyperpolarized compared to their surroundings and require intense ionic regulation (Chang, 2000). Depolarization of the mitochondria membrane is one indication of apoptosis and may be the leading cause of it as well.

Cytochrome C resides inside of the mitochondria mainly between the two membranes (intermembrane space) and may be utilized as an electron carrier. The permeability transition model suggest that its release is caused by permeability shifts, however, using photodynamic therapy, increased oxy radical production within the mitochondria will cause cytochrome c release without any notable increase in mitochondrial depolarization ψm (Chiu and Oleinik, 2001). Proper regulation of ROS and functioning of ATPase inside the mitochondria appears to be the difference between apoptosis and necrosis (Chang, 2005). Semantics aside, release of cytochrome c initiates the activation of a proteolytic cascade that is responsible for cell death. There are a number of possible ways a pro-death message may be sent to the cell body, most of which involve retrograde microtubule transport. The message may be relayed to the soma where

proteolytic activation occurs but inhibiting microtubule transport delays programed death (Carson et al., 2005). There are three major types of biological motor proteins myosin, kinesinand dynein in which transport is observed.

1.7MAP Tau and AD

Myosin is a great example of how calcium and ATP induce movement, but kinesin and dynein are the two motor proteins that are responsible for vesicular/vacuole transportation in a neuron. Microtubule associated proteins (MAPs) and their relationship to this system is crucial for proper transport. Microtubules are very unstable and require MAPs for stabilization

Several variations of neurological intracellular MAPs have been identified: MAP-1, MAP-2, and tau. The directionality (plus versus minus) of microtubules depends on which MAP is bound to it. MAP-2 is localized in dendrites and tau is localized in the axon. The ends of these microtubules are not bound together and essentially end in the cytoplasm, further yielding control to intracellular physiology. MAP-2 resides in dendrites whereas tau is only in the axon. Due to molecular configuration, kinesin transports anterogradely, while, for the same reason, dynein transports retrogradely. Dynein has been found to directly inhibit anterograde transport mechanisms (**The Cell**, chapter 11) making retrograde apoptosis a one way street. Microtubule activation proteins are named for their role in intracellular transport and because of this they have been studied as a possible cause for neurodegenerative diseases such as Alzheimer's disease. MAP tau helical filaments have been found in neurofibrillary tangles at the cellular level (Brionet al., 1985). Hyper phosphorylated tau protein in conjunction with phosphorylation of amyloid precursor protein causes a buildup of intracellular Aβ deposits which can be toxic to a neuron.

1.8The olfactory system

Now that apoE and intraneuronal calcium has been explored, the olfactory system, the model for the experiments presented in this paper, will be examined. The olfactory epithelium (OE) of a mouse is pseudostratified columnar epithelium. The OE is essentially a peripheral extension of specialized sensory neurons and like the CNS it has various cell types designed for injury recovery. Olfactory receptor neurons (ORNs) make up 75% of the total neuronal cell population whereas 15% of the population is supportive cells and the rest are globose basal cells (stem cells). The ORNs are similar to the neurons as seen in the CNS; main function is signal transduction/relay. The supporting cells deemed sustentacular cells (SUS) are located in the distal epithelial layer and also contain dendritic extensions with odorant receptors which are utilized in the initial stages of odor processing. Visually identified by their oval shape and larger nucei compartment, SUS cells have one function comparable to astroglia: cell maintenance (Hurtt et al., 1988; Schwob et al., 1995;Verhaagen et al.,1990; Verhaagen et al., 1989; Youngentobet al., 1997).

Previous research from this lab and others found that immunostaining of apoE in the OE is most prominent in the sustentacular cells post bulbectomy because the cells have a significant role in phagocytosis post olfactory bulb insult (Nathan et al., 2007; Suzuki et al., 1996). This suggests that, like astroglia in the CNS, synthesis of apoE is a necessary process for intra-tissue maintenance in the PNS. Sustentacular cells have an important role in the maintenance of the OE but macrophage recruitment is also a necessity when the severity of an injury is along the lines of ORN target ablation. Within 16 hours of OBX, macrophage recruitment may be observed in the OE of a mouse (Nan et al., 2001). Since the OE is a peripheral system itutilizes macrophages to perform some of the same functions that microglia utilize in the CNS: phagocytosis of dead

ORNs. The CNS also has a subpopulation of macrophages that has 2 phenotypic subtypes which are functionally dependent on the stimulus that activates its recruitment.

1.9Olfactory system: CNS versus PNS

Microglia and macrophages in the CNS differentiate into two different phenotypic subtypes that are considered "polarized" because they both represent opposing roles which are dependent upon the stimulus that cause them to differentiate from their resting/ramified precursor cells. The M2 microglia subtype function to repair unhealthy neurons and are stimulated by Th2 cytokines: IL (4,10,11), CD45, and SOCS-1. Activation of this M2 state is considered "alternative activation" and elicits an anti-inflammatory response. The "classical activation" of the resting precursor microglia differentiates into the M1 phenotype by a variety of factors including Th1 cytokines and more importantly with regard to AD: Aβ. Microglia's of the M1 phenotype are phagocytic and may be observed in areas of trauma and injury (Salemi et al., 2011). Since blood flow to the olfactory epithelium is from the anterior ethmoidal artery which is protected by the blood brain barrier, resident macrophages may have similar phenotypic variation in the OE as seen in the CNS.

The olfactory epithelium is unique because ORNs are an extension of cranial nerve 1 and is protected by the blood brain barrier, however the olfactory epithelium is considered a peripheral system. This is because the OE is directly manipulate by outside stimulus and contains epithelium, bowman's glands (which lie in the connective tissue below the ORN layers in an area called the lamina propria LP). Also globose basal cells (GBCs) proliferate and replace ORNs in response to ORN death which occurs continuously throughout the life of an organism. Considering the OE is subject to environmental insult and continuously repairs itself, the OE is a

part of the peripheral nervous system. It isresponsible for relaying signals from the environment to its CNS constituent the olfactory bulb (Calof and Murray, 1999).

1.10G-protein receptors in odorant perception

Like all neurological systems the olfactory system consists of a series of relay molecules that combines a plethora of signals from the OE to the final recipientthe granule layer of the OB where all of the information is received, processed and sent to other pathways in the cerebellum for odor perception. The dendritic extensions of the OE reside inolfactory mucus/cilia layers exposed to the nasal cavity.The cilia are ORN dendritic extensions and the mucus allows the capturing of olfactory stimulating molecules (scents) (*Figure 1*). Odor molecules stimulate G-protein receptors called G_{olf}. G proteins have 3 intracellular subunits γ (gamma),β (beta) and α (alpha). These subunits remain bound to G_{olf} withγbeing the receptor bound subunit which attaches to the β subunit which attaches to αsubunit. When all three of these subunits are attached to G_{olf}it is because the α is bound to GDP, however, upon ligand binding to G_{olf}, changes in conformation causes the release of heterodimer Golfγβ bound to Golfα to dissociate from the membrane bound receptor. Once the three subunits are released GDP is replaced with GTP causing the release of Golfα-GTP from G_{olf}γβ. G_{olf}α-GTP then reacts with a calcium activated chloride channel increasing intracellular calcium ion concentration and decreasing intracellular chloride ion concentration, polarizing the ORN enough to elicit an action potential. These ion channels are regulated by calcium binding to calmodulin which reduces the response of Golf to its ligand while phosphorylase strips a phosphate from GTP bound Golfα. The G_{olf}α-GDP complex re-binds to G_{olf}γβ and the membrane receptor Golf along with its subunits is restored to the inactive form.

Once an AP is elicited, signal transduction travels along the length of the axon through the OE proper into the lamina propria where the axon travels into a nerve bundle: through the fissures of cribiform plate of the ethmoid bone where the olfactory bulb resides (*Figure 2).* The axon extensions follow along the olfactory nerve fascicle and enter the glomeruli where signal transduction ends for ORNs and begins for tufted, mitral, periglomular and granule cells in the OB. The glomeruli are centers of synaptic transmission (*Figure 4*). Mitral cells aid is forming glomeruli and the mitral cell bodies reside between the external plexiform and the granular cell layers. The granular layer is composed of interneurons called granule cells which have dendritic extensions across the external plexiform layer. Tufted cells are found in the external plexiform layer while periglomular and tufted cells are found in the glomerular layers (*Figure 3*). The OB develops from the telencephalon and is part of the cerebral hemisphere. The most peripheral layer is not only composed of ORN extensions but also ensheathing glia and microglia.

Since olfactory system is a part ofboth the PNS and CNS it makes for a great model. Either by observing how the CNS responds to peripheral insult through TX lesioning or how peripheral neurons respond to target ablation via olfactory bulbectomy: both of which have been performed in this lab (Nathan et al., 1994; Nathan et al., 2000; Nathan et al., 2001; Nathan et al., 2007). For the experiments presented in this paper unilateral (right OB) olfactory bulbectomy is utilized along with immunohistochemistry to observe ORN death following insult only on one side of the OE (*Figure 5*). This allows for retrograde neuron death to be measured in a time dependent fashion.

1.11Aims of this study

The primary aim of this study is to examine how apoE and estrogen, in terms of neuroplasticity, modify the response of ORNs upon loss of axon function.

Aim 1: To examine apoE expression in the olfactory system of male versus female wild type (WT) mice. It is hypothesized that apoE quantity and expression will increase in dyingolfactory epithelium due to the accumulation of neuronal byproductsknown to increase in the days following OBX.Measurements include apoE, OE thickness, mature neurons degeneration/possible regeneration, and globose basal cell proliferation. Such investigations will be tested via neuroanatomical marking and comparison of apoE expression in the olfactory epithelium (OE) of WT males versus females using immunohistochemistry methods.

Aim-2: To examine apoE expression following target ablation. This results in destruction of the OE and instigates progressive OE death in WT ovariectomized females and females treated with estrogen. Various accepted time courses of adequate OE de-constitution will be analyzed and examined using the mouse olfactory system as a neurophysiological model, using the results to examine the response of the OE to OBX. Using immunohistochemistry followed by quantitative examination of OE thickness; and markers for cell division, and mature ORN; adequate comparisons will be established to allow for conclusions as to the effect of the sex, estrogen and apoE on neural tissue damage response and repair mechanisms concurrently and independent of each. Measurements include apoE, OE thickness, mature neurons degeneration/ regeneration, and globose basal cell proliferation.

Aim-3: To examine the effects of apoE deficiency on reconstitution of the olfactory epithelium following chemically induced lesioning in WT and apoE-KO mice in all experimental conditions

(male, female, OVX female and estrogen treated female). Olfactory nerve injury will be induced in both WT and KO mice using OBX an examination of the results will ascertain as to the impact of apoE deficiency on OE reconstitution. A comparison of mature ORN growth at a series of time points following OE destruction will demonstrate the effect of apoE loss on OE degeneration and regeneration. The results from these studies will help clarify the role of apoE and estrogen in the olfactory system, as well as its role in age-related neurological diseases and disorders, such as Alzheimer's disease (AD).

Materials and Methods

2.1 Olfactory bulbectomy

Breeding pairs of WT C57BL/6 strain and homozygous apoE KO mice (backcrossed a minimum of 10x) purchased from Jackson Laboratories, Bar Harbor, ME. ApoE genotype of the littermates was verified using polymerase chain reaction (PCR) technology and confirmed via immunoblotting visualization technique. Both WT and KO genotype mice were of the age range of 4-6 months (age range of an adult mouse).Twenty- four wild-type (WT) and twenty-four apoE KO genotype mice, separated by male, females, ovariectomized females with estradiol treatment, and ovariectomized females without estradiol treatment were obtained from the departmental mouse colony and were unilaterally bulbectomized (Costanzo and Graziadei,1983). The mice were first anesthetized usingKetamine HCl/XylazineHCl (50 mg/kg) (#K-113) was purchased from Sigma-Aldrich, St. Louis, MO. Appropriate dose is weight dependent. The right olfactory bulb was exposed by a sagittal incision exposing the skull slightly above the nose of the mouse. Once the olfactory bulb was located, a hole was drilled using aDremel 8000 10.8V Lithium-ion Cordlessa and109 Engraving Cutter purchased from Ace Hardware, Charleston, IL. The bulb was extracted using suction and replaced with Gelfoam™ purchased (Pharmacia &Upjohn, Kalamazoo, MI) to prevent the forebrain from entering the cavity. Time points used for the study on regeneration rates of the OE post injury were 3, 7, 21 days post- bulbectomy; with an equal number of WT and KO individuals (n=2) being used. As a control the contralateral (uninjured) epithelium for each mouse was measured for consistency. The uninjured epithelium for each treatment 3 days following injury was used as baseline values. This time course was chosen to span the experimentally accepted range of degeneration allowing degeneration of ORN to be

observed until OE thickness levels gradually regain thickness. Test subjects were anesthetized with ketamine/xylazine then gently restrained in a stereotaxic apparatus. The skull was exposed using a small midline incision. The area of the cranium where the right OB resides was identified then a hole was drilled. Using suction the right OB was extracted, the cavity filled using Gelfoam™ to prevent blood loss and frontal cortex invasion which could act as a target for regenerating neurons. The skin above the lesion was sutured and animals were allowed to recover from anesthesia under a heat lamp. To replenish electrolytes a subcutaneous injection of .9% NaCl was administered. Animals were housed in animal care facility until sacrifice at the chosen time (3, 7, and 21dpi).

2.2 Ovariectomy

Of the forty-eight mice experimented on two-thirds of them were female mice. Out of the thirty-six females, twenty-four of them were fully ovariectomized. Ovariectomy was performed five days prior to estrogen administration and olfactory bulbectomy: to insure total biological depletion of estrogen. The mice were first anesthetized using Ketamine HCl/XylazineHCl (50 mg/kg) (#K-113) was purchased from Sigma-Aldrich, St. Louis, MO. Appropriate dose is weight dependent. On each side of the mouse a small incision was made through all epidermal layers and muscle exposing the abdominal cavity. Once the ovaries were recognized they were expelled and the wounds were closed using surgical staples. Mice received .9%NaCl to replenish electrolytes and allowed to heal under a warm lamp, and then returned to the animal care facility.

2.3Estradiol silasiticimplant

17-β estradiol (E-8875) was purchased from Sigma-Aldrich, St. Louis, MO. A stock solution of estradiol was made by dissolving .25g of 17β estradiol into 5ml of corn oil. A 1ml working solution was then made by mixing 10μl of the stock solution and 990μl of corn oil. The

solution was then placed in SILASTIC laboratory tubing (508-008) was purchased from Dow Corning, Elizabethtown, KY. Each silastic capsule was cut to the length of 14mm with 2mm on each side used for aquatic sealant. Once the sealant on one side dried the silastic tube was filled with 20µl of working solution. Then the other end was sealed and allowed to dry. Implantation occurred 5 minutes prior to OBX and was allowed to equalize in PBS 24 hours prior to implant. The tubing was inserted in the peritoneum on the back just below the neck.

2.4Animal sacrifice and tissue preparation

The mice were first anesthetized using Ketamine HCl/XylazineHCl (50 mg/kg catalog #K-113Sigma-Aldrich, St. Louis, MO) and perfused transcardially with 4°C PBS for 5 min, followed by freshly prepared 4% paraformaldehyde (PBS) at 4°C. PrilledParaformaldehyde(#441244) was purchased from Sigma-Aldrich, St. Louis, MO. Turbinates were dissected and immersed in Cal-EX decalcifying solution for 1h, followed by 4% paraformaldehyde for 1h, and finally stored overnight in 30% sucrose (PBS) at 4°C. Turbinates were removed from solution and canine teeth were excised, followed by immersion in gelatin solution under vacuum for 1h. Samples were removed from solidified gelatin and frozen on dry ice, and were labeled and stored at -80°C. The OB samples were dissected and post-fixed in 4% paraformaldehyde for 1h, followed by storage overnight in 30% sucrose solution at 4°C. Sucrose (#S-9378) and gelatin (#G-1890-500) were purchased from Sigma, St. Louis, MO. Cal-EX decalcifying solution (#D0818-1L) and tissue-freezing medium (#15-183-13) were purchased from Fisher Scientific, St. Louis, MO. Tissue samples were frozen on dry ice, labeled, and stored at -80°C. Sample sections for both the turbinates and bulbs were cut at 10 µm thickness using a Reichert-Jung 2800 Frigocut E cryostat. Serial sections were thaw mounted using PBS onto slides pre-subbed in 2% gelatin and 0.5% chrome alum, then air dried and stored in a

labeled slide storage box. Experimental sections were dried in a 37°C oven prior to specify staining procedure.

2.5Nissl staining

Sections were washed in 0.1M PBS for 5 min, followed by delipidization with xylene three times for ten minutes each, then a hydration series of ethanol (70, 95, and 100 %) for ten minutes each. Samples were washed in DH_2O for 5 min, followed by staining with cresyl violet acetate for 4 min. Cresol violet acetate (#C5042-10G) was purchased from Sigma, St. Louis, MO. Sections were transferred to DH_2O wash for 10 min, and then differentiated in acidified 95% ethanol for 5min. This was followed by standard dehydration series of ethanol and delipidization in xylene for 30 min. Stained slides were coverslipped using permount.Permount (#SP15-500) was purchased from Fisher Scientific, St. Louis, MO.

2.6BrdU immunohistochemistry

Each post-bulbectomized test subject received an intraperitoneal injection of bromodeoxyuridine (BrdU), (50 mg/kg catalog #B9285was purchased from Sigma, St. Louis, MO), 24 hours prior to sacrifice to allow for an adequate time for cell division to occur. The contralateral OE of 3 days post injury test subjects were used as a baseline for marker protein levels for comparison and representation. Once the sections were on slides sections were washed in 0.1M PBS for 5 minutes and then permeabilized in 0.1% Triton X-100 for 30 minutes at room temperature. Hydrochloric acid (HCl) (#A144-500) was obtained from Fisher Scientific, St. Louis, MO. Denaturation was carried out using 2N HCl at room temperature for 60 minutes followed by neutralization with Hanks' balanced salt solution (HBSS) for 10 minutes. Nonspecific blocking of the sections was carried out with 5% bovine serum albumin (in PBS) within a humidified chamber, followed by a 10 minute rinse in 0.1 M PBS.Hanks' balance salt

solution (HBSS) (#14175-095) and fetal bovine serum (#26400-044) was purchased from Invitrogen, Grand Island, NY. An anti-BrdU primary antibody (1:1000 in blocking solution) was applied to the sections for incubation overnight at 4°C.Rat anti-bromodeoxyuridine (BrdU) primary antibody (#OBT0030CX) was purchased from Accurate, Westbury, NY. After a 10-minute PBS rinse, sections were incubated for 1 hour in Cy3-conjugated goat anti-rat IgG secondary antibody (1:500 in blocking solution) at room temperature.Cy3-conjugated goat anti-rat IgG secondary antibody (#112-165-068) was purchased from Jackson ImmunoResearch, West Grove, PA. Slides were rinsed in 0.1M PBS for 10 minutes and then cover slipped using Vectorshield mounting media.Anti-photobleach mounting media (#H-1000) was purchased from Vector Laboratories, Burlingame, CA.Immunoreactive cells were counted and digitally photographed using an Olympus BX50 microscope with appropriate fluorescence excitation filters.

2.7ApoE/OMP/SYN immunohistochemistry

Sections were washed in 0.1M PBS for 5 minutes and then permeabilized in 0.2% Triton X-100 for 30 minutes at room temperature. Next, they were rinsed with PBS and then subjected to a dehydration/rehydration series in ethanol (70, 95, 100, 95, and 70%) for 2 minutes each. Following a 5-minute rinse in PBS, nonspecific immunoreactivity was attenuated by incubation sections in 2.25% gelatin (in 0.1 M PBS) for 1 hour and rinsed again with PBS. The sections were incubated overnight at room temperature in a primary antibody diluted in its appropriate corresponding 4% serum (in 0.1M PBS). The primary antibodies included: anti-ApoE (1:4000Goat anti-human apolipoprotein E primary antibody catalog #178479 Calbiochem, San Diego, CA), anti-OMP (1:2000Goat anti-rat olfactory marker protein catalog #544-10001 primary antibody Wako.), anti-SYN (1:2000Rabbit anti-synaptophysin catalog #MRQ-40 Cell

MarqueCorporation, Hot Springs, AR). Following primary antibody incubation, sections were rinsed for 10 minutes in 0.1M PBS and incubated for 1 hour at room temperature with the appropriate secondary antibody: FITC-donkey anti-goat IgG (1:1000 catalog #707-165-003 Jackson ImmunoResearch, West Grove, PA.) and FITC-goat anti-rabbit IgG (1:1000catalog # 111-095-003 IgG Jackson ImmunoResearch, West Grove, PA). Dilutions were made in 4% corresponding serum: donkey serum (catalog #017-000-121Jackson ImmunoResearch, West Grove, PA) and goat serum (catalog #S-1000 Vector Laboratories, Burlingame, CA). Finally, slides were rinsed in 0.1M PBS for 10 minutes and then coverslipped using Vectorshield mounting media(Anti-photobleach mounting media catalog #H-1000 Vector Laboratories, Burlingame, CA). Specificity was determined by incubation with normal serum in place of the primary antisera, which resulted in no staining. Immunoreactive cells were digitally photographed on an Olympus BX50 microscope with appropriate fluorescence excitation filters. Saline and TX-irrigated mice from the two genotypes were processed on the same day with the same reagents to permit comparison of staining density.

2.8Microscopy and imaging

Immunocytochemistry was visualized with appropriate fluorescence excitation filters for fluorescent microscopy (Olympus BX-50). Tiff format images of the nasal septum of the turbinates or the medial edge of the OB were taken with a Pixara digital camera for microscopy at identical manual exposure settings for all experiments within each study. Bulbectomized mice from the two genotypes were examined under the microscope on the same day to ensure comparison of staining density.

2.9Morphometric analysis of OE

To examine if apoE has an impact on the normal remodeling that occurs in the OE after bulbectomy, morphometric analysis was performed, thickness of OE was measured using cresyl violet stained sections from WT and KO littermates.

2.10Quantification of BrdU

Stained BrdU–positive nuclei were quantified using computer-assisted morphometry and densitometry (Scion Image, Bethseda, MD) as described (Bauer et al., 2003; Nathan et al., 2001). Analysis was performed at two middle rostrocaudal levels of the turbinates controlled in all animals. Three anatomically matching areas were selected for cell counting throughout the extent of the OE, and all immunopositive cells were counted within the defined region. The data were expressed as labeling indices; that is, the ratio of the total number of BrdU-positive cells to the linear length of OE analyzed. Published studies have used this kind of quantification, since stereological analysis cannot be used in OE, due to its variation induced by lesioning (Baur et al., 2003; Holcomb et al., 1995; Schwartz et al., 1991).

2.11Quantification of apoE

Immunofluorescence was evaluated by fluorescence microscopy using 600X magnification. Pictures were taken from similar representative portions of the nasal septum of the OE of each animal from each time point. A complete set of pictures representing the entire time course was assembled into one panel using Adobe Photoshop 7.0 (San Jose, CA) and collectively adjusted for brightness and contrast. Following this adjustment, the panel of pictures was analyzed for differences in localization and levels of apoE present throughout the OE.

2.12Quantification of OMP

Images of OMP immunoreactivity in the OE were captured using a digital camera (Pixera, Los Gatos, CA) mounted to an Olympus microscope. Stained OMP–positive neurons were quantified using computer-assisted morphometry (Scion Image, Frederick, MD) as described (Teter et al., 2002). Analysis was performed in anatomically similar areas along the nasal septum of the OE by counting all the positively stained nuclei within the defined region. The data was expressed as labeling indices; that is, the ratio of the total number of OMP-positive cells to the linear length of OE analyzed (cells/100μm).

2.13Statistical analysis

All quantification procedures were repeated twice ±standard error and the data in individual experiments were presented as mean statistical analysis (ANOVA) was performed using SAS computer statistical program. The data was arranged as genotype (WT/KO), treatment (male/female/estrogen/no-estrogen) and days following insult (3,7 and 21). The day 0 data, since they are initial conditions, were observed using a separate test.

Results

3.1 ApoE in the OE following OBX

ApoE was qualitatively analyzed in the OE via immunohistochemical examination in mice 0, 3, 7 and 21 days post unilateral bulbectomy. ApoE was expressed in the sustentacular cells; mostly in the microvillar extensions that reside in the OE proper directly above the basal cells. In the lamina propria (LP), apoE was expressed in the endothelial cells surrounding the nerve bundles more so than in the ensheathing glia (EG) surrounding blood vessels (*Figure 6*).In non-injured epithelium apoE was rarely present in the ORN layers; however, in this study ORN death changes the distribution of apoE within the olfactory epithelium.

3.2 ApoE Expression and Sex

When the uninjured epithelium of female mice was observed under a microscope, both the OE and LP illustrate minimal apoE expression by comparison to other treatment groups prior to injury, however, after three days post injury (3dpi) the OE not only lost thickness but increased apoE levels. The olfactory epithelium of female mice 3dpi expressed apoE robustly along the basal lamina with dispersed pockets within the olfactory sensory neuron layers of the OE and diminutive visible apoE in the LP. One week after target ablation, the OE of female mice exuded fewer visible apoE along the basal lamina, limited expression in the LP and no pockets were observed in the OSN layer. ApoE was not prevalent in the OE along the basal lamina 3 weeks post injury, however, expression was visible in the olfactory propria close to the nasal lumen and in endothelial cells encompassing the blood vessel in the LP(*Figure 7*).

Unlike female mice, uninjured males expressed apoE in clusters along the basal lamina and in the lamina propria: a portrait of how apoE is naturally expressed in the OE of an average

male mouse. Expression of apoE in males 3 days post injury was unique in comparison to all other treatments at that time interval because of the masses of apoE expression isolated to one location within the OE and with pockets of expression in the LP. There was not a loss of apoE after 7 days, rather, a change in dispersion. The OE of males' one week post injury had fewer neurons in the OE indicated by the loss in thickness and fewer visible nissl bodies. ApoE at that time interval was seen throughout the OE and in the LP surrounding the basal lamina with positive expression around the blood vessels. ApoE was less uniformly dispersed in male mice 3 weeks post injury with large pockets of positive fluorescence in the OE proper and dispersed clusters of apoE in the LP(*Figure 7*). Most apoE in the OE resided along the basal lamina, however; pockets of positive staining were seen throughout the OSN layers.

Following injury the sex of the animal may determine how apoE reacted in the OE. The significance of apoE expression was not only in quantity of visible apoE in the OE but also its location. From 0 to 3 weeks post injury WT males had more apoE both in the OE proper and LP. The main difference between sexes was the dispersion of apoE in the OE; at 3dpi expression is evenly dispersed along the basal lamina in females but was throughout the ORN layers in males. After one week apoE remained close to the basal lamina in females and was diffused throughout the entire OE proper of males. The location of apoE in female mice at 21dpi was predominately near the olfactory cilia with no apoE above the basal lamina, whereas males contained clusters of apoE close to the olfactory cilia, basal lamina and in the areas between(*Figure 7*).

3.3 ApoE Expressionand estradiol treatment

Upon examination of the differences in apoE expression between animals with estradiol treatment and mice with no treatment there was little difference in apoE expression in the OE at

0 days post injury in estrogen treated mice and those without estradiol. Treated females exhibited uniform dispersion of apoE along the basal lamina with such definition to suggest it migrated into individual cells. The lamina propria of treated females yielded significant expression of apoE in the endothelial cells surrounding the blood vessels and some slight expression surrounding the olfactory nerve bundles. One week after injury the homogeneity of apoE expression remained consistent with 3 dpi in the OE, however, there was more fluorescence within the ORN layers in a thinning OE. The level of apoE in the lamina propria decreased at 7dpi with slight positive staining around the blood vessels. Throughout the ORN layers of treated mice, 21dpi, apoE appeared to be randomly expressed with a few positive receptor neurons close to the olfactory cilia region and others closer to the basal lamina. The lamina propria at 21dpi illustrated bright fluorescence mainly around the blood vessel and in other structural components surrounding the blood vessels.

When apoE was observed in ovariectomized untreated females clusters of apoE were expressed along the basal lamina and in the LP in unidentified structural components in uninjured epithelium. Three days after injury, in untreated females, there were pockets of apoE surrounding receptor neurons in a non-uniform distribution; there was also expression in the majority of the LP. One week post injury the majority of apoE resided in the OE proper with little to no expression in the LP; there was also 2 to 3 layers of well-defined cell bodies visible in the OE at this time interval. ApoE was more evenly dispersed throughout the entire OE proper in mice 3 weeks post injury, with more intense expression closer to the basal lamina and equally intense expression in the endothelial cells surrounding the blood vessel in the LP.

We examined the impact of estradiol replacement on OE reconstitution post OBX in ovariectomized mice. For this study, WT females were ovariectomized and either received

estradiol or did not. Overall, as for quantity, there did not seem to be a large difference of apoE between treatments. After 3 days, treated females expressed apoE along the basal lamina where basal globose cells resided, however, apoE surrounded individual olfactory receptor nissl bodies in untreated females. The localization of apoE expression in the OE proper 7dpi of treated and untreated females was about the same with more dispersion of apoE throughout the OE in untreated than in the treated females. After 3 weeks treated females exhibited sporadic expression of apoE with positive fluorescence surrounding ORN cell bodies throughout the OE, whereas the entire OE appeared to express apoE with brighter fluorescence above the basal lamina.

When all treatment groups were considered animals with estrogen appeared to have less apoE immunoflorescence than those with physiological estrogen levels or less at 3 and 7 days post injury with the dispersion of apoE more localized toward the basal lamina. On the 21st day following injury all female mice had similar quantities of apoE, however, WT males did not have as much apoE in its epithelium compared to any of the female treatment groups (*Figure 7*).

3.4Olfactory epithelium (OE) thickness

The uninjured olfactory epithelium is 7-8 layers of nissl (cell) bodies observed using a cresyl violet stain. Each neuron had an axon that projects through the basal lamina into the lamina propria where it travels to the olfactory bulb for signal transduction (*Figure 1*). This path traveled by the axon is crucial for the survival of the neuron.By unilaterally destroying the right olfactory bulb, the olfactory receptor neurons(ORNs) on the right (ipsilateral) side of the olfactory epithelium (OE) died, since, their signal pathway was depleted. Subsequently, the right

olfactory bulb was destroyed, the neurons in the epithelium necrotized, but, the left OE remained intact and was used as a control in each experiment (*Figure 5*).

3.5OE Thickness statistical analysis

The average of eight different random measurements was recorded for each mouse which was done for all epithelial thickness. This was done for mice of all independent variables; genotypes, treatments and time intervals. The mean value for each mouse represented one data point allowing data independence. Mean values, standard errors and a 3 way ANOVA was the sum of the statistical analysis performed for each dependent variable.

The mean values of 48 mice were recorded, (24 wild type and 24 knockouts, which equals 12 in each of the four treatments and 16 at each time period), and analyzed using a 3 way ANOVA via SAS. Results of the 3 way AVOVA revealed statistical significance between and among all main effects for all 3 dependent variables. Essentially all 2- way and 3-way interactions were also significant. Wild type mice had significantly greater epithelial thickness than did their KO counterparts. Similarly, WT animals had significantly more mature neurons and more dividing neurons than KO mice (*Table 1*). The OE thickness at 3 and 21 days were similar but greater than that at 7 days. The number of mature neurons was greatest at 3 days and fewest at 21 days. The number of developing neurons showed the same pattern as did mature neurons (*Table 2*). Mice the received estrogen had the largest epithelial thickness followed by females, females without estrogen and males. That same trend was seen in the number of mature neurons (OMP+) and the number of dividing neurons (BrdU +) (*Table 3*).

The data points for the uninjured olfactory epithelium were taken from the left OE, the contralateral uninjured epithelium 3days following unilateralbulbectomy in mice. The data

collected from the uninjured OE came from mice 3 days post injury for all dependent factors (OE thickness, OMP and BrdU). A 3 way ANOVA was done on the 0 day data independent of the other time intervals because this data represents the initial conditions. For OE thickness there was a significant correlation between genotypes for OE thickness and the number of mature (OMP+) neurons. When the 0 day data was combined with the experimental data, a full spectrum of the tissues' response to injury became more visible.

3.6OE thickness: ApoEand Sex

Comparing the overall epithelium thickness between genotypes in female mice following injury was the purpose of this experiment. With averages of all treatments groups and time intervals compiled, the overall OE of WT mice were 4.08μm thicker than KO mice and both had a standard error below 2. This illustrated a small difference between genotypes but that difference was significant (*Table 1*). The average OE of thickness WT females at initial conditions was 81.5μm thick whereas the OE of KO females was 81μm with no significant difference between the two genotypes. However, WT females 3 dpi had an average thickness of 42.13μm, 4.07μm thicker than its KO counterpart (38.06μm). The data for females 3dpi was significant. There wass a greater difference between genotypes in females 7dpi: WT females had a mean thickness of 29.98μm whereas KO females' mean thickness was 22.63μm: over a 7μm difference between the two. The difference in thickness 21dpi amongst genotypes was 7.3μm slightly higher than mice at 7dpi: on average the OE of WT females was29.98μm thick and KO females had an OE 22.63μm thick (*Figure 1*) (*Figure 8a)*(*Figure 9*). This trend was seen in all treatment groups but only in males did it come close to falling short.

For this experiment the differences between wild type and apoE KO male mice was illustrated. When observing the overall OE thickness between treatments, compiled between the

other dependent factors, males had the thinnest overall epithelium with a mean thickness of 24.41μm and a standard error of 1.4 (*Table 3*). Accounting for independent variables such as genotype and time, apoE KO mice became the contributing factor for males having had the lowest mean thickness. Three day post injury WT males had an average OE thickness of 27.64μm whereas KO males had an average of 20.28μm a 7.35μm difference which was far greater than that of 7dpi. One week following injury the OE of WT males had a thickness of 20.97μm and KO males had a thickness of 18.75μm, a 2.22μm difference. There was some recovery in the OE of WT males 3 weeks following injury with a mean thickness of 30.63μm, but, KO males had a thickness of 22.22μm at the same time interval, a less than modest recovery when compared to the other treatments (*Figure 8b)*(*Figure9*).

3.7OE thickness: ApoE and estradiol treatment

The next goal was to discover any measurable differences between animals treated with estrogen with and without apoE. By observing the treatment groups against all other independent factors, estrogen treated females had the thickest OE with a mean thickness of 41.04μm and a standard error of 2.29μm (*Table 3*). The neuroprotective effect of estrogen did not appear to be hindered by the lack of apoE. Three days following OBX apoE KO females had an average thickness of 48.1μm, 3μm thicker than WT estrogen treated mice (45.3μm). This was one of only two instances, in this experiment, where apoE KO mice had a greater OE thickness than its WT counterpart for a given time interval. Wild type treated females retained more OE thickness (35.37μm), 7dpi, than apoE knockout treated females (27.47μm); conversely in those four days KO mice lost 20.643μm of thickness whereas WT mice lost 9.933μm. The OE thickness of WT treated females (47.823μm) was greater than KO mice (42.23μm) 21 days following injury, however, the average thickness for KO mice increased by 14.73μm over the

course of 2 weeks; whereas WT mice only had an average thickness increase of 12.45μm(*Figure 8c) (Figure 9).* Similar occurrences between genotypes were also observed in untreated mice.

The next group of observations involvedovariectomized females without estradiol treatment of both genotypes. Similarly to their treated companions, untreated apoE KO mice maintained had greater OE thickness 3 days post injury (40.09μm), compared to WT mice (35.53μm). However, KO females had an average loss of thickness 23.75μm over the course of 4 days with an average thickness of 16.24μm at 7dpi. WT untreated females had an average OE thickness loss of 11.09μm with a thickness of 24.44μm at 7dpi. As seen with estrogen treated females, untreated apoE KO females overcame a greater deficit in OE thickness, over two weeks, with an average increase of 13.91μm contrary to untreated WT females with a 12.45μm, this difference was greater than the difference between the average OE thicknesses of both genotypes 3 weeks following injury (~30μm) (*Figure 8d) (Figure9*).

In determining which factor (apoE, estrogen, and sex) had a greater impact on the thickness of the OE after injury, all data for this dependent variable must be considered. Overall females had a greater OE thickness (11.56μm) disregarding genotype (*Table3*). Males had a greater loss of thickness 3 days post injury but KO males had a greater loss than WT males, a trend that was also seen with females. Females of both genotypes had a well-defined olfactory cilium layer, which was not seen in males of either genotype 3dpi. The olfactory receptor neuron cell bodies appeared to have less definition in males than in females; WT females and males along with KO males all had breaks in their ORNS layers at this time interval. The OE was broken up with little cell body definition and the nerve bundles were dilated for all treatments at 7 days post injury. There also appeared to be some residual olfactory cilium present in WT females at this time interval, however, the cilium was lacking at 21 dpi in females. The OE of

KO females at 21dpi was broken up in some areas which could also be seen in WT males. The OE of KO males 21 dpi consisted of 1 to 2 layers of ORNs (*Figure 8*).

3.8Olfactory marker protein in bulbectomized mice

The thickness of the olfactory epithelium depended on the presence of multiple cell lines; within the epithelium there are globular basal (stem) cells, immature receptor neurons, sustentacular cells, and mature receptor neurons. Olfactory neurons that maintained a synaptic connection with the glomeruli in the olfactory bulb also produced a protein called olfactory marker protein (OMP). By counting the number of mature olfactory receptor in the days following OBX, quantitative analysis of OE degeneration was calculated. In this study the differences in OMP expression between sex and estradiol treatment was compared across various time intervals.

3.9OMP statistical analysis

The injured data first statistically analyzed using a 3 way ANOVA, indicated a significant difference between WT and KO animals' mature neurons (OMP) count. The difference between genotypes was only a 2 OMP + neurons/ 100μm, and disregarded differences between time and treatments (*Table 1*). When combining treatments and genotypes, animals three days after injury exhibited the highest mean OMP count (36.57OMP neurons/100μm) with the mean OMP count decreasing with time: 10.81OMP+ neurons after 7days and 4.06 OMP + neurons after 21 days and none had a standard error greater than 2.1 (*Table 3*). Although there was a finite difference between genotypes and time, without the zero day data not much could be understood.

To reduce the quantity of mice experimented on; the initial data was derived from the uninjured side of the OE, 3 days post unilateral bulbectomy. The data for the 3 day uninjured OE was analyzed using ANOVA. There was a significant difference in initial mature neuron (OMP) count between genotypes. WT animals had an average of 93.25 mature neurons/ 100μm compared to KO animals which had 85.97 OMP + cells/ 100μm: both displayed a standard error below .5 (*Table 5*). When the data was compared exclusively across treatment groups there statistically were no significant correlations and the greatest average difference between two treatments was between females and females without estradiol treatment: a total of 4 OMP+ neurons /100μm (*Table 6*). By combining the zero day data with the injured data, a timeline of the activity in the OE after injury became observable.

There was a greater difference in OMP + neurons between genotypes in the initial data then there was between genotypes in the injured data. There were 7.3 more mature neurons in WT mice at initial conditions than in KO mice; however, there was only 1.8 more mature neurons in WT mice than in KO mice across all injured time intervals. Injured animals had the greater number of mature neurons after 3 days with a mean of 36.58 OMP + neurons; the average mature neuron count continuously decreased over time from the initial mature neuron count of 89.61OMP + neurons/100μm. Although there was no significant differences between treatments at zero days post injury, there were significant differences in the injured epithelium. On average estrogen treated mice had 24.63cells/100μm, the second highest OMP + cell count was female mice with 16.84cells/100μm, then the non-treated females with 14.69cells/100μm, and males on average had the fewest OMP + neurons with 12.45 cell/100μm (*Table 3*). One main focus of this study was to observe the differences of mature neuron loss following olfactory bulbectomy in various treatment groups at various time intervals in order to discover how physiological

alterations (sex, apoE, and estrogen) influenced neurodegeneration in mice. After combining the 0 day data with the injured data for each treatment in both genotypes, the effect of apoE on the OE could be measured.

3.10OMP: ApoE and Sex

First the differences between genotypes were examined in female mice with regard to mature olfactory neurons. The OE of WT females had an initial OMP cell count of 95.31cells/100μm, 9.8 more mature neurons on average than that of KO females (86.06cells/100μm). Three days following injury WT females illustrated a higher quantity of mature neurons (39.88cells/100μm) compared to KO females (33.31cells/100μm), however, within the three days, fewer mature neurons died in KO mice (52.75cell/100μm) than WT females (55.43cells/100μm). Mature neurons continued to die after one week with a mature neuron count of 9.25cells/100μm for WT females and 8.75cells/100μm for KO females, half a neuron difference; but, WT females still showed a greater loss of mature neurons than KO females. Three weeks following injury KO mice displayed an average of 2.88 cells expressing OMP, significantly fewer than the 7 OMP + neurons expressed in WT females(*Figure 10a*) (*Figure 11*).

Next the differences between genotypes and males mice with respect to mature olfactory neurons were surveyed. The OMP count at 0dpi in males followed the same trend seen in females, contrariwise, WT females had fewer mature neurons with 28.63 OMP+ neurons than KO males (30.5cells/100μm) a small difference yet statistically significant. KO males also lost fewer mature neurons in those first three days. On average the number of OMP + neurons in both genotypes was about 5 OMP+ neurons/100μm, with KO males illustrated slightly more

expression than their WT counterpart. After three weeks there remained only a little more than a one mature neuron difference between genotypes with KO males having average OMP + cell count of 1.625cells/100μm and WT animals having a count of 2.75cells/100μm(*Figure 10b*) (*Figure 11*).

When the data between sexes were compared, females displayed more neuroprotection than males, but the level of protection was not completely negated by a lack of apoE. WT mice had more mature neurons initially but showed more loss within one week following injury than KO mice did.However, WT mice had more OMP + neurons after 3 and 7 days, suggesting that apoE on some level increased neuroprotection in the OE (*Figure 11*). One important note was that not all neurons expressing OMP may have been alive, since OMP receptors were located on the plasma membrane, fragmented expression may be one indication of cell death. Contrary to what the numerical data suggested, the OE of KO female mice contained fissures lacking OMP expression in the ORN layers 3 dpi, and after one week entire nissl bodies were present in both genotypes, however, some neurons appear fragmented as if already dead. As for male mice, the WT treatments had better cell body definition after 3 days, whereas KO males did not, and what few neurons left after one week for both genotypes were well intact. Neurons expressing OMP 3 weeks post injury were well intact for both males and females. Although apoE did matter as the data advocated for animals 3 weeks after injury, sex had a greater impact on epitheliums overall response to OBX which made the further understanding of estrogen's role in neuroprotection a necessity (*Figure 10*).

3.11OMP: ApoE and estradiol treatment

In order to quantitatively measure thedifference between apoE and estrogen, OMP measurements were compared in mice treated with estradiol in both genotypes. The difference in OMP positive neurons between the two genotypes was greatest for uninjured tissue with estrogen treated WT females,which exuded an average OMP count of 92.38cells/100μm, almost 10 more neurons expressing OMP than in KO treated females (83.06cells/100μm). Treated WT females showed a greater loss of neurons after 3 days however they had a greater number of OMP + neurons (50.19cells/100μm) compared to their KO equivalent (47.19cells/100μm), exactly a 3 neuron difference. The variance of OMP + cells between genotypes after 7 days remained minimal. Both WT and KO mice illustratedan OMP + count near 19 cell/100μm. Three weeks following injury there was a significant difference in the epithelium's response to injury in both genotypes: WT treated females had an average of 9.31 OMP + neurons/100μm and KO mice with 3.44cells/100μm. The data shows that increased levels of estrogen decreased OMP + neuron death in bulbectomized OE, but, estrogen's neuroprotection capabilities were not limited by a lack of apoE (*Figure 10c*) (*Figure 11*).

The next goal was to measure any deleterious effects of losing estrogen or apoE in mice. In every treatment, KO mice consistently displayed fewer OMP + neurons in uninjured tissue than WT mice; this was also true for untreated females. The number of OMP+ neurons was greater in untreated apoE KO females (32.56cells/100μm) than in WT untreated females (30.38cells/100μm) after three days. Not only did KO animals express more OMP + neurons after 3dpi but WT animals had a greater loss in mature neurons in the first 3 days by comparison. The OE displayed about 10 mature neurons for both genotypes after one week. There were almost no mature neurons present in the OE of KO animals three weeks after OBX, whereas WT

untreated females had an average of 4.63cells/100μm (*Figure 11*). Regardless of genotype or treatment there were fewer OMP positive neurons 21dpi than at any other time interval (*Table 2*).

When estrogen treatment with the presence of apoE was compare, the number of mature neurons was lower in KO mice than WT mice in both treated and untreated mice in uninjured tissue making it difficult to argue that the subtle differences between genotypes was a direct response to the existence of apoE. There is thorough OMP expression among the ORN layers in WT animals with and without treatment than in KO animals. KO animals had pockets of non-expression 3 and 7dpi indicating continued ORN death at those times intervals. The 21 dpi data and images illustratedevidence that OMP + neurons continued to die in the weeks following 7dpi in all animals with and without estrogen. Estradiol treated animals consistently have more mature neurons than their untreated counterparts regardless of apoE. The only time apoE yielded a reasonable difference in the number of OMP + neurons was at 21 days post injury in both treatments (*Figure 10c*).

When all treatments and both genotypes were considered , mice without apoE appeared to have more cell death taking place 3 and 7 dpi than WT mice, a biological still shot of continuing ORN death between the four days. The number of actual living OMP positive cells may have been overshadowed by the residual OMP from neurons dying at those time intervals. This data supports the notion that estrogen did have neuroprotective qualities regardless of the presence of apoE and may also aid in the development of mature neurons as seen in the 21dpi data. As the quantity of estrogen in female's increased so seemedthe quality of neuroprotection, however, there appeared to be another factor in males that increased the rate of death because OMP levels in males were overall less than in untreated females. In summary, estrogen increased neuroprotection of mature neurons in both WT and KO animals almost equally, neither genotype

had a substantial recovery ofmature neurons, but WT mice showed a greater chance to recover than KO mice according to the data presented (*Figure 10*) (*Figure 11*).

3.12Bromodeoxyuridine (BrdU) in bulbectomized mice

There were small populations of globose basal cells (GBC) or stem cells that resided adjacent to the basal lamina. When the olfactory epithelium was damaged these cells proliferated and made new olfactory receptor neurons. The DNA of these dividing cells were labeled usingbromodeoxyuridine(BrdU) to allow a quantitative evaluation of neurogenesis over time. The goal of this study was to measure the differences between WT and apoE KO treatment groups for the number of dividing globose basal cells in the OE 0, 3, 7 and 21 days after olfactory bulbectomy.

3.13BrdU: statistical analysis

Once all of the mean BrdU+ count for all mice wascollected a 3 way ANOVA was conducted and the results revealed significant differences between genotypes, all treatments, and all time intervals in the injured OE. Through the contribution of all treatments and time intervals, WT animals had 4.89 dividing neurons/100μm and KO animals only had 3.04 GBCs/100μm illustrating a fundamental dissimilarity between genotypes (*Table 1*). When the genotypes and treatments were pooled to illustrate time as the sole independent factor, the trend seen in both thickness and mature neurons was true for BrdU positive neurons: 3 dpi mice had the largest number of dividing neurons (6.69GBC/100μm), 7 days with the second most with 3.6GBC/100μm, and 21 dpi with the fewest dividing basal cells (1.61GBC/100μm) (*Table 2*). To understand how the OE reacted to injury, measurements acquired of uninjured epithelium are equally as important.

Olfactory epithelium, when uninjured, still exhibits GBC proliferation because ORNs die and are replaced by newly regenerated neurons. For instance: the injured OE of WT animals (1.16GBC/100μm) have a greater mean BrdU positive count than their KO counterparts (.6GBC/100μm), but, BrdU is the only non-significant dependent variable between genotype. The 0 day data for the treatments show females, regardless of genotype, have more dividing neurons then males; however, estrogen does not increase the likelihood of basal cell proliferation in uninjured epithelium (*Table 5*). By comparing the zero day data with the injury data for each treatment, there can be a more inclusive understanding of how apoE may alter basal cell division.

3.14BrdU: ApoE and Sex

First the effect of apoE in BrdU expression was examined in female mice. The olfactory epithelium was constantly dying and regenerating so observing dividing basal cells in uninjured tissue of WT (1.1 cells/100μm) or KO females (.5 cell/100μm) was expected, however, the discrepancy between genotypes at this time interval only raised more questions. After the OB was displaced for 3 days the number of BrdU + cells increased for both genotypes: WT females had a dividing cell count of 8.5cells/100μm and KO females 5.4 cells/100μm. Cell division decreased 7 days post injury for both treatments, WT females displayed a BrdU + cell count of 4.2cells/100μm and KO females had a count of 2.9cells/100μm. Neurogenesis continued to decrease in each genotype 21dpi, however; WT females had more dividing neurons (2.6cells/100μm) than KO females (1.3cell/100μm)(*Figure 12a*) (*Figure 13*). ApoE increased the likelihood of observing neurogenesis in females from 0 to 21 days following injury in female mice.

Next, dividing basal cells were compared among genotypes in male mice. Dividing basal cells remained at their minimal in uninjured tissue for both genotypes but KO males had more dividing neurons than their WT equivalents: WT males presented .4 cells/100μm and KO males' .9cells/100μm. There was a sharp increase in basal cell division in both genotypes after three days; WT males had more cells dividing (4.4cells/100μm) than KO males (3.4cells/100μm). Cell division slowed in both WT and KO males after 7 days: WT males had more BrdU expression (3.5cells/100μm) than KO males (2.8cells/100μm).Three weeks post bulbectomy KO males contained a slightly higher rate of cell division (1.0cell/100μm) than WT males (.7cells/100μm). In the case of the male treatments, the presence of apoE appeared to increase GBC production with-in one week of injury, however, after three weeks any contribution to neurogenesis in male mice that apoE exhibited was lacking according to the data (*Figure 12b*) (*Figure 13*).

When the dividing basal cells were compare between sexes and genotypes, the average female mouse had 2.5 more dividing neurons than males regardless of genotype. This suggested a gender difference in favor of females having more neurogenesis at 3 days following injury, which was not the situation for the initial conditions(*Table 3*)(*Table 6*). In the days following injury the mean difference between genders was 1.52 dividing GBCs, a significant but small difference. Neurogenesis increased dramatically in WT females but only modestly in their KO counterparts 3 days post injury. Neurogenesis slowed after 3 days in all treatment groups but WT males had more dividing basal cells than KO males after 3 days, furthermore they illustrated more BrdU + cells 7dpi than either KO males or females. After 3 weeks WT females contained more neurogenesis than KO females, however, WT males had fewer dividing neurons than KO males. According to the data there was a gender bias in dividing neurons with females having

more overall neurogenesis than males; however, apoE is more useful to the preservation of the olfactory system of female mice than male mice (*Figure13*).

3.15BrdU : ApoE and Estradiol treatment

Our next focus was to determine what impact estradiol treatment has on the rate of neurogenesis among genotypes. The uninjured tissue of WT treated mice contained double the quantity of dividing neurons than the KO mice. There was a substantial increase in basal cell division at 3 dpi in WT treated females (13.7cells/100μm) and a modest increase by comparison in KO treated female mice (7.1cells/100μm). One week after injury the number of dividing basal cells in WT treated females reduced by 8.1cells/100μm (5.6cells/100μm), while KO treated females had a 3.4 cell/100μm decrease with a BrdU + cell count of 37. WT treated females had over double (3.4 cells/100μm) the number of dividing basal cells at 21 dpi than KO treated females (1.5cells/100μm). Without injury, treated WT mice had more neurogenesis than their KO counterparts which remained to be the case at every time interval proceeding OBX. The large genotypic differences in neurogenesis for treated mice were much larger than witnessed in untreated mice.

Next BrdU expression was compared in mice without estrogen or apoE in the days following injury. The differences between genotypes in uninjured tissuecan be compared to other treatments at this time interval: WT non-treated mice had a BrdU positive cell count of 1.2 cells/100μm and KO untreated females have .4 cell/ 100μm. WT untreated animals (7 cells/100μm) had more BrdU + cells than their KO complement (4 cells/100μm). One week post OBX untreated WT females had 3.8cells/100μm compared to the 2.3cells/100μm counted for KO untreated mice. Three weeks following OBX both genotypes had similar basal cell division

rates: WT untreated mice had 1.3cells/100μm and KO untreated mice had 1.1cells/100μm(*Figure 12c*) (*Figure 13*).The OE of untreated mice consistently had more neurogenesis occurring when apoE is present.

The possibility that estrogen increased neurogenesis in uninjured tissue may be nullified by the data showing a .7cell/100μm difference between untreated and treated WT mice in favor of untreated mice. Estrogen may not have impacted the initial BrdU count but apoE did according to the zero day data. Contrary to the zero day statistics, injured epithelium produced more dividing globose basal cells when estradiol was present than in animals without estradiol. Treated mice had far more dividing neurons than their untreated counterpart after 3 days; however, treated KO females had statistically the same number of BrdU positive neurons as untreated WT mice: the same was true at 7 and 21 days following injury. Untreated KO mice not only had fewer dividing neurons than their treated equivalent but also fewer compared to WT untreated mice. The presence of estrogen increased neurogenesis in animals with apoE but lostsome of its affect in mice lacking the lipid transport protein (*Figure 13*).

When neurogenesis was compared in all treatment groups a positive correlation was observed between estrogen levels and number of dividing neurons detected. Mice with above physiological levels of estrogen had more dividing neurons after injury than mice with just physiological estrogen levels (Female mice). Mice with no estrogen at all showed less neurogenesis than female mice. Lastly male mice had the fewest average number of dividing basal cells. The trend observed in WT mice is also true for apoE KO mice; the difference was fewer dividing neurons(*Figure 12*) (*Figure 13*).

Discussion

4.1 Unilateral Olfactory Bulbectomy (OBX)

The unilateral olfactory bulbectomy is an effective method for understanding neuron death because olfactory receptor neurons(ORNs) die semi-synchronously in a time dependent fashion. Previous works have shown nearly all ORNS die in the ipsilateral OE following the removal of its target (Costanzo &Graziade, 1983; Carson et al., 2005). In the days following olfactory bulbectomy (OBX), a decrease in mature neurons occurs while there is an increase in immature neurons on cytokinesis of globose basal cells (GBCs) (Costanzo &Graziadei, 1983 and Swartz-Levey et al., 1991). The initial GBC proliferation rates were high while the OE thickness decreased. The life span of the newly generated immature neurons is shortened due to the removal of its target (Schwob et al., 1991; Carr &Farbman, 1992;Carr &Farbman, 1993). Bulbectomy does not alter the sustentacular cells (Sus) or supporting basal cells (Holcomb et al., 1995). In fact,Sus cells along with macrophages function to clear cellular debris in both acute and chronic periods of cell death (Suzuki et al., 1996).

Theolfactory bulb (OB) contains axons of different length that travel along the olfactory tract fibers until their axon terminals enter a designated glomeruli. By ablating the OB, axons of mature neurons are severed at various locations not just the glomeruli but along the nerve fiber tract surrounding it (*Figure 4*). This may or may not cause enough insult to initially disrupt mitochondrial function in these axons. It is difficult to tell if apoptosis or necrosis is the cause of cell death because this type of insult varies among individual ORNs. Blebbing and fragmentation are the prognosis of apoptosis whereas necrotic cells swell and burst: both occur in this experiment and have a similar appearance under a microscope. For the sake continuity "cell

death" is the appropriate term. OBX not only impacts locale and intensity of apoE in the OE, but also thickness, olfactory marker protein (OMP) expression and GBC division, which were measured for various treatment groups.

4.2 ApoE expression following OBX

The first goal of this research was to understand how apoE expression may be altered by sex and estrogen treatment. ApoE is as a lipid transport protein that functions to aid normal sustentacular cell maintenance of ORNs such as the recycling of membrane degenerative byproducts (phospholipids) that then can be used for neurite growth in developing neurons. ApoE is not typically expressed robustly in the ORN layers in uninjured tissue suggesting that it is synthesized elsewhere: further evidence of its cellular reprocessing capabilities (Grainger et al., 2004; Nathan et al., 2007). The lamina propria has low levels of apoE expression in olfactory fascicles and higher levels in olfactory ensheathing glia (OEG) providing additional support that apoE is not synthesized by ORNs but rather glia cells surrounding the nerve fascicles. Provided the above information, animals knocked out for apoE were expected to have deficits in obtaining enough cholesterol for proper maintenance.

4.3 ApoE and Sex

ApoE reacted to tissue damage differently among sexes.The lack of apoE expression in the OE of WT female male and male mice in uninjured OE, compared to the days following injury, suggested that apoE is mostly present in the OE when needed. Since apoE transports lipids to regenerating tissue, 3 days post injury (dpi), apoE was observed along the basal lamina where globose basal cells were dividing and in the ORN layers where mature olfactory neurons were dying. However, male mice had the most apoE expression 3 dpi in the OE most likely

because male mice exhibit rapid neuronal loss once the olfactory bulb was removed. .Despite the further loss of thickness in female mice 7 days post injury, there was a decrease in apoE expression in the ORN layers and directly above the basal lamina, suggesting a decrease in both ORN death and GBC proliferation. Despite the loss of apoE in the OE proper, 7dpi results illustrated that there was a slight increase in apoE in the lamina propria, compared to 3 dpi, yielding evidence of apoE transportation into/out of the OE. Contrary, apoE expression in male mice remained visible in mice 7 dpi with a majority of OSNs dead. ApoE was most prevalent in the lamina propria of female mice 3 weeks post injury which was likely due to the need for more apoE recycling or providing lipids for axon outgrowth. The heavy staining throughout the OE in male mice 3 weeks post injury suggest that there was also regenerating and dying neurons. The sex of the animal appears to alter the rate of ORN death thereby altering the level of apoE expression at each time interval. .

4.4 ApoE and Estradiol treatment

This part of the experiment attempts to gage the differences of apoE expression between female's mice with and without estrogen. Resembling untreated female mice, treated females had a lower quantity of apoE in uninjured epithelium but showed an influx after 3 days juxtaposition to the basal lamina with little expression in the ORNs superior to the lamina: evidence that there are more dividing basal cells requiring cellular components than there are necrotizing receptor neurons. The LP has increased levels of apoE especially around the olfactory ensheathing glia indicating lipid transport in or out of the cell: between the decrease in thickness and increase in proliferation the flux may be bidirectional.Contrart to treated mice, the OE of untreated mice varied after 3 day. Iimmunofluorescence increased in the ORN layer then along the basal lamina suggesting more death then proliferation: there was also a large increase of apoE in the LP

indicating component reprocessing. In the 4 days following 3dpi, treated females acquired more apoE that is present along the basal lamina and above it which may have been caused by rapid ORN death and continued proliferation. In treated females the loss of apoE in the LP 7dpi coincided with the level of expression in the OE proper, because higher levels of apoE in the OE proper. In untreated females apoE loss in the LP occurred after 7 days with the majority of expression located in the OE proper. The level of apoE expression in the OE proper of treated females21dpi was unique only to treated females where there appeared to be higher levels of expression surrounding developing neurons (non-GBC), and in regions close to the OE cilia where mature neurons are typically found. There is ORN outgrowth at this time which requires apoE as seen close to the basal lamina; perhaps the expression closer to the cilia indicates further neurons death, but the increase in apoE in the LP does illustrate further need for lipid transportation. The OE of untreated female mice 21dpi had robust expression in OEGs of the LP and in the OE proper in the cells closest the lamina probably due to neurite outgrowth.

When sex and estrogen are examined together, a pattern becomes visible. Uninjured epithelium of treated and untreated animals contain similar levels of apoE expression, conversely, 3 days following injury yields a large increase in apoE expression in untreated mice compared to their treated The potential role for apoE produced by sustentacular cells in uninjured OE is to recycle debris (Nathan et al., 2007) by way of phagocytosis of macrophages (Fagan et al., 1998).The allocation of apoE in the OE is dependent on the tissue's response to injury. Mice with rapid loss of olfactory receptor neurons, male and untreated female mice, require more apoE than treated or OVX shammed females earlier on in the recovery process. Upon 7 days following injury apoE is vividly expressed in the OE proper of all treatments because this is where death and regeneration intersect. These results suggest there are proliferating basal cells, further dying

mature neurons and some developing neurons all of which increase apoE concentrations. The location of apoE in mice 21dpi is primarily isolated to developing neurons striving for maturity, and possibly expressed in dying neurons. What is unique about apoE immunohistochemistry in the OE is that it is a picture of the underlying mechanisms for which an organism salvages cellular debris, making it difficult to quantify due to its peregrine nature.

4.5Olfactory epithelium (OE) thickness

Olfactory receptor neurons are idiosyncratic to stimuli which allows for diversity in scent perception. The average lifespan of an individual ORN is 30 to 90 days and may be compromised by physical or chemical stressors (Mackay &Kittel, 1991). When massive cell death occurs in the OE by artificial means such as TX lesioning or olfactory bulbectomy (OBX), the OE behaves more like a community where GBCs start dividing and sustentacular cells along with macrophages aid in reducing cellular debris with the help of lipid transport proteins (apoE). Histologically, the simplest determinant of OE changes caused by injury is determined by measuring OE thickness.

4.6OE thickness statistical analysis

The statistics for the overall difference between uninjured OE thickness among WT and KO mice showed a statistically significant 2 micrometer difference and a testament to how a lack of apoE may alter the anatomy of the OE. Since the role of apoE is the reprocessing and transporting of lipids, a lack of such protein inhibits cellular components from maintaining the integrity of the OE at full potential. The initial thickness among treatments were close to 80 micrometers with little variance. Of the three dependent variables measured only thickness and OMP had a significant relationship among genotypes while BrdU was on the cusp of

significance. The data further suggested the need for apoE to fully maintain the epithelium's thickness.

The statistics for injured OE thickness also illustrated a significantly different among genotypes for all treatments; however there were also significant differences among all time intervals in all treatments. Females treated with estrogen had the thickest overall epithelium following injury, then OVX shams, untreated females and finally male mice. The 3 way ANOVA for injured data has a high model fit with approximately 90% of variation explained because all interactions are significant with the exception of BrdU for all three response variables. The primary aim of this discussion is to find a correlation between OE thickness as it relates to apoE, sex and estrogen.

4.7OE thickness: ApoE and Sex

The same pattern observed between WT females and males was also observed in their KO counterparts, the main difference was a thinner average OE thickness. Males had a greater loss of thickness 3 days after insult potentially becauseof greater physiological levels of estrogen: which has been proven to have neuroprotection properties. The thickness dropped for both sexes after one week; however, estrogen continued to show some impact on the overall thickness in WT females. On average, knockout females 7dpi had a much thinner OE thickness than WT females which may appear to contradict estrogen's contribution to the preservation of thickness, but it doesn't. Estrogen has been proven to increase basal cell proliferation in the OE after artificial injury (TX lesion) and increase neuron outgrowth in vivo (histology) and in vivo (cell culture). However, recovery appears to be stunted when the animal lacks the protein (apoE) responsible for transporting cellular components to dividing and growing neurons. Further

evidence of this is observed in WT versus KO males 21dpi where WT males show more resiliency.

4.8OE thickness: ApoE and Estradiol treatment

To test whether estrogen enables this level of neuroprotection, females with and without estradiol treatment were studied. Contradictory to male and female data, KO mice had a thicker OE than their WT companions in both treated and untreated females 3dpi. In treated mice, higher levels of estrogen allowed for neurons to maintain life and a lack of apoE did not appear to affect this. This may also be true for untreated mice considering the average difference between the two is greater in untreated then treated mice. After 3 days the OE of treated females is over 10 micrometers thicker than their untreated complement indicating a preservation of ORNs. Estrogen treated animals consistently had thicker OE in the weeks following OBX because of its ability to accelerate the process of neurogenesis and neurite outgrowth.

If the thickness of all treatments are observed one anomaly becomes clear, the OE thickness of untreated females was greater than that of males 3dpi. The rate at which ORN death occurs in male mice appears to be much greater 3 and 7dpi than in untreated female mice, an incongruity because males do produce low levels of estrogen and testosterone may be converted to estrogen by aromatase. Testosterone, a well-known androgen, is proven to cause neurological cell death when levels are increased well above the physiological norm. One mechanism proposed that elevated testosterone levels has deleterious effects on neurons in the CNS by increasing calcium levels and causing apoptosis(Estrada et al., 2006). Whether neuron death is accelerated by physiological levels of testosterone or not is beyond the scope of this project. Whatever alterations the physiology of male mice had on OE thickness appears to be time

dependent because the average OE thickness of WT males is within error of untreated females 21dpi.

4.9Olfactory marker protein (OMP) in bulbectomized mice

There is little contrast between OE thickness and mature neuron count as expected, since the primary caste of olfactory receptor neurons that die as a result of OBX are OMP positive. Olfactory marker protein is expressed primarily in functioning (mature) olfactory receptor neurons and expression starts when the axon of the ORN innervates the olfactory bulb (E. Danciger et al., 1989). A more recent study has found that OMP may have a role in odor detection and possibly signal transduction (Carr et al., 1998). These two studies along with others that involve target ablation of the OE consistently show a decrease of mature neurons with little to no recovery over time. This is why there is only a decrease in mature olfactory neurons over time.

On average the quantity of mature neurons in uninjured OE was much greater in WT mice then KO if compared to the differences in OE thickness. This suggests that ORN maturity is less likely observed in KO mice. Statistical analyses demonstrated that in injured animals, apoE improves the likelihood of prolonged mature neurons survival in the days and weeks following OBX. Naturally there will be fewer reported OMP positive neurons 3dpi in KO mice because the average OMP count initially was lower for that genotype. Previous studies have provided evidence that a lack of apoE or apoE 4 (Alzheimer's related isoform) may be detrimental to the life of neurons in the CNS (Masliah et al., 1995). ApoE has some neuroprotective qualities especially with regard to very low density lipoprotein receptors (VLDLR and apoer2). These receptors have multiple ligands including Reelin and apoE. When bound to Reelin these receptors couple with NMDA via PSD-95 causing activation of this

calcium channel thereby increasing plasticity. These receptors are believed to aid in endocytosis of lipidated apoE; however, it is uncertain how unbound apoE reacts with these receptors (Holtzman et al., 2012). These results suggest that apoE does increase synaptic plasticity in neurons approaching retrograde death. Through which mechanism is unclear.

4.10OMP: ApoE and Sex

When sexes were compared, apoE knockout or not, females have a greater number of mature neurons than their male counterparts. The average OMP count following OBX is largest 3days after: one possibility is estrogen and since physiological levels are much larger in females than in males so might the level of neuroprotection. As discussed regarding thickness, testosterone may alter the OE just enough to accelerate apoptosis. In one using male mice, 4-6 months of age, apoptosis occured in the OE following OBX. Using terminal deoxynucleotidyltransferasedUTP nick end labeling or (TUNEL assay which labels degrading DNA of dying cells) was expressed mostly in male mice between 1-3 days following OBX. This coincides with the results of this project because mature neurons in males 3dpi appeared fragmented and possibly in the process of being salvaged, which was not observed in females 3dpi (Holcomb et al., 1995). There is more fragmentation of mature neurons in female mice 7dpi, evidence of delayed apoptosis in female mice. Females had more mature neurons 21dpi than males; however in both sexes the nissl bodies appeared fully intact with the exception of dendritic and/or axonal extensions. An ORN lacking a dendritic extension and/or axonal projection is non-functioning. Once such a rigorous injury occurs there is a massive push to restore and repair the olfactory system and after 21days more resources may be appropriated to restore fallen neurons then are to recycle them as seen in almost all treatments of both genotypes.

With regards to females having more OMP positive neurons then males 21dpi, separate observation with estrogen is required.

4.11OMP: ApoE and Estradiol treatment

To test estrogen's effect on neuroprotection a similar experiment with OMP was set up using female mice with and without estradiol. The level of OMP in the uninjured OE expression was not altered in response to estradiol treatment as it was in mice lacking apoE. Females treated with estradiol showed less loss of mature neurons 3dpi than untreated animals. The number of mature ORNs became fewer in both treatments but estradiol treated mice had more than their untreated counterparts confirming that estrogen prolongs the life of a dying neuron. As time progressed the number of OMP positive neurons in the OE remained high in treated compared to untreated animals. Data for 21dpi revealed that treated animals maintained the life span of mature neurons but these neurons appeared to be non-functioning like those observed in male and female mice. There are several proposed mechanisms for which estrogen may increase the life span of a neuron programmed to die.

Among the proposed mechanism that describe why estrogen has neuroprotective properties an increase of blood flow in treated animals and in animals with normal physiological estrogen levels was found for ischemic brain injury (Pelligrino et al., 1998). Another proposal deals with intercellular signaling and found that estrogen activates mitogen-activated protein kinase (MAPK) signaling pathways via estrogen receptors allowing the facilitation neuroprotection (Watters et al., 1997; Singh et al., 1999; Singer et al., 1999). A second intracellular modification caused by estrogen is the increase of anti-apoptotic protein known as Bcl-xL inhibiting caspase mediated proteolysis (CJ Pike, 1999). The last possibility that seems plausible to occur in the OE and also coincides with the results found from male treatment

groups is the flow of calcium: estradiol inhibits the release of calcium from intracellular storage as well as alters the influx of calcium into the neurons (Chen et al., 2001). Estrogen is believed tpalter transcription of multiple proteins and perhaps all of these are reasonable possibilities for why estrogen increases plasticity in olfactory receptor neurons.

4.12Bromdeoxyuridine (BrdU) expression following OBX

Bromodeoxyuridine is a tyrosine analog that binds to DNA during the S phase: phase in the cell cycle where DNA replicates and base pairs are exposed. BrdU injections were administered 24 hours prior to sacrifice allowing a possible 1 to 2 cell cycles to occur. This explains the differences in brightness, because two sets of DNA in the S-phase bind to more BrdU causing an increase of immunofluorescence. The level of fluorescence is dependent on when the cell entered the S phase and the amount of time it takes for complete cytokinesis to occur and previous research has shown that regeneration occurs without the presence of apoE (Popko et al., 1993).

4.13BrdU statistical analysis

When the statistical analysis was observed for the data of uninjured OE of mice 3dpi presents no significant correlation between genotype, treatment and both combined. This is because GBC proliferation only transpires to replace dead ORNs a more random occurrence in the uninjured OE. It should be noted however that the differences between genotypes are virtually significant. ANOVA analysis presents much more randomness in uninjured OE than in injured.

All response variables in the injured OE are significant, except between all three independent variables because the number of dividing neurons per 100 micrometers is not a large figure in many of the treatments 7 and 21 dpi because data points fall numerically close. For

instance both WT females and untreated females had an average BrdU count of 1.3cells/100μm 21dpi. Also standard errors were relatively greater among dividing neurons than any other dependent variable. One example of this was untreated KO females which had an average BrdU count of 4cells/100μm but have a standard error of 1.4. These subtle differences do not repudiate all other significant correlations observed in dividing neurons.

Wild type animals had more neurons expressing BrdU than KO animals, a difference deemed significant by a 3-way ANOVA. The overall difference may not be arithmetically large but a lack of apoE does limit BrdU expression. Previous studies have proved that a lack of apoE can slow regeneration by decreasing the rate at which basal cells divide. In some instances, where females and untreated females KO animals had brighter BrdU expression, compared to their WT counterparts, indicatedBrdU bound to two sets of DNA (Nathan et al., 2001). Mice with apoE receive lipids for plasma membrane construction of the newly divided neuron and any interruption of that process (apoE KO) will slow down advancement toward cytokinesis.

4.14BrdU: ApoE and Sex

Comparing the data between genotypes illustrates more globose cell proliferation in mice with apoE than those without. Also when the sex of the animal is considered, the number of proliferating basal cells at in uninjured OE lacks a significant difference between the sexes. However, WT females had almost twice as many dividing basal cells 3 days post bulbectomy than the males. One week after injury the number of BrdU cells were still elevated for both males and females, but the number declined in the duration of four days. After 21 days the quantity of dividing neurons reduced to pre-injury values in WT males, but was still elevated in WT females. Overall WT females had more BrdU expression following unilateral OBX than did males. A previous study observing neuronal proliferation in the dentate gyrus of both male and

female rats found a ~ 45% increase in BrdU neurons in females compared to males. Not only does cell proliferation increase in females but even greater quantities of BrdU positive neurons observed in females during proestrus when estrogen is physiologically high in females(PatimaTanapat, 1999). Pinpointing estrogen as the contributor to increased cell proliferation post OBX.

4.15BrdU: ApoE andEstradiol treatment

When animals with estrogen were compared to those without, estradiol treated females on average had more dividing neurons than OVX untreated females. Three days following injury the number of proliferating neurons increased substantially for both treated and untreated females but treated has the greatest number of dividing neurons. Although the number of dividing neurons may decrease with time mice with estradiol treatment consistently have more dividing neurons than untreated animals. The expected time interval in which peak GBC proliferation has been observed is at 5dpi, which is not a data point in this study (Schwartz et al., 1991). If peak proliferation occurs after 5 days, then these results may not illustrate the full scope of proliferation among treatments. However, mice with estrogen do have more basal cell proliferation and the mechanism that sparks this phenomenon is still unknown but a few theories have been proposed.

It is believed that OBX does not induce GBC proliferation like it does in peripheral lesioning like TX lesioned animals (Roskams et al., 1998). Macrophage recruitment in the OE is activated to phagocytize apoptotic OSNs and release chemokines and growth factors (Kwong et al., 2004). Leukemia inhibitory factor is one such chemokine that is released by macrophages following ORN apoptosis and is responsible for inciting neurogenesis in bulbectomized mice

(Borders et al., 2007). One studied found that uterine LIF is a cytokine that's gene is regulated by estrogen in mice. LIF is very important to the reproductive system in mice and hamsters (Ding et al., 2008). The finding from another study suggests that physiological levels of estrogen increase macrophage activation rates (Routely et al., 2009). Contrary to the alterations of estrogen, testosterone limits immune responses and chemokine production (Rettew et al., 2008). Estrogen may increase or testosterone decreases the rate at which macrophages are activated and/or quantity of LIF released, the mechanism in which is currently unknown.

Conclusion

A synergistic look at the results suggests with that estrogen increases the life span of mature olfactory receptor neurons and the rate at which basal cell division occurs. Although the statistical analysis suggests that apoE increases neuron plasticity, which coincides with the results from previous studies. Previous research from Dr. Britto Nathan's lab illustrates the importance of apoE following TX lesioning of the OE. The data suggests that apoE is required for full recovery of OE in a time dependent fashion. This may possibly explain some of the discrepancy when dealing with a peripheral nervous system like the olfactory system. The OE is subject to outside stimuli and randomness when dealing with baseline values because if there is increased ORN death in apoE KO mice with an increased timescale of recovery, then there are greater chances of observing a thinner OE with less OMP positive neurons. In contrary to the variability of apoE in the OE, estrogen does increases ORN plasticity.

The mechanism for which estrogen increases the neuroplasticity of neurons in the brain mainly involves the hormones receptor. There are two well-understood estrogen receptors deemed estrogen receptor α (ERα) and estrogen receptor β (ERβ). They have homology in

various regions: a ligand binding domain that is involved in nuclear localization, N-terminal containing a region that interacts with transcription machinery, and 2 zinc finger that bind DNA. The "classical" approach for estrogen's regulation of a neuron, revolves around a genomic contrivance that binds to nuclear transcription factors thereby manipulating gene expression. Upon estrogen binding to its receptor homo- or heterodimers formation of these receptors may proceed. They can then bond to estrogen response elements (EREs) in DNA to promote gene expression. The reason for increased research on 1-β-Estradiol is for its ability to stimulate estrogen receptor mediated transcription, more so than 17-α-estradiol. ERα and ER β have shown to reside in the axons and dendrites in some neurons and glia in the hippocampus. With regards to the hippocampus, there have been a number of electrophysiological events recorded following an increase in estrogen (Woolley, 2007).

Whenever Alzheimer's disease and other dementia related diseases are the topic of research, it is import to discuss the center for memory formation: the hippocampus. The hippocampus, part of the limbic system, is well studied in Alzheimer's disease for it is the center of short and long term memory. The pyramidal cell layer of the hippocampus is divided into 3 regions: CA1, CA2, and CA3. Neuron connections exist between CA1 and CA3 neurons at a location called *Schaffer Collaterals.* There are also feedback loops that feed back into the CA3 region and multiple of other interneuronal connection that extend beyond these regions. However, much emphasis is on CA1 and CA3 region because of their interconnectivity and ability to modulate each other in a systematic way. Glutamate signal transduction is one of the major mechanisms for signal transduction among these neurons (**The Synaptic Organization of the Brain, 4th edition**).

The cells of the hippocampus rely on a phenomenon called long-term (LTP) potentiation which is what allows for memory storage. Increasing the estradiol concentration s in the hippocampus increases high frequency trains which increase LTP readings. This suggests that estrogen allows for an increase in female memory, just so long as there estrogen levels remain. This may be one reason women may be more susceptible to Alzheimer's disease following menopause.

According to the website alz.org Alzheimer's disease is more prevalent in females because they simply live longer, this may be a testament to the importance of estrogen in the life of a female. These results certainly suggest short term neuroprotective properties maintained by estrogen. Either through increased proliferation or decreased ORN death estrogen slows the progression of cell death whether the mechanism is through acute electrophysiological events or by an overhaul of genetic transcription. Since estrogen increases the capacity for LTP in the CA1 region of the hippocampus, improved memory may be expected. If there is any confusion about this, ask a married a man such as myself, how often his wife forgets.

Figures

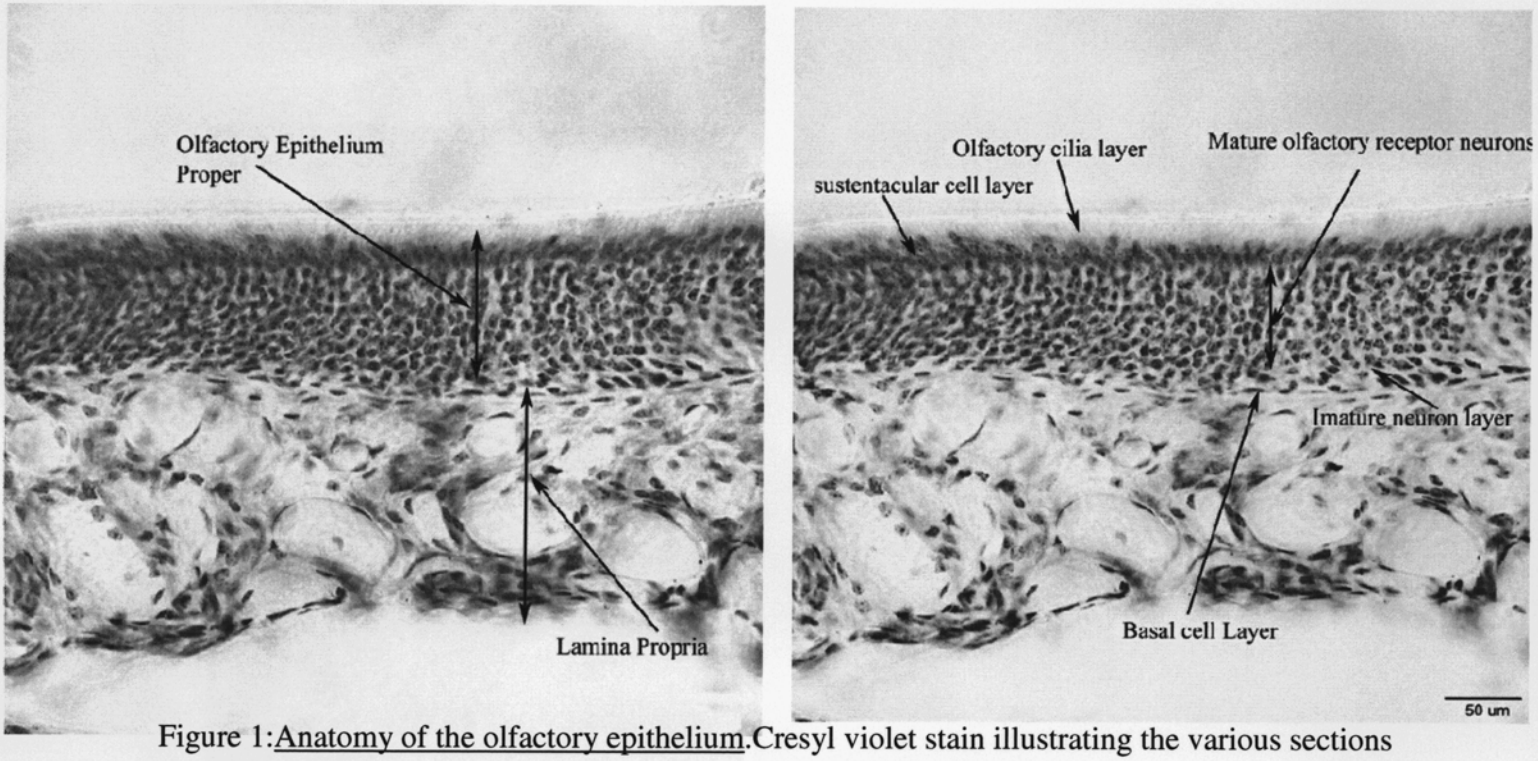
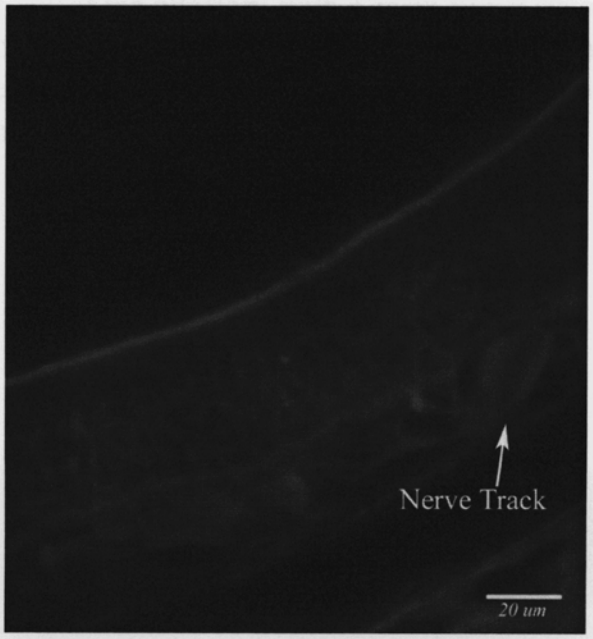


Figure 1:Anatomy of the olfactory epithelium.Cresyl violet stain illustrating the various sections (Left) and subsections (right) of the mammalian olfactory epithelium.



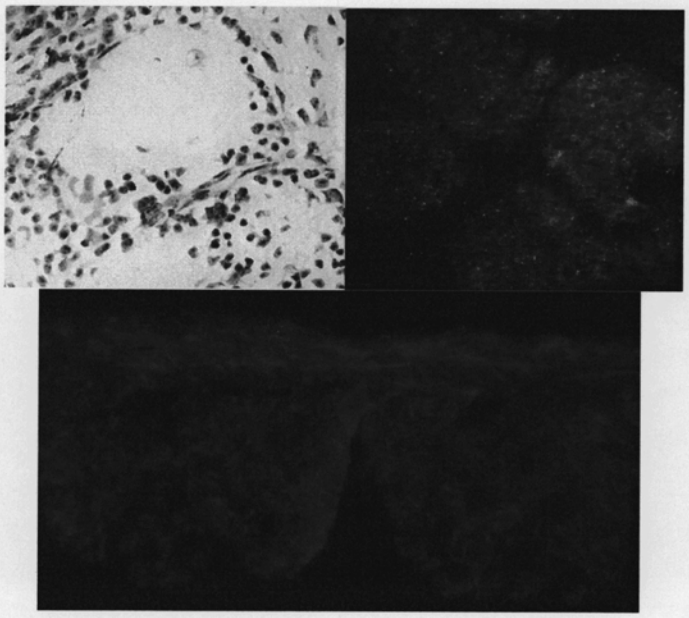


Figure 4: Immunohistochemistry of the olfactory bulb. **Top left** is a closer look at the Glomeruli in the OB using a CV stain. **Top Right** is a picture of the OB stained for synaptophysin (stains areas of synapsis) which illustrates areas of signal transduction. Each dot represents a synapsis. **Bottom**, also a glomeruli, but stained with OMP, illustrating the presence of mature neuron axons of ORNs protruding along olfactory nerve fascicles into the glomeruli.

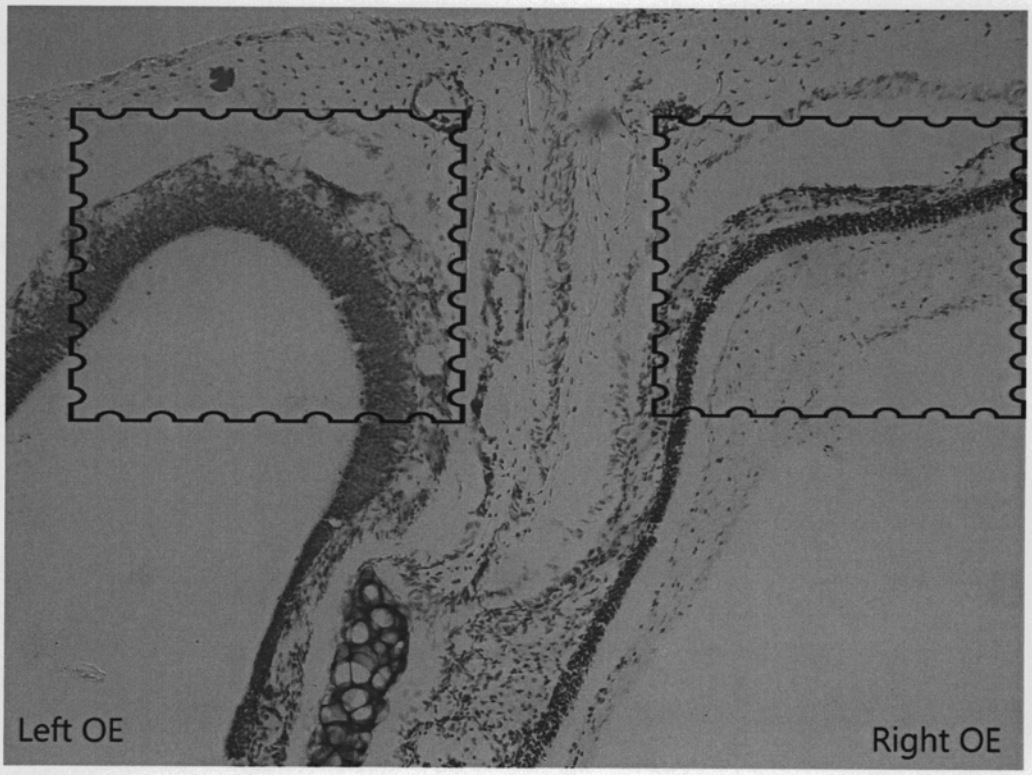


Figure 5: The epithelium following unilateral olfactory bulbectomy. The **Left OE** is the uninjured epithelium which maintained near physiological conditions since the left bulb remains fully intact. The **Right OE** has a large loss of receptor neurons cause by target ablation (OBX). The Boxes indicate where in the OE pictures/measurements were taken.

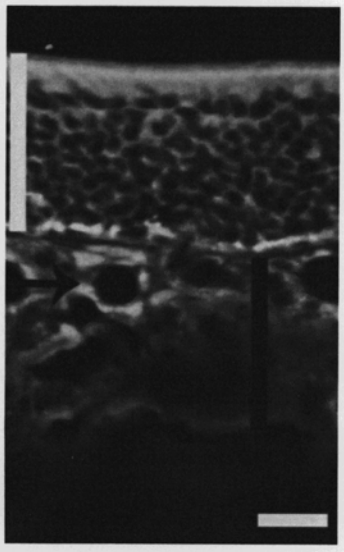


Figure 6:ApoE expression in the olfactory epithelium. Illustrates where apoE is generally expressed in the olfactory epithelium. The bright immunofluorescence along the basal lamina is most common. The White vertical bar is in the OE proper, the black vertical bar extends through the lamina propria, and the black arrow points at the olfactory ensheathing glia (OEG) surrounding the blood vessel. Scale bar= 50μm.

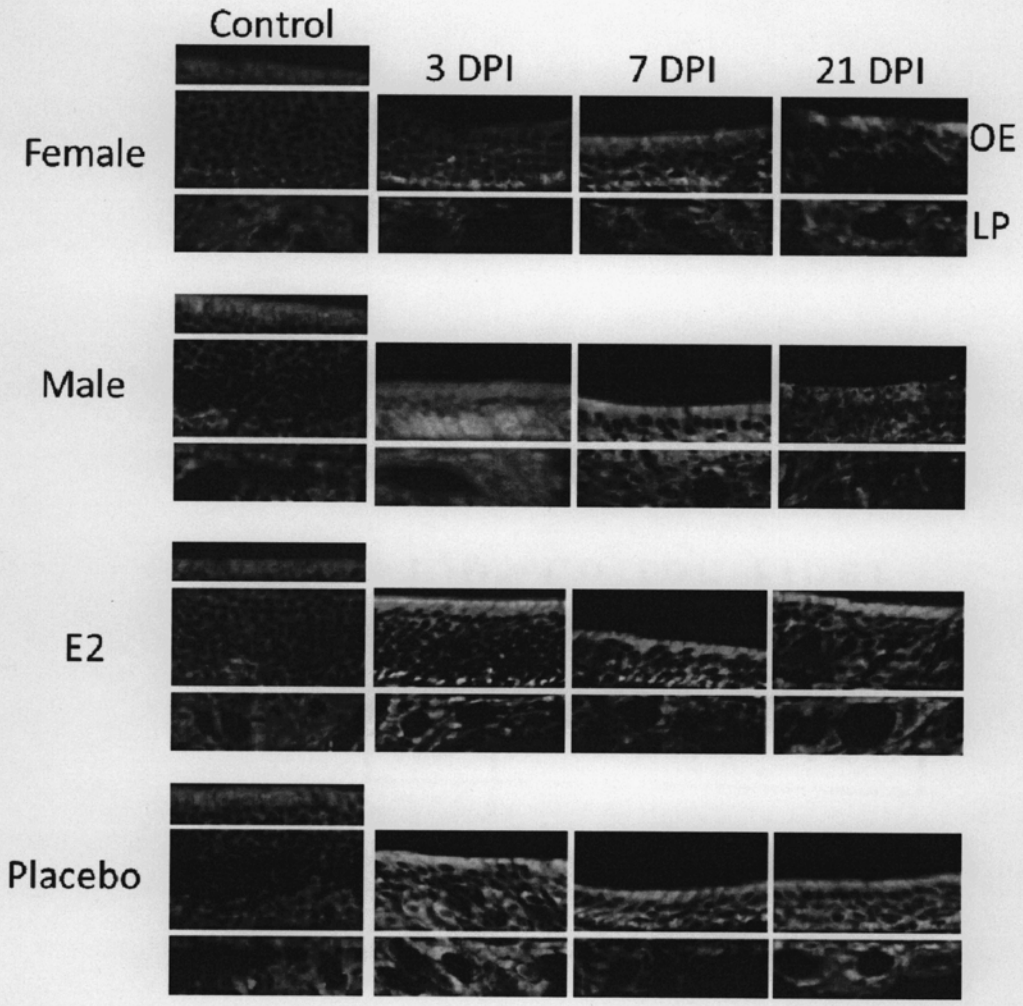


Figure 7: ApoE expression over time in all treatment groups.ApoE in WT treatment groups.

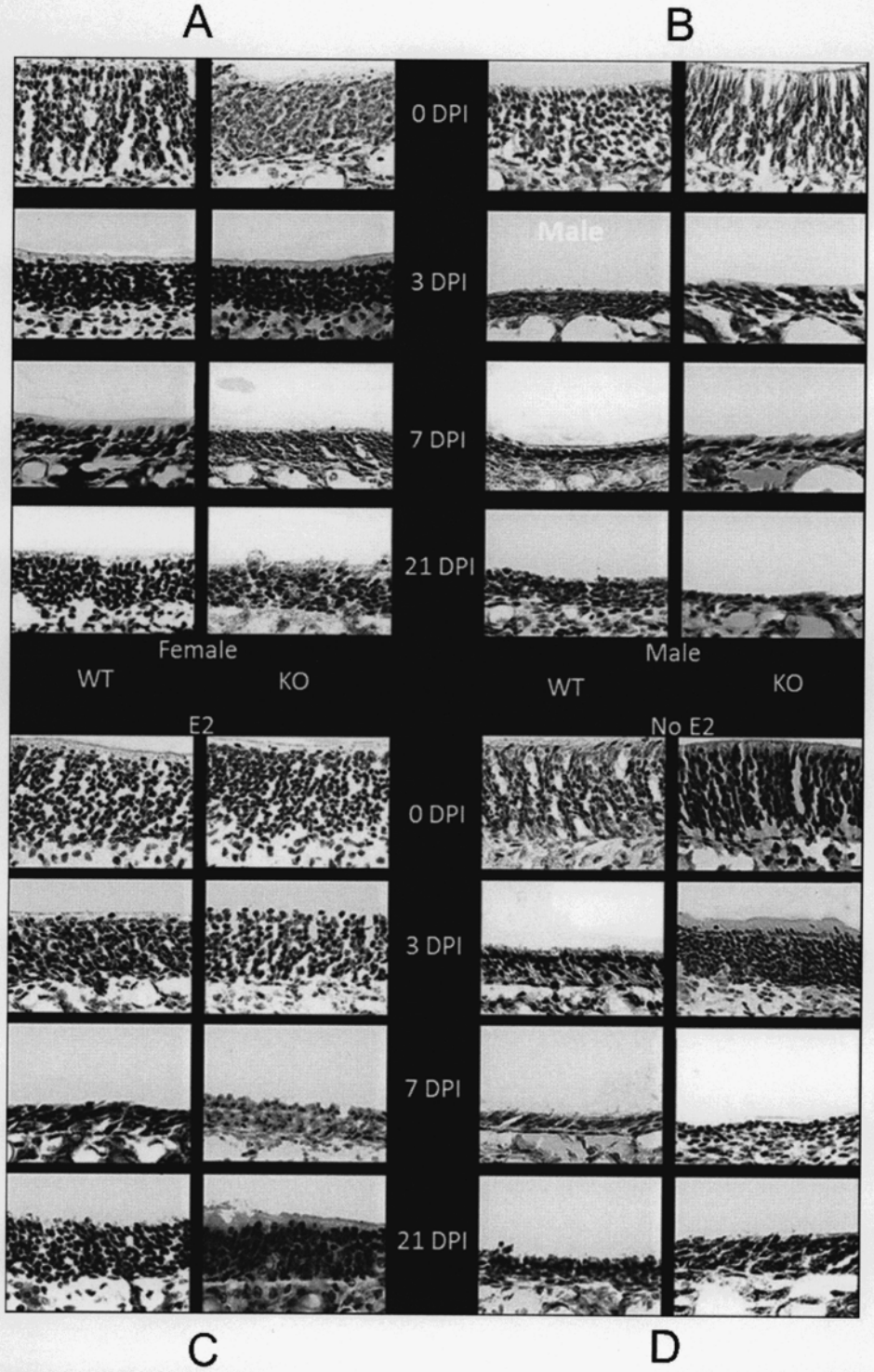

A
B
0 DPI
3 DPI
7 DPI
21 DPI
Male
Female
WT
KO
WT
KO
E2
No E2
0 DPI
3 DPI
7 DPI
21 DPI
C
D

Figure 8: OE Thickness over time in all treatment groups.An extensive look at the OE thickness in all treatments across all time intervals using a CV stain.

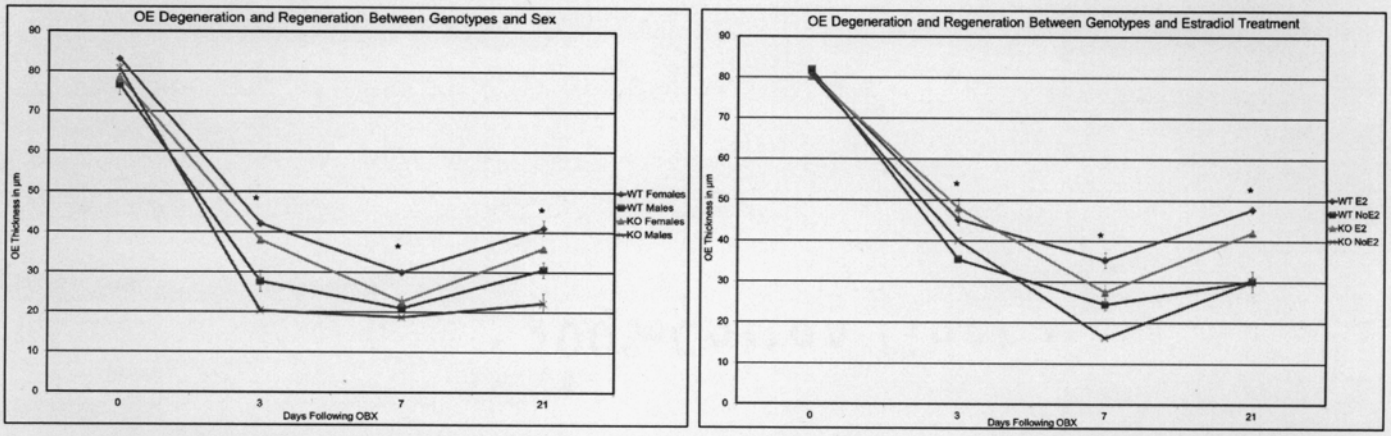


Figure 9: Comparing OE thickness among all treatments and genotypes over time: Line graphs illustrating the epitelium's thickness over a 21 day time course. The graph on the **left** represents male versus female mice of both genotypes whereas the graph on the **right** illustrates estradiol treated versus untreated mice of both genotypes.Astrisks represent sigficant data at each time interval.

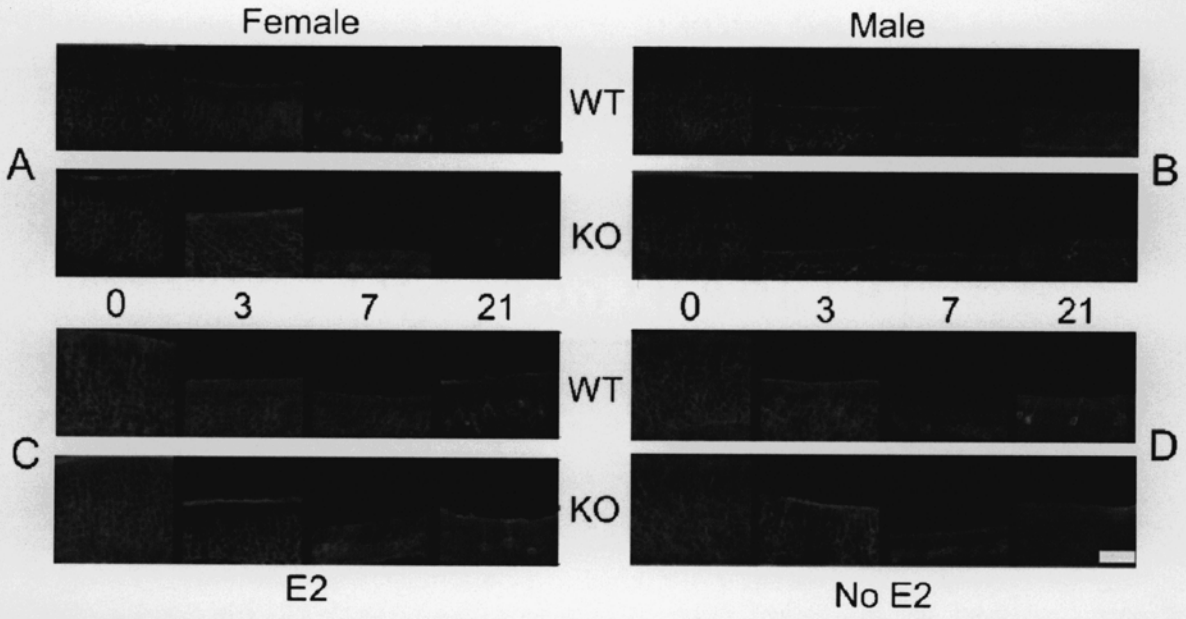


Figure 10:<u>OMP expression over time in all treatment groups</u>. A visual representation of OMP expression in the OE of each treatment at each time interval. C Scale bar= 30μm

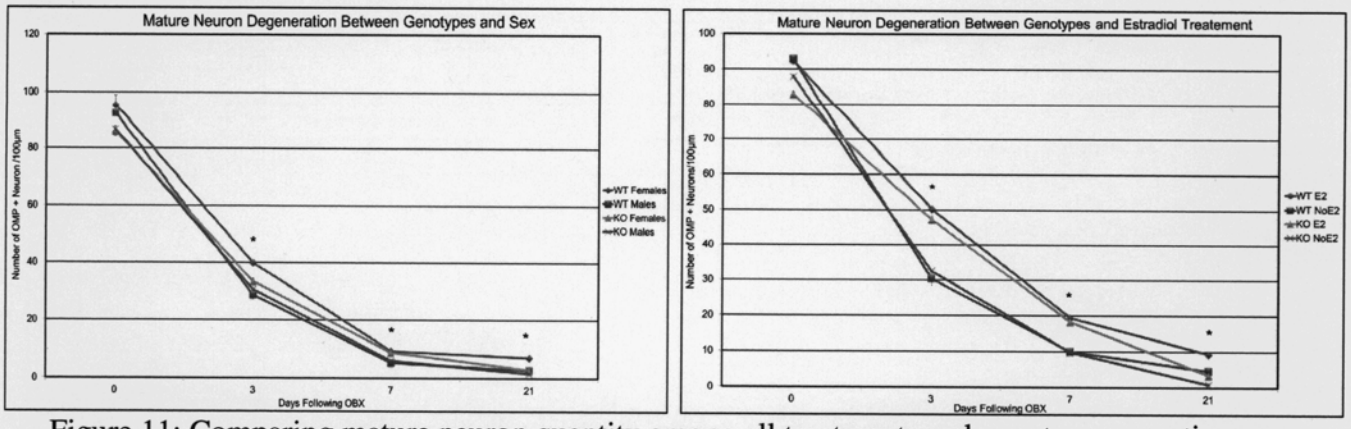


Figure 11: <u>Comparing mature neuron quantity among all treatments and genotypes over time:</u> Line graphs illustrating the changes in the number of mature ORNs in male versus female **Left** and treated versus untreated **Right.** Astrisks represent sigficant data at each time interval.

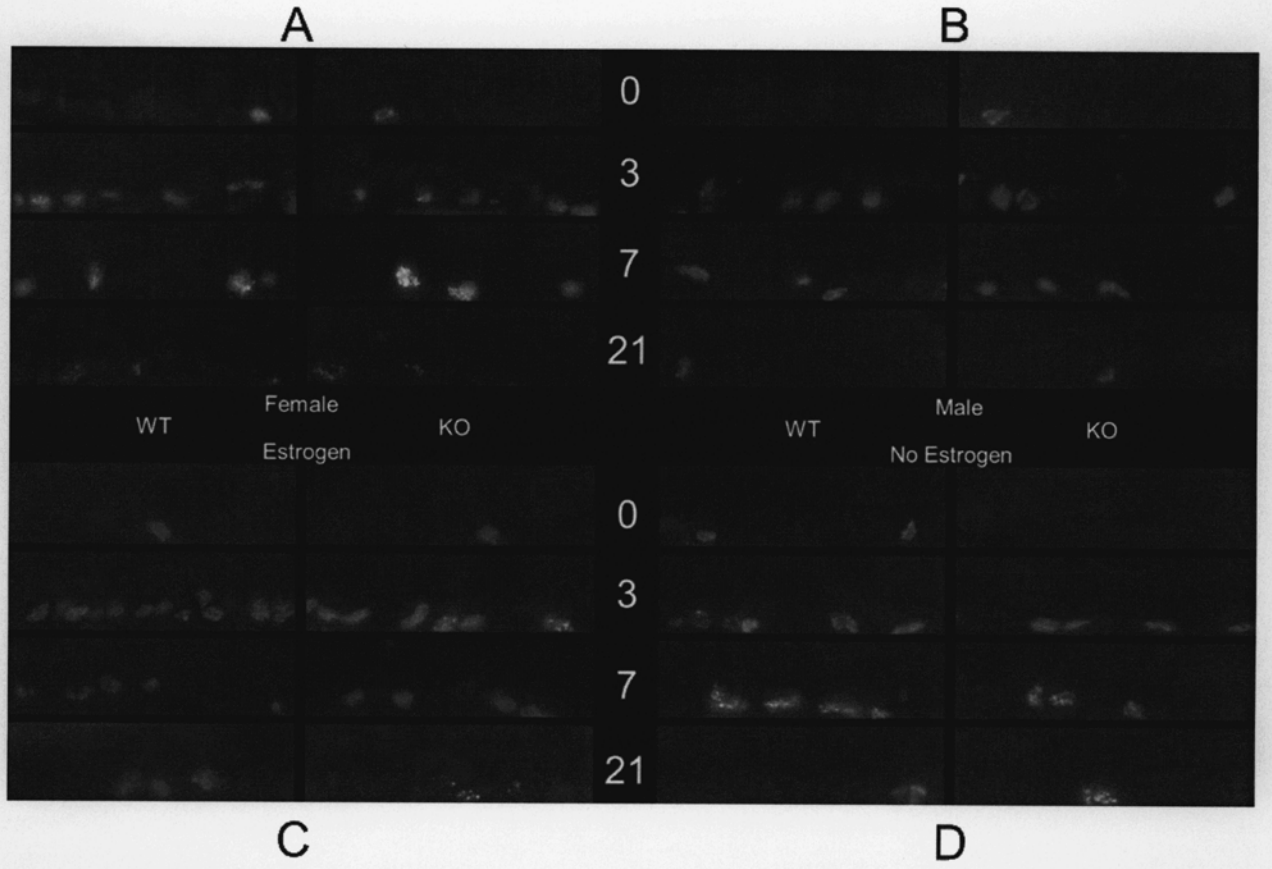


Figure 12: BrdU over time in all treatment groups: Visual portrayal of dividing globular basal cells along the basal lamina in all treatments.

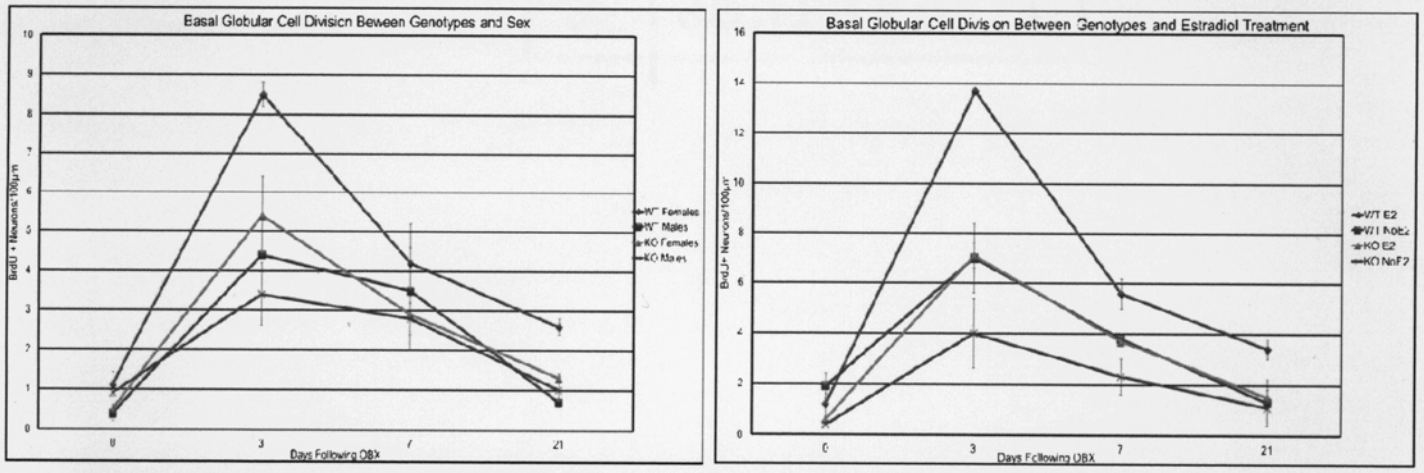


Figure 13:Comparing dividal basal cells among all treatments and genotypes over time: Line graphs comparing dividing basal cells. **Left** is a graph of male versus female mice varying levels of dividing neurons in the days following injury. **Right** is treated versus untreated mice.

Tables

Table 1: Dependent variables between genotypes in injured epithelium: Illustrates the difference between genotypes: Wild type (WT) and apoe Knockout (KO) mice. Data is pooled from the average of all time intervals and treatment groups. Asterisk * represents significant data.

Dep. Variables	**OE Thickness**		**OMP**		**BrdU**	
Genotype	WT	KO	WT	KO	WT	KO
Mean	*34.26	*30.18	*18.05	*16.26	*4.89	*3.04
std error	1.2	1.12	0.87	0.82	0.45	0.36

Table 2: Dependent variables between treatment groups in injured epithelium : Illustrates the difference between treamtent groups. Data is pooled from the average of all time intervals and genotypes groups. Asterisk * represents significant data.

Dep. Variables	**OE Thickness**			
Treatment	Female	Male	E2	No E2
Mean	*34.97	*23.41	*41.04	*29.45
Std error	2.07	1.357	2.29	2.34
	OMP			
	Female	Male	E2	No E2
	*16.84	*12.45	*24.63	*14.69
	4.3	3.7	5.39	3.71
	BrdU			
	Female	Male	E2	No E2
	*4.15	*2.63	*5.83	*3.25
	0.73	0.45	1.19	0.67

Table 3: Dependent variables between time intervals in injured epithelium: Illustrates the difference for each time interval following injury. Data is pooled from the average of all treatment groups and genotypes groups. Asterisk * represents significant data.

Dep. Variable	**OE Thickness**		
Days	3	7	21
Mean	*37.14	*24.48	*35.03
std error	1.52	1.55	2.05
	OMP		
	3	7	21
	*36.58	*10.81	*4.06
	2.01	1.27	0.69
	BrdU		
	3	7	21
	*6.69	*3.6	*1.61
	0.84	0.31	0.25

Table 4: Dependent variables between genotypes in uninjured epithelium: Illustrates the difference between genotypes: Wild type (WT) and apoe Knockout (KO) mice. Data is pooled from the average of all time intervals and treatment groups. Asterisk * represents significant data.

Dep. Variables	OE Thickness	
Genotype	WT	KO
average	*80.92	*78.63
Std Error	0.38	0.33
	OMP	
	WT	KO
	*93.25	*85.97
	0.5	0.5
	BrdU	
	WT	KO
	1.15	0.6
	0.14	0.08

Table 5: Dependent variables between treatment groups in uninjured epithelium: Illustrates the difference between treatment groups. Data is pooled from the average of all time intervals and genotypes groups. Asterisk * represents significant data.

Dep. Variables	OE Thickness			
Treatment	Female	Male	E2	No E2
Means	79.56	79.21	79.06	81.25
Std Error	0.66	0.63	0.7	0.35
	OMP			
	Female	Male	E2	No E2
	86.47	89.56	87.72	90.47
	3.08	0.99	1.56	0.84
	BrdU			
	Female	Male	E2	No E2
	0.8	0.65	0.9	1.15
	0.17	0.12	0.15	0.28

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