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PROGRESSIVE LOSS OF SYNAPTIC INTEGRITY IN HUMAN APOLIPOPROTEIN E4 TARGETED REPLACEMENT MICE AND ATTENUATION BY APOLIPOPROTEIN E2

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Abstract—Inheritance of the *APOE4* allele is a well established genetic risk factor linked to the development of late onset Alzheimer's disease. As the major lipid transport protein in the central nervous system, apolipoprotein (apo) E plays an important role in the assembly and maintenance of synaptic connections. Our previous work showed that 7 month old human apoE4 targeted replacement (TR) mice displayed significant synaptic deficits in the principal neurons of the lateral amygdala, a region that is critical for memory formation and also one of the primary regions affected in Alzheimer's disease, compared to apoE3 TR mice. In the current study, we determined how age and varying *APOE* genotype affect synaptic integrity of amygdala neurons by comparing electrophysiological and morphometric properties in C57BL6, apoE knockout, and human apoE3, E4 and E2/4 TR mice at 1 month and 7 months. The apoE4 TR mice exhibited the lowest level of excitatory synaptic activity and dendritic arbor compared to other cohorts at both ages, and became progressively worse by 7 months. In contrast, the apoE3 TR mice exhibited the highest synaptic activity and dendritic arbor of all cohorts at both ages. C57BL6 mice displayed virtually identical synaptic activity to apoE3 TR mice at 1 month; however this activity decreased by 7 months. ApoE knockout mice exhibited a similar synaptic activity profile with apoE4 TR mice at 7 months. Consistent with previous reports that *APOE2* confers protection, the apoE4-dependent deficits in excitatory activity were significantly attenuated in apoE2/4 TR mice at both ages. These findings demonstrate that expression of human apoE4 contributes to functional deficits in the amygdala very early in development and may be responsible for altering neuronal circuitry that eventually leads to cognitive and affective disorders later in life. **© 2010 Society for Vascular Surgery. Published by Elsevier Ltd. All rights reserved.**

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Abbreviations: AD, Alzheimer's disease; apo, apolipoprotein (refers to the protein or isoform); *APOE*, designation for human apolipoprotein E gene; C57, murine wild type strain; KO, knock out; LDLR, low density lipoprotein receptor; LTP, long term potentiation; sEPSC, spontaneous excitatory post-synaptic currents; TR, targeted replacement.

Key words: APOE, targeted, replacement, mice, synaptic, integrity.

APOE4 carriers represent one quarter of the population in most developed countries and have the highest risk for contracting late onset Alzheimer's disease (AD) compared to non-*APOE4* carriers (Rubinsztein and Easton, 1999). In contrast, *APOE2* carriers represent approximately 15% of the population and theoretically deter Alzheimer's disease compared to presence of the *APOE3* allele (Corder et al., 1994). Humans are the only species to express three isoforms of apolipoprotein (apo) E, each differing by a single amino acid (Weisgraber, 1994). This singular difference has dramatic effects on apoE receptor kinetics (Bohnet et al., 1996) which may explain apoE isoform specific effects on synaptic transmission. Maintaining synaptic integrity is arguably the most important CNS function needed to avoid AD. We define synaptic integrity as a functional synaptic unit with unimpaired neuronal transmission. The loss of synapses or decline in synaptic function is the strongest correlate of cognitive decline in AD (Terry et al., 1991). Multiple studies in animals show that deficits in synaptic transmission occur prior to the detection of any pathognomonic hallmarks of AD (i.e. plaques and tangles) (Buttini et al., 2002; Mucke et al., 2000; Raber et al., 2000). Since cognitive deficits can be detected in *APOE4* carriers very early in life (Acevedo et al., 2010; Reiman et al., 2004; Snowdon et al., 2000), determining when these deficits begin could provide very useful information for treating the disease. Furthermore, understanding how apoE2 expression confers protection against AD illuminates an entirely new research strategy for development of novel therapeutics.

Numerous groups have used the human apoE targeted replacement (TR) model to study the role of apoE in AD (Trommer et al., 2005; Blain et al., 2006; Osorio et al., 2007; Yun et al., 2005; Bales et al., 2009). Previously, we showed that young adult apoE4 TR mice displayed reduced excitatory synaptic transmission, dendritic arborization and spine density in the lateral amygdala compared to apoE3 TR mice, and that these deficits occurred in the absence of any pathological hallmarks such as gliosis, amyloid deposition or neurofibrillary tangles (Wang et al., 2005). In support of this, Dumanis et al. found that spine density was reduced in cortical layers II/III of apoE4 mice (vs. apoE3 mice) that became progressively worse with age (Dumanis et al., 2009). Behavioral studies demonstrate spatial memory deficits in young apoE4 TR mice compared to apoE3 mice (Grootendorst et al., 2005) that

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also became worse with age (Bour et al., 2008a) lending further support to apoE4 deficits in synaptic integrity. Furthermore, Trommer et al. found that young apoE4 mice exhibit reduced hippocampal long term potentiation (LTP) in the dentate gyrus compared to apoE3 mice (Trommer et al., 2004). In contrast, Korwek et al. found enhanced LTP in the CA1 field of apoE4 mice (Korwek et al., 2009), demonstrating that the effect of apoE4 on neuronal transmission is regionally specific.

The amygdala is a limbic structure that participates significantly in memory formation (Akirav and Richter-Levin, 2002; Fried et al., 2001; McGaugh, 2004) and like the hippocampus is one of the earliest structures to undergo neurodegeneration in AD (Hamann et al., 2002; Cuenod et al., 1993). The amygdala also regulates neural processes that govern affective states, such as depression and anxiety which are very prevalent in AD. For example, Hamann et al. found significant impairment in fear conditioning in AD patients, suggesting a loss of synaptic integrity in the amygdala (Hamann et al., 2002). Behavioral studies in mice have also shown that apoE differentially impacts multiple measures of anxiety (Siegel et al., in press; Raber, 2007; Bour et al., 2008b; Grootendorst et al., 2005).

Herein we examine both the effect of age and *APOE* genotype on synaptic integrity using the human apoE TR mice. We used apoE2/4 heterozygous mice to measure the effect of apoE2 expression on synaptic integrity since apoE2 homozygous mice develop type III hyperlipidemia (Sullivan et al., 1998), a chronic pro-inflammatory state that can confound interpretation of CNS phenotypes. Analysis of “wild type” C57BL6 (C57) mice provided us an opportunity to make evolutionary comparisons between murine and human apoE while apoE knockout (KO) mice were used to assess the absence of apoE on synaptic integrity in the amygdala. Overall, our data suggest that age and *APOE* genotype have a significant impact on regulating synaptic transmission and neuronal morphology.

EXPERIMENTAL PROCEDURES

Preparation of animals

The TR mice were created by gene targeting as described previously (Sullivan et al., 1997). Briefly, the construction of the TR mice differ from other types of apoE transgenic mice in that human *APOE* genomic fragments were used to replace the mouse *Apoe* gene via homologous recombination. All lines of apoE TR mice contain chimeric genes consisting of mouse 5′ regulatory sequences continuous with mouse exon 1 (non-coding) followed by humans exons (and introns) 2–4. Thus, all three lines of apoE TR mice regulate apoE gene expression in the same fashion. The present study uses mice that have been backcrossed to C57B16 mice eight times and therefore are >99.6% C57B16. The animals are genotyped using an allele-specific PCR approach based on Hixson and Vernier (Hixson and Vernier, 1990). All mice are maintained on a normal chow diet (ND) consisting of 4.5% (w/w) fat and 0.2% (w/w) cholesterol (Prolab Isopro, Agway Inc., DeWitt, NY, USA). The animals were handled in accordance with guidelines approved by the Duke and VA Animal Care and Use Committee, which includes minimizing the number of animals used and their suffering. All experiments were performed on age (1 or 7 months) and sex (male) matched animals for each genotype. TR mice were either homozygous for *APOE3* (3/3), *APOE4* (4/4) or heterozygous for *APOE2* and *APOE4* (2/4). Age and sex matched C57B16 and apoE KO mice were also used in this study.

Slice preparation

Acute coronal amygdala slices (350 μm thick) from the TR mice were prepared as described previously (Wang et al., 2005; Klein and Yakel, 2006). Briefly, mice were anaesthetized with 200 mg/kg 2,2,2-tribromoethanol (Vector Laboratories, Inc., Burmingham, CA, USA) and decapitated. Brains were quickly removed and placed in ice-cold oxygenated artificial cerebral spinal fluid containing (mM): 126 NaCl, 2.5 KCl, 1.3 $MgCl_2$, 2.5 $CaCl_2$, 1 NaH_2PO_4, 25 $NaHCO_3$ and 10 glucose. Upon removal of the cerebellum, the brain was hemi-sected and the right hemisphere was glued to the Vibratome 1000 Plus (Vibratome, Campden, England) stage and immersed in cooled oxygenated artificial cerebral spinal fluid.

Electrophysiology

All experiments were conducted single-blinded, where the researcher performing the electrophysiological recordings and data analysis were unaware of the *APOE* genotype. After a 1 hour incubation period single slices were transferred to the recording chamber. Whole-cell patch-clamp recordings were performed in lateral amygdala neurons using patch pipettes with resistances of 3–5 MΩ filled with internal solution (IS) containing (in mM): 140 potassium gluconate, 0.5 $CaCl_2$, 2 MgATP, 2 $MgCl_2$, 5 EGTA, and 10 Hepes (pH 7.2–7.3). For morphological studies, 0.3% biocytin (Sigma-Aldrich, St. Louis, MO, USA) was added to the IS on the day of the experiment. Slices were superfused at room temperature (18–22 °C) with artificial cerebral spinal fluid. We clamped cells at −70 mV and recorded spontaneous excitatory post-synaptic currents (sEPSCs) using an Axopatch 200B amplifier, filtered at 1 kHz, and sampled at 10 kHz using pClamp 10.1 software (Molecular Devices Inc, Sunnyvale, CA, USA). We measured the sEPSCs interval and amplitude using Mini Analysis software (Synaptosoft, Inc. Decatur, GA, USA) and statistical analyses was performed using Origin software (Microcal, Northampton, MA, USA). Averaged data are presented as means±SEM. Statistical significance ($P<0.05$) was assessed using a Student’s *t*-test. Firing properties and electrical excitability were assessed using whole-cell current-clamp mode. Hyper- and depolarizing step pulses were delivered at 1 s durations ranging from −0.2 to 0.2 nA in 25 pA increments.

Biocytin staining

At the end of the recording session, the patch-pipette was gently retracted from the filled neuron and the brain slice was immediately transferred to a solution containing 4% paraformaldehyde in phosphate buffered saline (PBS), pH 7.4 (USB Corp., Cleveland, OH, USA) and stored at 4 °C. Slices were incubated for 1–3 days with avidin-biotin-peroxidase complex (Vectastain Elite ABC kit, Vector Laboratories, Inc., Burmingham, CA, USA) diluted in PBS containing 1% Triton X-100. Slices were then stained with diaminobenzidine (Vector Labs) solution freshly prepared in PBS. The reaction was stopped by rinsing with deionized water and the stained slices were mounted on gelatin subbed slides, air-dried overnight and cover slipped using standard techniques. The morphology of the biocytin-filled neurons was examined using a light microscope (either a Nikon EP3000 or Zeiss Axio ImagerD2), then traced with Neurolucida software (under a 40× objective) and analyzed with Neuroexplorer software (MicroBrightField, Colchester, VT, USA).

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