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Acetylcholine receptor and behavioral deficits in mice lacking apolipoprotein E

Jessica A Siegel, B.A.^{a,#}, Theodore S Benice, B.S.^{a,#}, Peter Van Meer, M.S.^a, Byung S Park, Ph.D.^b, and Jacob Raber, Ph.D.^{a,c,d,*}

Jessica A Siegel: siegelj@ohsu.edu; Theodore S Benice: benicet@ohsu.edu; Peter Van Meer: petervmeer@yahoo.com; Byung S Park: parkb@ohsu.edu

^aDepartment of Behavioral Neuroscience, Oregon Health & Science University, 8131 SW Sam Jackson Park Rd., Portland, OR 97239, USA

^bDepartment of Public Health & Preventative Medicine, Oregon Health & Science University, 8131 SW Sam Jackson Park Rd., Portland, OR 97239, USA

^cDepartment of Neurology, Oregon Health & Science University, 8131 SW Sam Jackson Park Rd., Portland, OR 97239, USA

^dDivision of Neuroscience ONPRC, Oregon Health & Science University, 8131 SW Sam Jackson Park Rd., Portland, OR 97239, USA

Abstract

Apolipoprotein E (apoE) is involved in the risk to develop sporadic Alzheimer's disease (AD). Since impaired central acetylcholine (ACh) function is a hallmark of AD, apoE may influence ACh function by modulating muscarinic ACh receptors (mAChRs). To test this hypothesis, mAChR binding was measured in mice lacking apoE and wild type C57BL/6J mice. Mice were also tested on the pre-pulse inhibition, delay eyeblink classical conditioning, and 5-choice serial reaction time tasks, which are all modulated by ACh transmission. Mice were also given scopolamine to challenge central mAChR function. Compared to wild type mice, mice lacking apoE had reduced number of cortical and hippocampal mAChRs. Scopolamine had a small effect on delay eyeblink classical conditioning in wild type mice but a large effect in mice lacking apoE. Mice lacking apoE were also unable to acquire performance on the 5-choice serial reaction time task. These results support a role for apoE in ACh function and suggest that modulation of cortical and hippocampal mAChRs might contribute to genotype differences in scopolamine sensitivity and task acquisition. Impaired apoE functioning may result in cholinergic deficits that contribute to the cognitive impairments seen in AD.

Keywords

Acetylcholine; apolipoprotein E; Alzheimer's disease; muscarinic; stress; attention

*Correspondence should be addressed to Dr. Jacob Raber, Department of Behavioral Neuroscience, Oregon Health & Science University, 8131 SW Sam Jackson Park Rd., Portland, Oregon 97239, USA. Telephone: (503) 494-1524. Fax: (503) 494-6877. raberj@ohsu.edu.

[#]These authors contributed equally to this manuscript

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1. Introduction

Apolipoprotein E (apoE) plays a critical role in lipid transport and metabolism in the brain (Mahley, 1988) and is involved in CNS repair after injury (Arendt et al., 1997). The gene encoding for apoE is polymorphic in humans, yielding apoE2, apoE3, and apoE4 isoforms (Mahley, 1988). ApoE interacts with the brain acetylcholine (ACh) system in an isoform-specific manner in humans, such that apoE4 is associated with decreased nucleus basalis neuronal activity (Salehi et al., 1998) and choline acetyltransferase activity in the cortex and hippocampus compared to apoE2 and apoE3 (Allen et al., 1997; Lai et al., 2006; Poirier et al., 1995). Compared to apoE3, apoE4 is also associated with an increased risk of developing sporadic Alzheimer's disease (AD) (Farrer et al., 1997; Saunders et al., 1993). Deterioration of the brain ACh system in the basal forebrain, cortex, and hippocampus is a hallmark of AD (Geula and Mesulam, 1996; Svensson et al., 1997; Whitehouse et al., 1982). As cognition is highly modulated by the ACh system (Berger-Sweeney, 2003), apoE-isoform specific effects on ACh function may be directly related to the development of cognitive impairments associated with AD. These differential effects of apoE could be due to lack of function or gain of misfunction effects. Therefore, mice deficient in apoE are valuable to define the potential role of mouse apoE in cholinergic function.

Experiments using animal models also support a role for apoE in the maintenance of ACh function, but the data are incomplete. Mice lacking apoE (*ApoE*^{-/-}) show decreased cholinergic functioning compared to wild type (*ApoE*^{+/+}) mice (Buttini et al., 2002; Chapman and Michaelson, 1998; Gordon et al., 1995; Kleinfeld et al., 1998). However, not all studies have found such effects (Anderson and Higgins, 1997; Bronfman et al., 2000; Krzywkowski et al., 1999). Thus, the relationship between apoE and the ACh system remains elusive. As most previous studies have focused on enzymatic and structural markers of ACh function, it is unclear whether apoE affects the number or function of ACh receptors in the brain.

There are two classes of ACh receptors; nicotinic and muscarinic (Cummings, 2000). The M₁ and M₂-type muscarinic ACh receptors (mAChRs) are two of the most highly expressed ACh receptors in brain (von Bohlen and Dermietzel, 2002) and are expressed in the cortex and hippocampus (Palacios, 1982; Porter et al., 2002; Probst et al., 1988; Schwab et al., 1992), two areas important for cognitive function and conditioned learning (Christian and Thompson, 2003). M₂ receptors function as presynaptic autoreceptors, regulating ACh release (Cummings, 2000). Furthermore, mAChR binding and function is impaired in AD (Claus et al., 1997; Koch et al., 2005). Pre-pulse inhibition (PPI), the delay eyeblink classical conditioning (DEBCC) task, and the 5-choice serial reaction time task (5-SRTT), are behavioral tasks sensitive to alterations in ACh function. PPI is a measure of sensorimotor gating that is impaired in AD (Ueki et al., 2006) and mAChR antagonism causes PPI impairments in rats (Jones and Shannon, 2000) that are reversed by acetylcholinesterase inhibitors (Ballmaier et al., 2002). DEBCC is a type of associative learning that is also impaired in AD and is affected by mAChR blockade in rabbits (Woodruff-Pak et al., 2002; Woodruff-Pak and Hinchliffe, 1997) and mice (Takatsuki et al., 2002). Finally, attention, as measured by the 5-SRTT, increases ACh levels (Arnold et al., 2002) and the ability to perform well on attention tasks is diminished with mAChR antagonism in rats (Gill et al., 2000; Mirza and Stolerman, 2000). Attention is also one of the earliest cognitive domains to deteriorate in AD (Perry and Hodges, 1999). Thus, the PPI, DEBCC, and the 5-SRTT tests can be used to determine ACh functioning in rodents and model cognitive deficits associated with early AD.

As apoE is important for ACh function, *ApoE*^{-/-} mice might have lower M₁ and M₂ mAChR densities than *ApoE*^{+/+} mice. Such changes might be associated with altered

performance on tasks sensitive to ACh function and increase the sensitivity of *Apoe*^{-/-} mice to ACh challenges. In this study we examined the role of apoE in ACh function by determining mAChR binding in the cortex, hippocampus, and cerebellum of *Apoe*^{+/+} and *Apoe*^{-/-} mice and assessing behavioral performance on tasks sensitive to alterations in ACh function under baseline conditions and following an ACh challenge with scopolamine, a mAChR antagonist. To rule out non-specific effects of scopolamine on sensitivity to the stimuli used in the behavioral tests, we measured the effect of scopolamine on stimulus sensitivity thresholds. Since the stress hormone corticosterone can modulate DEBCC performance (Shors et al., 1992), we also measured corticosterone levels induced by one day of DEBCC training after scopolamine administration. We hypothesized that, compared to *Apoe*^{+/+} mice, *Apoe*^{-/-} mice would show reduced mAChR binding in the cortex and hippocampus and that as a result of this decreased binding would also show impaired performance on PPI, DEBCC, and the 5-SRTT tests. We further hypothesized that disruption of ACh transmission by scopolamine would impair performance to a greater degree in *Apoe*^{-/-} mice than in *Apoe*^{+/+} mice, thus supporting a role for apoE in central ACh function.

2. Methods

2.1. Animals

Three- to 5-month-old naïve male C57BL/6J *Apoe*^{+/+} and *Apoe*^{-/-} mice, bred in our colony, were used for all experiments with the exception of the 5-SRTT in which both female and male mice were used. The mice were maintained on a 12 h light/dark schedule (lights on at 06:00). Behavioral testing took place during the light cycle. Lab chow (PicoLab Rodent Diet 20, #5053; PMI Nutrition International, St. Louis MO, USA) and water were given *ad libitum*. Separate groups of animals were used for each procedure. All procedures conformed to the standards of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee of the Oregon Health and Science University.

2.2. Muscarinic acetylcholine receptor saturation binding

Receptor saturation binding experiments were performed using cortical, hippocampal, and cerebellar membrane preparations from *Apoe*^{+/+} (*N* = 16) and *Apoe*^{-/-} (*N* = 17) mice using radioligands specific for M₁ ([³H]pirenzepine) or M₂ ([³H]AF-DX-384) mAChRs (see supplementary data (Vaucher et al., 2002; Watson et al., 1986)). The number of binding sites (*B_{max}*) and the equilibrium dissociation constant (*K_d*) were determined according to the Hill equation (Whiteaker et al., 2000), using non-linear regression analysis performed in Graphpad Prism 4.0 (Graphpad, San Diego CA, USA).

2.3. Foot-shock and acoustic startle

Mice were tested for foot-shock (*N* = 8) or acoustic startle thresholds (*N* = 8) following saline or scopolamine injections to rule out potential genotype differences in sensitivity to stimuli or scopolamine (see supplementary data).

2.4. Behavioral tests

Separate groups of *Apoe*^{+/+} and *Apoe*^{-/-} mice were used for the behavioral tests. Sensorimotor gating was measured in the PPI test (*N* = 73; (Plappert et al., 2004)), associative learning was measured in the DEBCC test (*N* = 44; (Bao et al., 1998; Lee and Kim, 2004)), and attentional behavioral measured in the 5-SRTT (*N* = 96; (Robbins, 2002); see supplementary data for detailed methods). Mice were administered either saline or

various doses of scopolamine to determine the involvement of ACh muscarinic transmission in each test.

2.5. Plasma corticosterone levels

A separate group of mice ($N = 14$) were given injections of saline or scopolamine and one day of paired DEBCC training to determine the plasma corticosterone response immediately following the DEBCC (see supplementary data).

2.6. Statistical analysis

Analysis of variance (ANOVA) was used to assess the effects of genotype and scopolamine, where appropriate, on the number (B_{max}) and affinity (K_d) of M_1 and M_2 mAChRs, sensory thresholds, PPI, corticosterone levels, and performance on the 5-SRTT. Repeated measures ANOVA was used for the DEBCC and learning on the 5-SRTT (repeated factor: training day and training level, respectively). Duncan's post-hoc tests were performed to compare between groups. The Kaplan-Meier method was used to assess the median training level at which mice were unable to learn the 5-SRTT (dropout level). Fisher's Exact Test assessed the effects of genotype on dropout rates at each level of training. Only significant interactions are reported. $P < 0.05$ was considered significant for all tests.

3. Results

3.1. Decreased M_1 and M_2 mAChRs in $Apoe^{-/-}$ mice

The B_{max} and K_d values for M_1 receptors in the cortex and hippocampus (M_1 receptors are not found in the cerebellum (von Bohlen and Dermietzel, 2002)) and M_2 receptors in the cortex, hippocampus, and cerebellum are given in Table 1. The number of M_1 mAChRs (B_{max}) was greater in the hippocampus than the cortex of both genotypes ($F(1, 13) = 7.20$, $p = 0.03$). $Apoe^{-/-}$ mice had less M_1 mAChRs in the cortex and hippocampus than $Apoe^{+/+}$ mice ($F(1, 9) = 41.02$, $p < 0.001$; Figure 1A and 1C, respectively). The M_1 mAChR binding affinity (K_d) did not differ between the brain regions or the genotypes ($F(1, 9) = 0.58$, $p = 0.47$ and $F(1, 9) = 1.89$, $p = 0.20$, respectively).

$Apoe^{-/-}$ mice had fewer M_2 mAChRs than $Apoe^{+/+}$ mice in the cortex (Figure 1B) and hippocampus (Figure 1D), but had equivalent number of receptors to $Apoe^{+/+}$ mice in the cerebellum (genotype $\times$ region interaction; $F(2, 14) = 40.4$, $p < 0.001$). $Apoe^{-/-}$ mice had lower K_d values (increased receptor affinity) in the cortex (Figure 1B) and hippocampus (Figure 1D) compared to $Apoe^{+/+}$ mice, but had equivalent M_2 receptor K_d values to $Apoe^{+/+}$ mice in the cerebellum (genotype $\times$ region interaction; $F(2, 14) = 4.40$, $p = 0.03$).

3.2. Shock and acoustic sensitivity were unaffected by genotype or scopolamine

Shock and acoustic sensitivity thresholds were assessed to rule out potential effects of genotype and scopolamine administration on sensitivity to the CS and US stimuli used in DEBCC. There was no difference between the genotypes or scopolamine- or saline-treated mice in either shock sensitivity threshold (Figure 2A) or acoustic sensitivity threshold (Figure 2B).

3.3. Impaired PPI in $Apoe^{+/+}$ and $Apoe^{-/-}$ mice following scopolamine

There was an effect of scopolamine treatment on the average startle response to the 110 db stimuli, with saline-treated mice showing a lower startle response compared to 2.4 mg/kg scopolamine-treated mice ($F(3, 65) = 4.29$, $p < 0.01$; Figure 3). There was also an effect of genotype on the average startle response to the 110 db stimuli, with the $Apoe^{-/-}$ mice

showing a lower startle response compared to *Apoe*^{+/+} mice ($F(1, 65) = 24.84, p < 0.01$; Figure 3A).

Percent response during the pre-pulse trials was the outcome measure for the PPI test, with 100% response indicating complete inhibition of the startle response. There was an effect of treatment on the percent PPI, with *Apoe*^{-/-} and *Apoe*^{+/+} mice treated with 0.8 mg/kg or 2.4 mg/kg scopolamine showing reduced PPI compared to *Apoe*^{-/-} and *Apoe*^{+/+} mice treated with 0.2 mg/kg scopolamine or saline ($F(3, 65) = 7.45, p < 0.01$; Figure 3B). Because the genotypes and treatment groups differed in baseline startle responses to the 110 db stimulus, we performed an analysis of covariance (ANCOVA) with average baseline startle as the covariate. When baseline startle was included as a covariate, the effect of treatment remained significant, indicating that the differences in baseline startle did not account for differences in PPI ($F(3, 64) = 7.47, p < 0.001$).

3.4. Enhanced sensitivity of *Apoe*^{-/-} mice to scopolamine-induced DEBCC impairments

The lowest dose of scopolamine affecting both genotypes equally in the PPI experiments, 0.8 mg/kg was selected for the DEBCC experiment (Figure 4). The percent CRs increased over the 5 DEBCC training days in the paired training condition while the percent CRs remained the same over the training days in the unpaired training condition, showing that *Apoe*^{-/-} and *Apoe*^{+/+} mice only learned with paired DEBCC training (training day $\times$ training type interaction; $F(4, 120) = 6.46, p < 0.001$). Furthermore, scopolamine decreased CRs in the DEBCC performance only in the paired training condition (scopolamine $\times$ training day interaction; $F(1, 30) = 6.09, p = 0.02$). Scopolamine had a specific effect on DEBCC performance during paired training only in the *Apoe*^{-/-} mice, with a reduction in percent CR with scopolamine in the *Apoe*^{-/-}, but not in the *Apoe*^{+/+}, mice (genotype $\times$ scopolamine interaction; $F(1, 4) = 4.80, p = 0.036$; Figures 4B and 4C). The scopolamine effect was on average greater in the paired training condition compared to the explicitly unpaired condition, but the genotype $\times$ scopolamine $\times$ training type interaction did not reach significance ($F(1, 30) = 3.49, p = 0.07$).

3.5. Scopolamine increased plasma corticosterone levels in *Apoe*^{-/-}, but not *Apoe*^{+/+}, mice after one day of paired DEBCC training

Corticosterone levels were assessed in plasma immediately following one day of paired DEBCC training in scopolamine (0.8 mg/kg) or saline treated *Apoe*^{+/+} and *Apoe*^{-/-} mice. There was an effect of scopolamine injection only in the *Apoe*^{-/-} mice, with scopolamine increasing plasma corticosterone levels after one day of DEBCC training compared to saline (genotype $\times$ scopolamine interaction; $F(1, 8) = 6.77, p = 0.03$; Figure 5).

3.6. *Apoe*^{-/-} mice unable to acquire performance on the 5-SRTT

As none of the male *Apoe*^{-/-} mice in the first testing group acquired the 5-SRTT, we added female mice based on the finding that girls with attention deficit hyperactivity disorder (ADHD) show less severe inattention than boys with ADHD (Gershon, 2002). There were no differences between male and female mice in the median dropout training level (Kaplan-Meier analysis; $p = 0.50$, log-rank test), dropout rates at each individual level of training (Fisher's Exact Tests), or in the number of days required to pass each level of training amongst those mice that passed the test to the final criterion (repeated measures ANOVA; $F(1, 17) = 0.97, p = 0.34$). Thus, male and female mice were pooled for genotype comparisons. A 2-bottle choice test in a separate group of *Apoe*^{+/+} and *Apoe*^{-/-} mice revealed no difference between genotypes or sexes in sucrose solution preference over water (see supplementary Figure 1), suggesting that potential differences in 5-SRTT performance were not due to differences in motivation to receive the sucrose reward.

Approximately half of the *ApoE*^{+/+} mice in the first testing group were able to acquire the 5-SRTT to the final testing criterion. To avoid the potential confound of a selection bias, we tested additional groups of *ApoE*^{+/+} and *ApoE*^{-/-} mice and added female mice to obtain an accurate estimate of the percentage of mice that were unable to acquire performance. The median dropout training level was lower amongst *ApoE*^{-/-} mice than *ApoE*^{+/+} mice (*ApoE*^{+/+} median level; criterion 2: *ApoE*^{-/-} median level; nosepoke training: $p < 0.001$, log-rank test). There was a difference in dropout rates between *ApoE*^{+/+} and *ApoE*^{-/-} mice at the level of magazine training ($p < 0.001$, Fisher's Exact Test) and nosepoke training ($p < 0.001$, Fisher's Exact Test), with *ApoE*^{-/-} mice dropping out significantly more than *ApoE*^{+/+} mice (Table 2). None of the *ApoE*^{-/-} mice were able to learn the 5-SRTT to the level where attention could be assessed while 28% of the *ApoE*^{+/+} mice reached this final criterion level (criterion 6; Figure 6).

3.7. Scopolamine impaired performance on the 5-SRTT

There was no difference between male and female *ApoE*^{+/+} mice on the average percent of correct responses (correct responses/correct responses + incorrect responses) or percent omissions (trials without a response/total trials) prior to treatment administration during the final criterion of the 5-SRTT ($F(1, 17) = 0.17$, $p = 0.69$ and $F(1, 17) = 1.19$, $p = 0.29$, respectively). There was also no effect of sex on the average number of days required to reach asymptotic performance on the final criterion ($F(1, 17) = 0.01$, $p = 0.92$). There was no difference in the percent correct responses or omissions during the 3 saline treatments ($F(2, 34) = 2.86$, $p = 0.07$ and $F(2, 34) = 1.09$, $p = 0.35$, respectively). Thus, percent correct responses and omissions were averaged for all saline treatments for comparisons with performance during scopolamine. The percent correct responses during 1.4 mg/kg scopolamine treatment were lower than the percent correct responses during saline or 0.2 mg/kg scopolamine treatment ($F(3, 51) = 8.40$, $p < 0.001$; Figure 7). Scopolamine did not affect the percent omissions ($F(3, 51) = 0.94$, $p = 0.443$). Male and female mice were equally affected by the treatments, as indicated by no main effect of sex and no interaction between sex and treatment.

4. Discussion

ApoE is involved in the risk for the development of sporadic AD (Farrer et al., 1997; Saunders et al., 1993) and ACh neurotransmission is deleteriously affected in AD (Geula and Mesulam, 1996; Svensson et al., 1997; Whitehouse et al., 1982). However, the potential role of apoE in brain ACh function has been in dispute for the last decade (Anderson and Higgins, 1997; Bronfman et al., 2000; Chapman and Michaelson, 1998; Fisher et al., 1998; Gordon et al., 1995; Krzywkowski et al., 1999). Our data are in agreement with those studies that have suggested a role for apoE in brain ACh neurotransmission (Fisher et al., 1998; Gordon et al., 1995). The main outcomes of our study were: 1) *ApoE*^{-/-} mice had reduced number of M₁ and M₂ mAChRs and increased M₂ mAChR affinity in the cortex and hippocampus compared to *ApoE*^{+/+} mice, 2) DEBCC learning was affected by systemic mAChR blockade only in *ApoE*^{-/-} mice, 3) mAChR blockade induced positive modulation of the hypothalamic-pituitary-adrenal (HPA) axis response to a single day of paired DEBCC training only in *ApoE*^{-/-} mice, and 4) *ApoE*^{-/-} mice were unable to acquire performance on an operant test of attention. We did not observe a genotype difference in PPI, a task that is not learned. Thus, the effects of reduced mAChRs and increased sensitivity may only affect performance on learned tasks. Our behavioral data demonstrate a marked perturbation of performance on several tasks in *ApoE*^{-/-} mice which, when combined with the neurochemical findings, suggests a potential relationship between cholinergic deficits and behavioral impairments in *ApoE*^{-/-} mice. Furthermore, this study suggests that apoE may

alter ACh neurotransmission throughout life, potentially affecting cognition in the context of AD.

Compared to *Apoe*^{+/+} mice, *Apoe*^{-/-} mice were equally capable of acquiring the eyelid CR in the DEBCC test. However, when mAChR-mediated ACh neurotransmission was challenged by a moderate dose of scopolamine (0.8 mg/kg), learning was significantly disrupted only in *Apoe*^{-/-} mice. Importantly, sensitivity of both genotypes to either shock or noise stimuli was unaffected by scopolamine, and *Apoe*^{+/+} and *Apoe*^{-/-} mice were equally affected by the same doses of scopolamine in PPI, indicating a specific effect on DEBCC learning. This suggests that ACh transmission in the DEBCC brain circuitry of *Apoe*^{-/-} mice is more vulnerable to disruption. A higher dose of scopolamine may have been required to impair DEBCC performance in *Apoe*^{+/+} mice due to greater mAChR densities. The necessary circuitry for DEBCC CR acquisition is located in the cerebellum (Christian and Thompson, 2003). However, our data suggest that the cerebellum may not be the site involved in the differential sensitivity of *Apoe*^{-/-} and *Apoe*^{+/+} mice to effects of scopolamine in the DEBCC test. There was no difference in either the number or affinity of M₂ mAChRs in the cerebellum of *Apoe*^{-/-} and *Apoe*^{+/+} mice. However, this does not rule out other potential changes in cerebellar ACh signaling in *Apoe*^{-/-} mice, such as differences in ACh innervations or ACh release.

In addition to disrupting DEBCC learning, the HPA axis response to one day of DEBCC training was augmented by scopolamine only in *Apoe*^{-/-} mice. Brain ACh plays an important role in HPA axis regulation. Central mAChR agonism with carbachol stimulates HPA axis activity in rats (Bugajski et al., 2007). Our present data also support a role for ACh in HPA axis regulation, since scopolamine administration increased plasma corticosterone levels in *Apoe*^{-/-}, but not *Apoe*^{+/+}, mice after one day of DEBCC training. The effect in *Apoe*^{-/-} mice is in apparent contrast to the stimulatory effect of mAChR agonists on the HPA axis, however other evidence suggests that hippocampal mAChRs serve to down-regulate the HPA axis (Bhatnagar et al., 1997).

Apoe^{-/-} were unable to acquire performance on the 5-SRTT, a finding that was not due to motor deficits, as previous studies have shown normal motor abilities in these mice (Raber et al., 1998). However, potentially high anxiety levels amongst the *Apoe*^{-/-} mice may have led to poorer performance, as previous findings suggest that *Apoe*^{-/-} mice are more anxious than wild type controls (Raber et al., 2000). Thus, the potential effect of anxiety in 5-SRTT performance in *Apoe*^{-/-} mice cannot be ruled out. A high dose of scopolamine reduced the percent of correct responses in *Apoe*^{+/+} mice, but not to the degree predicted. The mice that were treated with scopolamine were trained in the 5-SRTT for an average of 60 days, and this extensive training may have led to task performance resistant to disruption. Scopolamine administered at an earlier point during 5-SRTT training may have yielded more robust effects.

72% of the *Apoe*^{+/+} mice were unable to acquire the 5-SRTT task under the specified experimental conditions. The criteria for dropping out of the 5-SRTT used in this study (more than 10 days on a single training level) may have been too stringent for C57BL/6J mice. Although previous studies using similarly difficult training criteria in mice have not reported similar dropout rates (de Bruin et al., 2006; Humby et al., 1999; Patel et al., 2006; Young et al., 2007; Young et al., 2004), the large dropout rates found in the current study suggest that dropouts may have occurred in these studies. Thus, it is important to consider that only including animals that successfully complete the training could enter bias into studies of attention by selecting the best performing animals. We believe it is important to include the information gained from animals that fail to learn tasks in future studies of attention in animal models.

ApoE^{-/-} mice had fewer M₁ and M₂ mAChRs and increased M₂ mAChR affinity in the cortex and hippocampus compared to *ApoE*^{+/+} mice. This finding suggests impaired presynaptic and postsynaptic ACh system integrity in *ApoE*^{-/-} mice, with increased M₂ mAChR affinity potentially compensating for reduced M₂ mAChR number. This is in contrast to Krzywkowski et al., who reported no genotype differences in these receptors in *ApoE*^{+/+} and *ApoE*^{-/-} mice using autoradiography (Krzywkowski et al., 1999). This discrepancy suggests that the saturation binding technique used in our study may be more sensitive than autoradiography. Interestingly, there was no difference in the number or affinity of M₂ mAChRs in the cerebellum between *ApoE*^{+/+} and *ApoE*^{-/-} mice, suggesting that apoE-dependent effects are region specific. The cerebellar cholinergic projections originate in the vestibular nuclei and the pedunculopontine tegmental areas (Jaarsma et al., 1997) while the cortex and hippocampal cholinergic projections originate in the basal forebrain (Gaykema et al., 1991). However, the mechanism whereby apoE modulates the number of mAChRs in a brain region-selective manner is unclear. ApoE can influence the lipid and cholesterol content of cell membranes which could influence the trafficking, insertion, and internalization of surface receptors, as has been previously suggested (Bongers et al., 2004). Previous studies report a significant age-dependent decrease in cortical and hippocampal ACh fiber innervation only in older *ApoE*^{-/-} mice (Buttini et al., 2002). Thus, the non-significant difference in fiber density in younger mice likely does not underlie the decreased mAChR number in *ApoE*^{-/-} mice, but rather it is likely a direct effect of apoE loss on mAChRs expression. However, the precise interaction between apoE and mAChRs remains to be elucidated.

It should be noted that in addition to mAChRs, nicotinic acetylcholine receptors (nAChRs) are also important for central cholinergic function. In humans and rodents, nAChR agonists improve attention, perception, and memory (for a review, see: (Paterson and Nordberg, 2000)). Furthermore, nAChR signaling is thought to modulate other transmitter systems, such as dopamine, serotonin, and noradrenalin signaling via presynaptic nAChRs (Paterson and Nordberg, 2000). In AD, cortical nAChRs are reduced compared to age matched controls (Paterson and Nordberg, 2000), although this decrease is not *APOE* genotype-dependent (Lai et al., 2006; Reid et al., 2001). Previous studies have not found nAChR binding differences between wild type and *ApoE*^{-/-} mice (Krzywkowski et al., 1999) suggesting that apoE may not modulate nAChR binding. However, nAChR signaling plays an essential role in central ACh neurotransmission and potential changes in nAChR binding in *ApoE*^{-/-} mice cannot be excluded.

The cholinergic system is not the only system affected in *ApoE*^{-/-} mice. Noradrenergic terminals (Chapman et al., 2000) and histamine H₃ receptors (Bongers et al., 2004) in the cortex and hippocampus are reduced in *ApoE*^{-/-} mice compared to *ApoE*^{+/+} mice, although these mice show similar expression of dopaminergic and serotonergic terminals and histamine H₁ receptors (Bongers et al., 2004; Chapman et al., 2000). As the neurotransmitter system alterations in *ApoE*^{-/-} mice are not selective for the ACh system (Raber, 2004) and effects of altered noradrenergic or histaminergic H₃ neurotransmission on mAChR binding and behavior cannot be ruled out, interpretation of the behavioral data should be made with caution until the precise involvement of these non-cholinergic systems in these tests is elucidated. In addition, apoE is known to have other neurobiological effects that could influence behavior (for a review, see: (Raber, 2004)). For example, apoE deficiency is associated with reduced neurite outgrowth in cultured cells and in brains of AD patients (Raber, 2004). Also, human apoE4, the apoE isoform that confers risk to develop AD, is associated with decreased synapse density in the hippocampus and increased oxidative stress (Raber, 2004). ApoE also interacts with microtubule-associated proteins to affect cytoskeletal remodeling (Mahley and Rall, 2000). Although the precise mechanisms

are not clear at this time, apoE plays a role in neuroprotection against various insults (Mahley and Rall, 2000; Raber, 2004).

In conclusion, this study supports the hypothesis that apoE influences ACh neurotransmission. We observed perturbed behavioral performance under baseline conditions in the 5-SRTT as well as in the DEBCC task following scopolamine challenge. Furthermore, a lack of apoE was associated with decreased M₁ and M₂ mAChRs in the cortex and hippocampus, suggesting that apoE influences ACh transmission in brain areas vulnerable to the effects of neurodegenerative processes in healthy aging and AD. The DEBCC paradigm and tests of attention have both been used to differentiate AD from normal aging (Perry and Hodges, 1999; Woodruff-Pak et al., 1990), and *APOE* genotype might critically contribute to this effect. We suggest that cholinergic changes associated with *APOE* genotype might underlie the observed behavioral disruptions. Increased efforts are warranted to understand the molecular mechanisms underlying the effects of apoE on ACh function as they may lead to the discovery of biomarkers to allow early detection of disease processes that involve declines in ACh neurotransmission.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Muscarinic acetylcholine receptor binding reduced in the cortex and hippocampus of *Apoe*^{-/-} mice compared to *Apoe*^{+/+} mice
 (a) Cortical and (c) hippocampal M₁ receptor binding (B_{max}) was reduced in *Apoe*^{-/-} mice compared to *Apoe*^{+/+} mice. (b) Cortical and (d) hippocampal M₂ receptor binding (B_{max} and K_d) were reduced in *Apoe*^{-/-} mice compared to *Apoe*^{+/+} mice. $n = 3-4$ membrane preparations per genotype and brain region.

Figure 2. No difference in stimulus sensitivity levels between *Apoe*^{+/+} and *Apoe*^{-/-} mice treated with scopolamine or saline

(a) There was no difference in foot-shock startle threshold or (b) acoustic startle threshold between the genotypes. * $p < 0.05$ versus all other groups. $n = 3-4$ mice per genotype and treatment for each experiment.

Figure 3. Increased baseline startle response and impaired pre-pulse inhibition in *Apoe*^{+/+} and *Apoe*^{-/-} mice treated with scopolamine

Mice were treated with 0.2 mg/kg, 0.8 mg/kg, 2.4 mg/kg scopolamine, or saline, 15 min prior to testing. (a) Baseline startle responses were lower in mice treated with saline compared to mice treated with 2.4 mg/kg scopolamine. (b) Pre-pulse inhibition was impaired in mice treated with 0.8 mg/kg and 2.4 mg/kg scopolamine compared to those treated with 0.2 mg/kg scopolamine and saline. $n = 7-11$ mice per genotype and treatment.

Figure 4. Delay eyeblink classical conditioning learning impaired by scopolamine in *Apoe*^{-/-} but not *Apoe*^{+/+} mice

(a) Analysis intervals of delay eyeblink classical conditioning (DEBCC) trials used to obtain percent conditioned response (CR) outcome measures. A CR was counted when the root mean square amplitude (RMS Amplitude V) during the conditioned stimulus (CS) interval was > 4 standard deviations above the average amplitude of the pre-CS interval for at least 3 consecutive milliseconds. Percent CR was calculated from the number of CRs divided by the number of valid trials. Valid trials excluded any trial with average RMS amplitude > 0.2 V or with a CR during the α interval. (b) Paired DEBCC learning was not affected by 0.8 mg/kg scopolamine in *Apoe*^{+/+} mice (c) but was affected in *Apoe*^{-/-} mice. There was no evidence of pseudoconditioning in the explicitly unpaired control groups. $n = 5-7$ mice per genotype and treatment for paired conditioning, $n = 3$ mice per genotype and treatment for unpaired conditioning.

Figure 5. Plasma corticosterone levels increased by scopolamine in *Apoe*^{-/-} but not *Apoe*^{+/+} mice
 After one day of delay eyeblink classical conditioning, *Apoe*^{-/-} mice treated with 0.8 mg/kg scopolamine had higher plasma corticosterone levels compared to saline treated *Apoe*^{-/-} mice and scopolamine or saline treated *Apoe*^{+/+} mice. **p* < 0.05 versus all other groups. *n* = 3–4 mice per genotype and treatment.

Figure 6. *Apoe*^{-/-} mice failed to acquire performance on the 5-choice serial reaction time task All of the *Apoe*^{-/-} mice failed to acquire the task beyond criterion 2 whereas 72% of the *Apoe*^{+/+} mice failed to acquire the task to criterion 4. Because training level was progressive and dropout was an event, we mimicked this data as time to event and applied the Kaplan-Meier method. Median dropout levels differed between *Apoe*^{+/+} mice (criterion 2) and *Apoe*^{-/-} mice (nosepoke training; Kaplan-Meier Analysis). More *Apoe*^{-/-} mice failed at the level of dipper training and nosepoke training compared to *Apoe*^{+/+} mice (Fisher's Exact Tests). *n* = 37 male/31 female *Apoe*^{+/+} mice and 13 male/15 female *Apoe*^{-/-} mice at the beginning of testing.

Figure 7. Impaired performance on the 5-choice serial reaction time task in *Apoe*^{+/+} mice treated with scopolamine

Mice were treated for 3 consecutive days with the following treatments 15 min prior to testing on criterion 6: saline, 1.4 mg/kg scopolamine, saline, 0.8 mg/kg scopolamine, saline, and 0.2 mg/kg scopolamine. Percent of correct responses did not differ between the saline treatments and were pooled for analysis. Data are shown as the mean percent of correct responses over the 3 days of treatment. Treatment with 1.4 mg/kg scopolamine reduced the percent of correct responses. Scop = scopolamine. * $p < 0.05$ versus treatment with saline or 0.2 mg/kg scopolamine. $n = 12$ male/7 female mice.

B_{max} and *K_d* values (mean ± S.E.M) for M₁ and M₂ muscarinic acetylcholine receptor binding in the cortex, hippocampus, and cerebellum of *Apoe*^{+/+} and *Apoe*^{-/-} mice.

Table 1

Genotype	Brain Region	<i>B_{max}</i> (fmol/mg protein)	<i>K_d</i> (pM)	<i>B_{max}</i> (fmol/mg protein)	<i>K_d</i> (pM)
<i>Apoe</i> ^{+/+}	Cortex	732.9 ± 66.5	5897.3 ± 790.4	1481.3 ± 54.5	9011.3 ± 697.8
	Hippocampus	755.6 ± 38.8	5544.7 ± 544.7	965.2 ± 82.5	11 476 ± 538.0
	Cerebellum	N.E. ^a	N.E. ^a	150.0 ± 8.5	5143.0 ± 925.6
<i>Apoe</i> ^{-/-}	Cortex	380.4 ± 32.2 *	5147.0 ± 324.2	527.3 ± 48.4 *	6235.5 ± 265.3 *
	Hippocampus	579.2 ± 13.2 *	7282.3 ± 940.9	557.7 ± 74.6 *	9958 ± 307.2 *
	Cerebellum	N.E. ^a	N.E. ^a	161.0 ± 7.7	5437.0 ± 799.2

^a N.E. = No evidence of receptor binding

^b mAChR = muscarinic acetylcholine receptor

* *p* < 0.05 by ANOVA compared to *Apoe*^{+/+} mice for the same receptor in the same brain area *n* = 3–4 experiments per brain region and receptor

Proportion of *Apoe*^{+/+} and *Apoe*^{-/-} mice dropping out compared to passing each training level of the 5-Choice Serial Reaction Time Task

Table 2

Genotype	Training Level					
	DT ^a	NP ^b	C1 ^c	C2 ^c	C3 ^c	C4 ^c
<i>Apoe</i> ^{+/+}	0/68	3/65	28/37	14/23	2/21	2/19
<i>Apoe</i> ^{-/-}	10/18	9/9	4/5	4/1	1/0	0/0
Fisher's Exact Test	**	**	NS ^d	NS ^d	NS ^d	NS ^d

^aDT = dipper training

^bNP = nosepoke training

^cC1-C4 = Criterion 1 – criterion 4

^dNS = Non-significant

Data expressed as number of mice dropping out/passing

** *p* < 0.001 by ANOVA compared to *Apoe*^{+/+} mice for the same level of training.