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Long-term effects of methamphetamine exposure on cognitive function and muscarinic acetylcholine receptor levels in mice

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Abstract

Exposure to methamphetamine during brain development impairs cognition in humans and rodents. In mice, these impairments are greater in females than males. Genetic factors, such as apolipoprotein E genotype, may modulate the cognitive effects of methamphetamine. Methamphetamine-induced alterations in the brain acetylcholine system may contribute to the cognitive effects of methamphetamine and may also be modulated by apolipoprotein E isoform. We assessed the long-term effects of methamphetamine exposure during brain development on cognitive function and muscarinic acetylcholine receptors in mice, and whether apolipoprotein E isoform modulates these effects. Mice expressing human apolipoprotein E3 or E4 were exposed to methamphetamine (5 mg/kg) or saline once a day from postnatal day 11-20 and behaviorally tested in adulthood. Muscarinic acetylcholine receptor binding was measured in the hippocampus and cortex. Methamphetamine exposure impaired novel location recognition in female, but not male, mice. Methamphetamine-exposed male and female mice showed impaired novel object recognition and increased number of muscarinic acetylcholine receptors in the hippocampus. The cognitive and cholinergic effects of methamphetamine were similar in apolipoprotein E3 and E4 mice. Thus, the cholinergic system, but not apolipoprotein E isoform, might play an important role in the long-term methamphetamine-induced cognitive deficits in adulthood.

Keywords

Methamphetamine; acetylcholine; cognition; hippocampus; apolipoprotein E; mouse

Introduction

The use of amphetamines, including methamphetamine (MA), during pregnancy has increased over the past two decades in the United States (Terplan *et al.*, 2009). Exposure to MA during brain development increases the risk of birth defects (Forrester and Merz 2007) and being born small according to gestational age (Smith *et al.*, 2006). MA exposure has also been shown to cause physiological and cognitive impairments in infants that persist into adolescence (Struthers and Hansen 1992; Chang *et al.*, 2004; Smith *et al.*, 2008). Children

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exposed to MA *in utero* show reduced spatial memory (Acevedo *et al.*, 2008a), visual motor integration, attention, and verbal memory (Chang *et al.*, 2004, 2009).

Animal studies examining the effects of MA exposure during brain development mirror the findings in humans. Rodent hippocampal development occurs during the first 3 postnatal weeks and models human hippocampal development during the third trimester (Clancy *et al.*, 2007a,b; Winzer-Serhan 2008). Wild type (WT) rats and mice exposed to MA during postnatal hippocampal development show impaired spatial learning and memory (Vorhees *et al.*, 2000, 2007; Williams *et al.*, 2002., 2003a,b; Acevedo *et al.*, 2007) and sensorimotor gating (Acevedo *et al.*, 2007) in adulthood. The long-term cognitive effects of MA at a dose of 5 mg/kg are more severe in WT female than WT male mice, suggesting enhanced sensitivity to the neurotoxic effects of MA in females (Acevedo *et al.*, 2007). This dose of MA also impairs hippocampal integrity in WT mice, as suggested by reductions in microtubule-associated protein-2 levels (Acevedo *et al.*, 2008b).

Importantly, peak levels of MA in the cortex and hippocampus do not differ between male and female wild type mice following a 5 mg/kg dose of MA at postnatal day 20 (Acevedo *et al.*, 2008). Therefore, in the current experiments, we used the same dosing and injection paradigm as used in our previous studies (Acevedo *et al.*, 2007, 2008b). Although we aimed to study effects of methamphetamine during hippocampal development, we recognize the limitations of comparing exposure of mouse or rat pups to drugs of abuse during postnatal hippocampal development to *in utero* exposure of human fetuses during prenatal hippocampal development. On the other hand, postnatal exposure does remove the potential confound of altered maternal care due to drug administration and withdrawal. Indeed, early life parental care can affect cognitive function later in life (Bredy *et al.*, 2004; Rice *et al.*, 2008). Postnatal exposure to MA also controls for potential variations in the dose of MA that each pup would receive *in utero* depending on the size of the litter. Finally, rats exposed to MA during postnatal development compared to *in utero* exposure show altered nociception in adulthood, suggesting a unique effect of MA exposure during postnatal brain development (Hruba *et al.*, 2010).

Although there are consistent effects of MA exposure during brain development on cognition, there are individual differences in the degree of these impairments. For example, despite overall group differences between MA-exposed and unexposed children on the Behavior Rating Inventory of Executive Function (BRIEF) and a measure of spatial memory, some of the MA-exposed children were rated on the BRIEF and performed as well on the spatial memory task as unexposed children (Piper, *et al.*, 2010). Individual differences in the degree of cognitive impairment following MA exposure suggest that other factors, such as genetic factors, may modulate the susceptibility to cognitive impairment following *in utero* MA exposure. Given its role in neuronal repair after injury (Mahley 1988; Arendt *et al.*, 1997), apolipoprotein E (apoE) may be one factor influencing the degree of brain damage resulting from *in utero* MA exposure. ApoE plays a role in lipid transport and metabolism in the brain and exists as 3 major isoforms in humans; apoE2, apoE3, and apoE4 (Mahley 1988). ApoE modulates responses to CNS injury (Buttini *et al.*, 1999; Lomnitski *et al.*, 1999) and might also modulate susceptibility to cognitive impairments following exposure to MA during brain development. Compared to apoE3, apoE4 is associated with an increased risk of developing Alzheimer's disease (Saunders *et al.*, 1993) and cognitive impairments following environmental challenges (Guangda *et al.*, 1999; Crawford *et al.*, 2002; Nathoo *et al.*, 2003; Brichtova and Kozak 2008). Children carrying the $\epsilon 4$ allele show impaired cognition compared to children without the $\epsilon 4$ allele (Acevedo *et al.*, 2009). Mice lacking apoE show impaired acquisition of a test of attention (Siegel *et al.*, 2009) and mice expressing apoE4 in neurons (Raber *et al.*, 1998) or astrocytes (van Meer *et al.*, 2007) show impaired spatial learning and memory.

Little research has examined the effects of apoE in the context of drugs of abuse. However, studies do show that apoE4 is associated with increased cognitive impairments in alcoholic patients with Wernicke-Korsakoff syndrome (Muramatsu *et al.*, 1997) and with smaller hippocampal volumes in female alcoholics (Bleich *et al.*, 2003). ApoE3 is associated with a history of alcohol withdrawal seizures compared to apoE2 (Wilhelm *et al.*, 2007). Furthermore, alcoholic patients carrying one ϵ 4 allele showed better visual memory than patients carrying one ϵ 2 allele (Bartres-Faz *et al.*, 2002). Mice lacking apoE show enhanced ethanol conditioned place preference compared to WT mice, suggesting that apoE modulates the rewarding effects of ethanol in a mouse model (Bechtholt *et al.*, 2004). Thus, apoE modulates the way in which alcohol affects the brain and cognition. However, the effects of apoE on MA-induced brain and cognitive impairments have not yet been examined. As previous studies in our lab have shown that MA exposure during hippocampal development alters adult cognitive function in WT mice (Acevedo *et al.*, 2007), the goal of the current study was to replicate this procedure in mice expressing apoE isoforms to determine the potential ability of apoE to modulate the effects of MA on cognition.

MA exposure during brain development affects multiple neurotransmitter systems, including the dopamine and serotonin systems (Won *et al.*, 1992, 2002; Weissman and Caldecott-Hazard 1993; Heller *et al.*, 2001a,b; Crawford *et al.*, 2003; Schaefer *et al.*, 2007). Although less well known, MA also affects the brain acetylcholine (ACh) system. MA acutely increases ACh levels in adult mice (Dobbs and Mark 2008), alters ACh receptors in cell culture and adult rats (McCabe *et al.*, 1987; Garcia-Rates *et al.*, 2007), and reduces choline acetyltransferase, the enzyme responsible for synthesizing ACh, in adult humans (Kish *et al.*, 1999; Siegal *et al.*, 2004). There are two types of ACh receptors, nicotinic and muscarinic (Caulfield and Birdsall 1998), both of which are altered by MA (McCabe *et al.*, 1987; Garcia-Rates *et al.*, 2007). The M₁ and M₂ muscarinic subtypes are expressed at high levels in the hippocampus and cortex, two areas of the brain important for cognitive function (Deacon *et al.*, 2002; Alexander *et al.*, 2007). As the ACh system is involved in cognition (van Hest *et al.*, 1990; Muir *et al.*, 1994; Voytko *et al.*, 1994; Lin *et al.*, 1998; Mirza and Stoleran 2000), potential MA-induced alterations in ACh receptors may contribute to the cognitive impairments observed following MA exposure during brain development.

The ACh system is also modulated by apoE. Mice lacking apoE show lower muscarinic ACh receptor (mAChR) binding in the cortex and hippocampus than WT mice (Siegel *et al.*, 2009) and apoE4 is associated with lower nucleus basalis neuronal activity (Salehi *et al.*, 1998) and choline acetyltransferase activity than apoE2 and apoE3 (Poirier *et al.*, 1995; Allen *et al.*, 1997; Lai *et al.*, 2006). In this study, the effects of MA exposure during hippocampal development on cognition and ACh receptor binding in adulthood were assessed in male and female mice expressing apoE3 or apoE4.

Methods

Subjects

Human *APOE* targeted replacement mice expressing human apoE3 or apoE4 under control of the mouse apoE promoter, on the C57BL/6J background (Sullivan *et al.*, 1997; Knouff *et al.*, 1999), were provided by Dr. Patrick Sullivan for breeding. Mice were housed and bred in our colony using homozygous mating. Lab chow (PicoLab Rodent Diet 20, #5053; PMI Nutrition International, St. Louis, MO) and water were freely available. Pups were weaned and group housed according to sex on postnatal day (PD) 22. Litters were provided soft foods during the injection period and 2 weeks after weaning to maintain stable weight gain. The mice were kept on a 12 hr light/dark schedule (lights on at 06:00h). Behavioral testing took place during the light cycle. 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.

Injections

(d)-MA hydrochloride (5 mg/kg), obtained from the Research Triangle Institute (Research Triangle Park, NC) through the National Institute on Drug Abuse drug supply program, was diluted with 0.9% sodium chloride (saline: SA) to the appropriate concentration. This dose was used based on previous findings that it causes long-term cognitive impairments in WT mice exposed during hippocampal development (Acevedo *et al.*, 2007). A total of 18 litters were used for the current study; 10 apoE3 litters and 8 apoE4 litters. Male and female pups were weighed and given a single i.p. injection of 0.1 mL containing MA ($n = 44$) or SA ($n = 40$) daily at 10:00h, from PD11-21, based on previous findings that exposure to MA during this period results in long-term cognitive impairments in WT mice and rats (Williams *et al.*, 2002; Acevedo *et al.*, 2007). A within-litter injection design was used to balance the number of MA and SA injections within a litter and across sexes.

Weight gain and maternal care

Due to time and resource limitations, 11 of the 18 litters were chosen at random to be included in the weight gain and maternal care measures. The pups ($n = 59$ pups; 5 apoE3 and 6 apoE4 litters) were weighed each day to measure weight gain over the injection period. These pups were individually labeled and video recorded from PD8-21. One hour video recordings were obtained at hours 1, 4, and 12 post injection time (10:00h). Maternal nursing and grooming were scored every 4 minutes within each hour. For individual pups, one point was given if nursing or grooming was observed (Bredy *et al.*, 2004) and the percent of maternal nursing and grooming out of the total possible observations was calculated and averaged across all days for each post injection hour.

Behavioral testing

Litters born within a 2-week period were combined into cohorts for behavioral testing beginning on PD90. Mice ($n = 84$) were singly housed 48 h prior to testing and all mice were tested consecutively using the following sequence of tests: novel location and novel object recognition, rotarod, pre-pulse inhibition (PPI), and the 5-choice serial reaction time task (5-SRTT). The tests are described in more detail below.

Novel location and novel object recognition—Mice were individually habituated to a lit open arena with clear Plexiglas walls (40.6 cm $\times$ 40.6 cm; Hamilton-Kinder, Poway, CA) for 5 min for 3 consecutive days. On the fourth day, the mice were trained in 5 consecutive 10 min trials. The first 3 were familiarization trials with 3 objects placed within the arena (one in each of 3 corners). For the novel location recognition test (fourth trial), one object was moved to a novel location in the arena. The same object was moved for all mice. For the novel object recognition test (fifth trial), one object was replaced with a novel object. The same object was replaced for all mice. A 4 min inter-trial interval elapsed between all trials. The time spent exploring each object during all trials was analyzed using a multiple body point video tracking system that can track the nose-point of a mouse ((Benice and Raber 2008); Ethovision XT, Noldus, Leesburg, VA). Exploration was counted when the nose was in a defined object zone 3 cm surrounding an object. The time spent exploring each object, as a percentage of the total exploration time, was calculated. The difference between the percent time spent exploring the object in the novel location (trial 4) and the percent time spent exploring the same object in its original location (trial 3) was calculated to measure novel location recognition. The percent time exploring the novel object as compared to the

two familiar objects in trial 5 was calculated to measure novel object recognition. The arena was cleaned with 70% ethanol between trials to remove residual odors.

Rotarod—Sensorimotor function was assessed using a rotarod. The rod had a diameter of 7 cm and was placed 64 cm above the floor of a chamber (Hamilton-Kinder, Poway, CA). The rod initially rotated at 5 rpm and accelerated by 5 rpm every 15 sec. Mice were tested in 3 consecutive trials for 3 days. The average length of time on the rod (latency to fall) was measured. The rod was cleaned with 70% ethanol between mice to remove residual odors.

Pre-pulse inhibition—Mice were placed in an enclosure within a startle monitor sound-attenuated chamber and startle response amplitudes were measured with a force transducer (Hamilton-Kinder, Poway, CA). Following a 5 min acclimation period, mice were exposed to 3, 40 ms acoustic stimuli (120 db). The testing phase consisted of 20 ms pre-pulses (70-80 db) followed by 50 ms delays and 40 ms acoustic stimuli (120 db). Random inter-trial intervals were used between trials (15-30 sec). PPI was calculated using the following formula: % response = $100 \times ((S-PS)/S)$, where S was the mean startle amplitude without a pre-pulse and PS was the mean startle response following a pre-pulse.

5-Choice serial reaction time task—Operant learning and attention were assessed in the 5-SRTT. First, mice ($n = 82$) were food restricted to 80% of their free-feeding weight and tested in a 2-bottle preference test for sucrose solution as previously described (Siegel *et al.*, 2009). Mice were tested for 20 min once a day in operant testing chambers ((Robbins 2002); Med Associates., St. Albans VT, USA). Sucrose solution (5%, 20 μ L) was used as the reward.

5-SRTT training and testing was performed as previously described (Siegel *et al.*, 2009), with minor modifications. For training of the mice to retrieve a reward from the magazine (magazine training), the magazine light was illuminated and the reward was dispensed. Upon consumption, the magazine light was turned off and a 15 s inter-trial interval elapsed. For training of the mice to poke their nose in a hole to receive a reward (nosepoke training), all 5 holes in the chamber were illuminated and a nosepoke into any hole resulted in reward. Following consumption and a 2 s inter-trial interval, all of the holes were re-illuminated. For magazine and nosepoke training, mice were required to perform 40 responses for 2 consecutive days before advancing to the next level. Mice that failed to learn nosepoke training (more than 15 days without advancing) were returned to magazine training.

Level testing followed nosepoke training. For level testing, one of the 5 holes was illuminated and a nosepoke into the lit hole was rewarded. An incorrect nosepoke or failure to respond during the stimulus duration of the hole illumination (omission) resulted in a 5 sec timeout (the house light was illuminated and the mice received no rewards). Mice progressed through levels 1, 2, 3, 4, 5, and 6 (20, 10, 8, 4, 2, and 1 sec stimulus duration, respectively). Mice were required to perform at least 10 correct nosepoke responses at a maximum latency of half the stimulus duration for 2 consecutive days to advance to the next level. The number of days to pass a training level and the level at which a mouse failed to learn the task (more than 15 days without advancing) were measured. Mice that failed to learn a level were taken back to nosepoke training. Subsequent failure on any level resulted in removal from testing (dropout). At level 6, attention was measured by calculating the percent of correct responses and omissions before and during treatment with scopolamine, a general mAChR antagonist. Once asymptotic performance was reached (percent correct responses with 90-110% of average percent correct from previous 5 days), the mice were given a single i.p. injection of saline or scopolamine (1.4, 0.8, or 0.2 mg/kg) each day prior to testing. All mice began with 3 consecutive days of saline injections, followed by 3 consecutive days of scopolamine 1.4 mg/kg, 3 days of saline, 3 days of scopolamine 0.8 mg/

kg, 3 days of saline, and finally 3 days of scopolamine 0.2 mg/kg. If asymptotic performance was not reached after 55 days on level 6, mice with fewer than 15 total responses for more than 60% of the days or 115 days on level 6, whichever occurred first, were removed from testing (dropouts).

Muscarinic receptor binding

Receptor saturation binding experiments were performed following the 5-SRTT, using cortical ($n = 39$) and hippocampal ($n = 48$) membrane preparations and radioligands specific for M_1 ($[^3H]$ pirenzepine) or M_2 ($[^3H]$ AF-DX-384) mAChRs (Watson *et al.*, 1986; Vaucher *et al.*, 2002; Siegel *et al.*, 2009). Following behavioral testing, bilateral hippocampus and cortex were dissected and freshly frozen. Tissue preparations were homogenized in 1 mL of ice cold buffer (140 mM NaCl, 0.2 mM KCl, 10 mM $MgCl_2$, 0.5 mM EDTA, 50 mM Tris, pH = 7.5), centrifuged at $16\,000 \times g$ for 25 min, washed in ice cold buffer, and re-centrifuged. The washing procedure was repeated 4 times and the membrane pellet was stored at $-80^\circ C$ until use. Tissue pellets were re-suspended in ice-cold binding buffer (10 mM NaH_2PO_4 , 10 mM Na_2HPO_4 , 1 mM $MgCl_2$, pH = 7.4) and protein concentrations measured (BCA protein assay kit, Pierce, Rockford IL, USA).

Tissue samples (50 μL) were incubated for 1 h in triplicate (hippocampus) or duplicate (cortex) in a total volume of 200 μL , containing binding buffer (50 μL), radioligand (50 μL), and atropine or water (50 μL). For the cortical binding, 8 different increasing concentrations of $[^3H]$ pirenzepine (312.5 pM to 40 nM) or $[^3H]$ AF-DX-384 (390.6 to 50 nM) were used. Single point saturation binding was performed for hippocampal preparations due to a limited amount of tissue (64.0 nM $[^3H]$ pirenzepine and 66.8 nM $[^3H]$ AF-DX-384). To measure non-specific binding, 2.5 μM atropine was added. Following incubation, 5 mL of ice cold binding buffer was added and tissues were bound to filter paper (Beckman, Fullerton CA, USA) using a cell harvester (Brandel, Gaithersburg MD, USA). Filters were placed in tubes and 5 mL of scintillation fluid (Optiphase 2, Perkin Elmer) was added before counting radioactivity using a beta counter (LS 6000SC Beta counter, Beckman, Fullerton CA, USA). The maximal number of binding sites (B_{max}) and the equilibrium dissociation constant (K_d ; for cortical binding only) were determined according to the Hill equation (Whiteaker *et al.*, 2000), using non-linear regression analysis and Graphpad Prism 4.0 software (Graphpad, San Diego CA, USA).

Data analysis

Analysis of Variance (ANOVA) was used to assess the effects of treatment, genotype, and sex on weight gain, performance in the behavioral tests, and the B_{max} and K_d of the mAChRs in the hippocampus and cortex. Repeated-measures ANOVA was used to assess maternal care and the effects of scopolamine administration on performance in the 5-SRTT (repeated factors: post-injection hour and scopolamine dose). Lower-bound estimates of epsilon were used for all repeated-measures analysis of variance tests. Four mice were removed from the novel location recognition analysis due to influential outlier scores (greater than two standard deviations from the mean. One cohort of mice (representing all genotypes, sexes, and treatments, $n = 16$) was removed from the novel object recognition analysis due to technical equipment problems during this phase of testing. The Kaplan-Meier method was used to assess the median training level at which mice were unable to learn the 5-SRTT (dropout level). The Fisher's Exact Test was conducted to assess dropout rates at each level of training in the 5-SRTT. Duncan's post-hoc tests were performed to compare groups where appropriate. Only significant interactions are reported. To examine the potential role of litter in mediating the effects of MA treatment, the outcome measures that showed a significant effect of MA treatment were re-analyzed with litter as the

experimental unit. Data within the text are reported as mean $\pm$ SEM. All statistical tests were conducted with a two-tailed significance alpha level of .05.

Results

Weight gain and maternal care

MA- and SA-injected pups were weighed each day during the injection period to monitor weight gain. MA-treated mice gained less weight than SA-treated mice ($F(1, 51) = 4.37, p < 0.05$; Figure 1). In addition, apoE3 mice (1.90 ± 0.15) gained less weight than apoE4 mice (3.05 ± 0.10 ; $F(1, 51) = 43.03, p < 0.01$). There was no effect of sex on weight gain during the injection period.

As maternal care can influence behavior of the offspring in rodents (Bredy *et al.*, 2004), we measured maternal feeding and grooming to assess potential differences in the maternal care that MA- and SA-exposed pups received during injections. During the baseline period (PD8-10), there was no significant main effect of treatment, sex, or genotype on maternal feeding. Similarly, during the injection period (PD11-21), there was no significant main effect of treatment, sex, or genotype on maternal feeding. However, there was a significant genotype $\times$ post-injection hour interaction ($F(1, 51) = 5.16, p < 0.05$). ApoE3 mice received more maternal feeding than apoE4 mice at both 1 hour (apoE3: 66.37 ± 1.77 ; apoE4: 61.36 ± 1.65) and 4 hours (apoE3: 70.06 ± 1.67 ; apoE4: 65.18 ± 1.35) post injection (Table 1 describes significant effects for all outcome measures). There was no difference in maternal feeding between apoE3 and apoE4 mice at 12 hours post injection.

During the baseline period, there was no effect of treatment, sex, or genotype on maternal grooming. However, there was a significant genotype $\times$ post-injection hour interaction ($F(1, 51) = 4.48, p < 0.05$). ApoE3 mice (3.73 ± 1.28) received more maternal grooming than apoE4 mice (0.82 ± 0.26) at 4 hours post injection time prior to the start of injections (PD8-10). There was no difference in baseline maternal grooming between apoE3 and apoE4 mice at 1 hour and 12 hours post injection time. There was no main effect of treatment, sex, or genotype on the percent of maternal grooming observations during the injection period (PD11-21).

Novel location and novel object recognition

The percent time spent exploring an object in a novel versus familiar location was used as a measure of novel location recognition (Benice and Raber 2008). There was no significant main effect of treatment, sex, or genotype on the total amount of time spent exploring the objects over the 5 trials (Figure 2A). There was also no significant main effect of treatment, sex, or genotype on novel location recognition. However, MA-treated female mice spent less time exploring the object in the new versus the old location than SA-treated female mice, as indicated by a significant sex $\times$ treatment interaction ($F(1, 73) = 7.04, p = .01$; Table 2). Furthermore, paired t-tests collapsed across genotype for each group showed that MA-treated female mice did not show a significant difference in the percent time exploring the object in the new versus the old location (Figure 2B), indicating impaired novel location recognition.

The percent time spent exploring a novel object was used as a measure of novel object recognition (Benice and Raber 2008). There was no significant main effect of treatment, sex, or genotype on the percent time spent exploring the novel object over the 10 minute trial (Table 2). Previous studies suggest that the majority of object exploration occurs in the first few minutes of the novel object recognition test (Dix and Aggleton 1999; Mumby *et al.*, 2002). Furthermore, the total time spent exploring the objects within each minute over the 10 minute trial decreased in the current study (repeated measures ANOVA; $F(9, 540) =$

5.85, $p < 0.01$), suggesting that most of the exploration of the novel object occurred during the first part of the trial. Therefore, we also examined novel object recognition in the first 5 minutes of the 10 minute trial. Over the first 5 minutes of the trial, MA-exposed mice spent significantly less time exploring the novel object than SA-exposed mice ($F(1, 60) = 4.48$, $p < 0.05$; Table 2). Furthermore, an ANOVA conducted with planned *a priori* contrasts between each of the objects, collapsed across genotype and sex, showed that SA-exposed mice spent more time exploring the novel object over the other two objects while MA-exposed mice did not (Figure 2C). There was no significant effect of sex or genotype on novel object recognition.

Rotarod

As motor coordination can affect performance on cognitive tests, sensorimotor function was measured using the latency to fall in the rotarod test (Soderling *et al.*, 2003). There was no significant main effect of treatment, sex, or genotype on sensorimotor function in the rotarod test (Table 2).

Pre-pulse inhibition

Sensorimotor gating was assessed using PPI. A 100% response in this test indicates complete inhibition of the startle response during pre-pulse trials. There was no significant main effect of treatment or sex on the baseline acoustic startle response or PPI. However, compared to apoE4 mice, apoE3 mice showed an increased baseline acoustic startle response to the 120 db stimulus ($F(1, 76) = 6.05$, $p < .02$; Table 2) and increased PPI ($F(1, 76) = 7.38$, $p < .01$; Table 2).

Attention behavior

All mice showed preference for a 5% sucrose solution over a 10% and 20% sucrose solution in a 2-bottle preference test ($F(2, 156) = 462.40$, $p < 0.001$; data not shown). Therefore, the 5% sucrose solution was used as the reward in the 5-SRTT. There was no significant main effect of treatment or genotype on the median dropout training level in the 5-SRTT (Kaplan-Meier analysis; $p > 0.45$ for both treatment and genotype, log-rank test). There was also no significant main effect of treatment or genotype on the dropout rates at each individual level of testing. The median dropout training level was lower among female than male mice (female median level; level 1: male median level; level 6: $p < 0.01$, log-rank test; Figure 3A). There was also a significant difference in dropout rates between male and female mice at level 1 ($p < 0.001$, Fisher's Exact Test). Female mice (53%) dropped out more than male mice (11%).

For the mice that remained in the 5-SRTT through level 6, there was no significant main effect of treatment, sex, or genotype on percent correct responses during level 6 testing prior to scopolamine injections. There was no significant main effect of treatment or sex on percent omissions during level 6 testing. However, prior to scopolamine injections, apoE3 mice showed more omissions than apoE4 mice ($F(1, 37) = 5.75$, $p < 0.02$; Table 2).

Scopolamine-induced attention impairments

The effects of scopolamine, a general mAChR antagonist, on attention were assessed during level 6 of the 5-SRTT among the mice that did not dropout. There was no significant difference in the percent correct responses or omissions during the 3 saline administration periods ($F(1, 16) = 1.06$, *NS* and $F(1, 16) = 3.64$, $p = 0.074$, respectively). Thus, percent correct responses and omissions were averaged for all saline injection days for comparisons with performance during scopolamine injections. There was an effect of scopolamine dose on the percent correct responses. For all mice, the percent correct responses were lower

during both the 1.4 mg/kg and 0.8 mg/kg scopolamine injections compared to saline injections ($F(1, 16) = 8.22, p < 0.01$; Figure 3B). There was no significant main effect of treatment, sex, or genotype on percent correct responses. However, there was a significant treatment $\times$ sex $\times$ genotype interaction ($F(1, 16) = 4.82, p < 0.05$). Thus, the effects of sex and treatment were examined in each genotype. In apoE4 mice, there was no significant effect of sex or treatment on percent correct responses during the saline and scopolamine injections. In the apoE3 mice, there was a treatment $\times$ sex interaction ($F(1, 6) = 9.23, p < 0.02$). When this interaction was explored further, there was no significant effect of treatment in female apoE3 mice, but there was a significant effect of treatment in the male mice. However, the group sizes at this point in the 5-SRTT were low (apoE3 SA male: $n = 1$; apoE3 MA male: $n = 3$), rendering this significant effect of treatment as un-interpretable.

Scopolamine treatment also affected percent omissions. For all mice, the percent omissions were higher during scopolamine 1.4 mg/kg treatment than saline, scopolamine 0.8 mg/kg, and scopolamine 0.2 mg/kg treatment ($F(1, 16) = 14.29, p < 0.01$; Figure 3C). There was no significant main effect of treatment on percent omissions following the scopolamine or saline injections. There were more omissions in male mice (75.8 ± 3.1) than female mice (61.0 ± 4.3 ; $F(1, 16) = 16.41, p < 0.01$) and in apoE3 mice (72.9 ± 3.6) than apoE4 mice (66.2 ± 4.3 ; $F(1, 16) = 9.20, p < 0.01$).

Muscarinic receptor binding

In the hippocampus, there was no main effect of sex or genotype on the number ($B_{\max}$) of M_1 mAChRs. However, MA-treated mice had more M_1 mAChRs (higher $B_{\max}$) than SA-treated mice ($F(1, 16) = 11.24, p < 0.01$; Figure 4). There was no significant main effect of treatment, sex, or genotype on the number of M_2 mAChRs in the hippocampus, although there was a trend for apoE4 mice to have more M_2 mAChRs than apoE3 mice ($F(1, 16) = 4.32, p = 0.054$; Table 3). As the mice used for the binding studies were of different ages when they finished the 5-SRTT, we also analyzed the binding data using age and 5-SRTT dropout status as a covariate. When age or dropout status were included as covariates, the results for hippocampal binding remained unchanged.

There was no significant main effect of treatment, sex, or genotype on the number or affinity (K_d) of either M_1 or M_2 mAChRs in the cortex (Table 3). When age or dropout status were included in the analyses as covariates, the results for cortical binding remained unchanged.

Litter analysis

In order to control for the potential effects of litter, we used litter as the experimental unit in the analyses where MA treatment showed a significant effect. Genotype was not included in these analyses as there were no significant interactions between MA treatment and genotype. When litter was used as the experimental unit, there was no longer a significant effect of MA treatment on weight gain (Table 4 shows effects of litter analysis for all measures). For novel object recognition, the effect of sex became significant when litter was used as the experimental unit ($F(1, 41) = 5.46, p < 0.02$), with male mice showing greater novel object recognition than female mice. The effect of MA treatment on novel object recognition remained significant. The effects of MA treatment and sex remained unchanged for novel location recognition and hippocampal M_1 mAChR $B_{\max}$ when litter was used as the experimental unit (Table 4).

Discussion

Exposure to MA during brain development causes long-term cognitive impairments in humans and rodents (Struthers and Hansen 1992; Vorhees *et al.*, 2000; Williams *et al.*,

2002., 2003a,^b; Chang *et al.*, 2004., 2009; Acevedo *et al.*, 2007, 2008a; Vorhees *et al.*, 2007.). Consistent with these findings, here we show that mice expressing human apoE3 or apoE4 have long-term cognitive impairments following MA exposure during hippocampal development. We also show that MA exposure during hippocampal development increases hippocampal M₁ mAChR levels in adulthood, supporting long-term effects of MA on the cholinergic system.

Similar to our findings in WT mice (Acevedo *et al.*, 2007), both male and female MA-exposed mice showed deficits in novel object recognition while only female MA-exposed mice showed deficits in novel location recognition. This effect was independent of apoE isoform and supports an increased susceptibility of female mice to the effects of postnatal MA on performance of a hippocampus-dependent test (Save *et al.*, 1992). The mechanism underlying this increased susceptibility in female mice is not yet understood, although data suggest that female mice metabolize MA at a slower rate than male mice, potentially increasing the neurotoxicity of MA (Acevedo *et al.*, 2008b). Alternatively, male sex hormones may protect against MA-induced insults on brain development in mice. Androgens are protective against other brain challenges, such as β -amyloid toxicity (Pike 2001) and apoE4 expression (Raber *et al.*, 2002). Future studies are warranted to determine the potential protective effect of androgens against the detrimental long-term effects of postnatal MA exposure.

The time period of MA exposure was designed to target both hippocampal and mAChR development (Aubert *et al.*, 1996; Winzer-Serhan 2008). In the mouse brain, mAChRs increase during the first three postnatal weeks of life (Aubert *et al.*, 1996), the same period during which the hippocampus develops (Bayer *et al.*, 1982; Altman and Bayer 1990; Clancy *et al.*, 2001., 2007a). Consistent with these mouse data, there is an increase in mAChRs and hippocampal development in the human fetal brain during the third trimester (Ravikumar and Sastry 1985; Rice and Barone 2000). In children, *in utero* MA exposure results in smaller hippocampal volumes (Chang *et al.*, 2004). In adult rodents, postnatal MA exposure results in altered expression of brain derived neurotrophic factor and nerve growth factor (Skelton *et al.*, 2007), decreased dendritic spine density (Williams *et al.*, 2004), and decreased microtubule-associated protein-2 levels (Acevedo *et al.*, 2008b) in the hippocampus. These findings demonstrate the long-term effects of MA exposure on the hippocampus, a brain structure important for cognitive function (Save *et al.*, 1992; Deacon *et al.*, 2002). The current results corroborate these findings and also suggest that postnatal MA exposure in mice causes long-term effects on the muscarinic receptor system. To the best of our knowledge, this is the first study to show long-term changes in the cholinergic system following MA exposure during brain development. These cholinergic changes might have important implications for MA-induced cognitive impairments. Therefore, future studies seem warranted to examine the potential effects of MA exposure on the cholinergic system at various ages and in multiple species.

The increase in hippocampal mAChRs in adulthood following postnatal MA-exposure indicate that altered mAChR function might contribute to the long-term cognitive effects of MA exposure during brain development. Increases in hippocampal M₁ mAChRs may be a compensatory mechanism for other MA-induced cholinergic changes, such as decreases in ACh synthesis or release. mAChR changes and potential changes in other areas of the cholinergic system may be directly related to the MA-induced cognitive impairments. Novel object recognition is a cortex-dependent task (Bussey *et al.*, 1999) and is disrupted by mAChR antagonism (Sambeth *et al.*, 2007). Although no changes in cortical mAChRs were found in the current experiment, potential MA-induced alterations in ACh release or choline acetyltransferase levels may relate to impaired novel object recognition in MA-exposed mice. mAChR and other cholinergic changes in the hippocampus following MA exposure

may relate to impaired novel location recognition in MA-exposed female mice. Novel location recognition is a hippocampus-dependent task (Save *et al.*, 1992) and performance in mice is impaired by mAChR antagonism (Murai *et al.*, 2007). While MA-exposed male mice showed increased hippocampal M₁ mAChRs, they did not show novel location recognition impairments, suggesting that female mice may be more sensitive to the cognitive effects of altered cholinergic function than male mice. Indeed, adult female rats show greater decreases in hippocampal ACh levels following a cholinergic insult (Hortnagl *et al.*, 1993) and show consistently lower levels of ACh release into the hippocampus (Masuda *et al.*, 2005) compared to adult male rats. In contrast to adulthood, multiple studies suggest that male rodents are more sensitive to the long-term cognitive effects of neonatal cholinergic insult than female rodents (van Hest *et al.*, 1990; Arters *et al.*, 1998; Timofeeva *et al.*, 2008). However, adolescent female rats show greater cognitive impairments following exposure to the cholinergic neurotoxin 192 IgG saporin on PD1 and 3 than male rats (Ricceri *et al.*, 2002), suggesting increased susceptibility of female rodents to cholinergic manipulations early in life under specific conditions.

Previous studies from our lab show that postnatal MA exposure increases brain histamine (HA) levels in neonates (Acevedo *et al.*, 2008b) and that increased HA might mediate the MA-induced cognitive impairments in adulthood, as blockade of HA activity blocked the long-term effects of postnatal MA (Acevedo *et al.*, 2007). The cholinergic system is modulated by the histaminergic system (for a review, see; (Bacciottini *et al.*, 2001)). HA release from the tuberomammillary nucleus to the medial septum increases ACh release from the medial septum to the hippocampus (Bacciottini *et al.*, 2001). Thus, MA-induced increases in neonatal HA and potential subsequent increases in neonatal or adult ACh may lead to long-term changes in both the HA and ACh system, thus altering ACh receptor levels and cognitive performance in adulthood. Future studies are warranted to investigate other cholinergic alterations that occur in neonates and adults following postnatal MA exposure in mice and how these changes potentially relate to MA-induced cognitive impairments and the increased susceptibility of female mice to these impairments.

There was no effect of postnatal MA exposure on PPI or attention, as assessed in the 5-SRTT. Both of these tasks are considered to be “ACh-dependent,” as altered cholinergic function affects both PPI (Jones and Shannon 2000; Ballmaier *et al.*, 2002) and 5-SRTT performance (Gill *et al.*, 2000; Mirza and Stolerma 2000; Arnold *et al.*, 2002). The long-term cholinergic effects of postnatal MA exposure may not be sufficiently severe to cause impairments in PPI and 5-SRTT performance, but such impairments might be revealed at earlier time points following MA exposure. Alternatively, the lack of cholinergic receptor changes in other brain areas, such as the cortex, may leave sensorimotor gating and attention behavior intact in adulthood following postnatal MA exposure. Although not measured in the current study, MA exposure during brain development has been shown to alter both dopaminergic (Heller *et al.*, 2001a,b; Crawford *et al.*, 2003) and serotonergic (Won *et al.*, 1992, 2002; Weissman and Caldecott-Hazard 1993; Schaefer *et al.*, 2007) function immediately after exposure and later in life in both cell cultures and rodents. As both of these neurotransmitters interact with the cholinergic system (Day and Fibiger 1992; Gaykema and Zaborszky 1996; Kenny *et al.*, 2000; Bitner and Nikkel 2002; Berlanga *et al.*, 2005), the effects of MA on cognition may be partially due to long-term changes in the dopamine and serotonin systems. Future studies are warranted to examine the interaction between these systems following exposure to MA during hippocampal development to understand potential mechanisms underlying MA-induced cognitive impairments.

In contrast to the current findings, previous studies in WT mice show impairments in PPI following postnatal MA exposure (Acevedo *et al.*, 2007). Differences in PPI impairments might suggest a protective effect of human apoE against the effects of postnatal MA

exposure on sensorimotor gating. This difference in behavioral impairments between WT and apoE3 or apoE4 mice may be a result of apoE functionality. ApoE is involved in the restoration of neuronal function and neurite outgrowth following brain injury (for a review, see; (Raber 2004)). Astrocytes synthesize apoE and secrete an apoE-cholesterol complex that circulates to injured regions within the brain where the cholesterol is used for membrane and synapse formation (Poirier 1994). Mouse apoE and human apoE may differ in trafficking and promotion of neurite outgrowth and species differences in ability of apoE to promote neurite outgrowth may account for differential brain repair and cognitive function following MA administration during brain development.

The lack of apoE effects in the current study suggests that apoE does not influence effects of MA on the brain or behavior. However, it is important to keep in mind that only one aspect of the cholinergic system was investigated in this study and that apoE isoform may modulate the effects of MA on other aspects of the cholinergic system or on other neurotransmitter systems. Relatively few studies have examined how apoE genotype might interact with the effects of drugs of abuse, and those that have focus mainly on alcohol (Muramatsu *et al.*, 1997; Bartres-Faz *et al.*, 2002; Bleich *et al.*, 2003; Bechtholt *et al.*, 2004; Wilhelm *et al.*, 2007). Thus, the current findings might contribute important information to the field of MA abuse and exposure. As the number of pregnant women using MA continues to increase (Terplan *et al.*, 2009), it is becoming more important to identify which factors influence the effects of MA on the brain and cognition in exposed children. The current findings suggest that apoE genotype may not be a factor influencing differences in cognitive impairments observed in children following exposure to MA *in utero* (Piper B. *et al.*, 2010).

Some limitations of the current study should be noted. First and foremost, only 18 litters were used, and thus each pup was considered the experimental unit. Studies have found that litter effects on the outcome measures can be large and inflate the possibility of a Type I error in statistical analyses (Holson and Pearce, 1992). Due to this limitation and based on the recommendations of Holson and Pearce (1992), we re-analyzed all analyses in which MA showed an effect with litter as the experimental unit. We found that the only effect that changed was that of MA treatment on weight. However, this is likely due to the fact that genotype was consistent within a litter, and the apoE4 mice gained more weight over the injection period than the apoE3 mice. Weight gain may also depend on how much overall feeding the dam provided to the pups. The effect of MA treatment on novel location and novel object recognition, as well as the mAChR binding, remained significant when litter was used as the experimental unit.

In summary, the results from this study show that exposure to MA during a time period equivalent to the third trimester of human fetal gestation impaired novel location recognition in female mice and impaired novel object recognition in both male and female mice. MA exposure also altered cholinergic function by increasing hippocampal M₁ mAChR levels in adulthood. As the ACh system is important for cognitive function throughout life (van Hest *et al.*, 1990; Muir *et al.*, 1994; Voytko *et al.*, 1994; Lin *et al.*, 1998; Mirza and Stolerman 2000), MA-induced ACh receptor alterations may contribute to the long-term cognitive impairments following MA exposure during brain development, especially in female mice. Future studies are warranted to examine the long-term effects of postnatal MA on other aspects of ACh function and their potential contribution to the long-term effects of postnatal MA on cognitive function.

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Figure 1.

Weight gain from postnatal day 11 to 20 in mice injected with saline or methamphetamine collapsed across apolipoprotein E genotype. There was a main effect of treatment on weight gain, with saline-exposed mice gaining more weight over the injection period compared to methamphetamine-exposed mice. * $p < 0.05$ saline-treated mice higher than methamphetamine-treated mice Data are expressed as mean + S.E.M.

Figure 2.

Percent time exploring objects and preference to explore a familiar and novel object in a novel location novel object recognition test. (A) There was no effect of methamphetamine treatment, sex, or genotype on the total amount of time spent exploring the objects over the 5 testing trials. Data are collapsed across sex and apolipoprotein E genotype. (B) All groups except the methamphetamine-treated female mice explored the object more in the new location than the old location. Data are collapsed across apolipoprotein E genotype. $*p < 0.05$ percent time spent exploring the object in the new location was greater than the percent time spent exploring the object in the old location. (C) Saline-exposed mice explored the novel object more than the other two familiar objects while methamphetamine-exposed mice

did not show this exploration preference for the novel object. Data are collapsed across sex and apolipoprotein E genotype. $*p < 0.05$ percent time spent exploring the novel object was greater than percent time exploring the other two familiar objects. Data are expressed as mean $\pm$ S.E.M.

Figure 3.

Dropout rates and performance during scopolamine injections on the 5-choice serial reaction time task. (A) Female mice dropped out of the 5-choice serial reaction time task at a lower median level (level 1) than male mice (level 6). Data are collapsed across treatment and apolipoprotein E genotype. (B) Percent of correct responses during level 6 testing were reduced by scopolamine 1.4 mg/kg and 0.8 mg/kg injections compared to saline injections in all mice. (C) Percent of omissions during level 6 testing were increased by scopolamine 1.4 mg/kg injections compared to saline, scopolamine 0.8 mg/kg, and scopolamine 0.2 mg/kg injections in all mice. * $p < 0.05$ compared to indicated treatments. Data are expressed as mean + S.E.M.

Figure 4.

M₁ muscarinic acetylcholine receptor levels in the hippocampus collapsed across apolipoprotein E genotype. Methamphetamine-treated mice had increased number (B_{max}) of M₁ muscarinic receptors in the hippocampus compared to saline-treated mice. * $p < 0.05$ methamphetamine-treated mice higher than saline-treated mice. Data are expressed as mean + S.E.M.

Table 1
Summary of Significant Effects of Treatment, Sex, and APOE Genotype

Factor	Test	Measure	Effect
MA Exposure (MA vs SA)	Weight gain	Body weight gain	Decreased in MA
	Novel location recognition	Novel location recognition	Decreased in MA-female
	Novel object recognition (5 min)	Novel object recognition	Decreased in MA
	5-SRTT	Percent correct responses during scopolamine injections	Increased in MA-apoE3
	mAChR binding	M1 hippocampal mAChRs	Increased in MA
Sex (Males vs Females)	5-SRTT	Median dropout training level	Increased in male
	5-SRTT	Dropout rates on level 1	Decreased in male
	5-SRTT	Percent correct responses during scopolamine injections	Decreased in male apoE3
	5-SRTT	Percent omissions during scopolamine injections	Increased in male
APOE Genotype (ApoE4 vs ApoE3)	Maternal care	Feeding (hours 1 and 4)	Decreased in apoE4
	Maternal care	Grooming (hour 4)	Decreased in apoE4
	Weight gain	Body weight gain	Increased in apoE4
	PPI	Baseline acoustic startle to 120 db stimulus	Decreased in apoE4
	PPI	PPI to 120 db stimulus	Decreased in apoE4
	5-SRTT	Percent omissions on level 6	Decreased in apoE4
	5-SRTT	Percent omissions during scopolamine injections	Decreased in apoE4

Note. MA = methamphetamine, SA = saline, ApoE = apolipoprotein E, 5-SRTT = 5-choice serial reaction time task, mAChR = muscarinic acetylcholine receptor, PPI = pre-pulse inhibition. All effects are mice of the same treatment, genotype, or sex compared to group listed.

Table 2

Performance on the behavioral tests

Test	Measure	Males		Females			
		ApoE3	ApoE4	ApoE3	ApoE4	ApoE3	ApoE4
		SA	MA	SA	MA	SA	MA
Novel Loc.	Novel Loc. Rec.	14.9 ± 4.2	16.8 ± 7.0	7.6 ± 4.2	13.4 ± 4.2	21.1 ± 8.3	21.2 ± 3.9
Novel Object	Novel Object Rec. 10 Min	42.11 ± 2.72	38.3 ± 4.01	49.70 ± 3.80	34.97 ± 6.12	30.46 ± 4.88	34.43 ± 6.49
Novel Object	Novel Object Rec. 5 Min	46.33 ± 4.82	39.74 ± 5.59	53.92 ± 6.41	33.96 ± 7.74	33.96 ± 5.63	30.69 ± 5.37
Rotorod	Latency to Fall (s)	60 ± 2.9	53.7 ± 2.4	56.7 ± 2.5	54.2 ± 1.0	58.9 ± 2.4	61.2 ± 2.0
PPI	Baseline Startle (N)*	2.1 ± 0.5	2.7 ± 0.5	1.5 ± 0.3	1.8 ± 0.4	1.8 ± 0.3	1.1 ± 0.4
	PPI*	67.9 ± 6.0	67.9 ± 7.2	57.3 ± 5.4	57.9 ± 8.5	68.9 ± 6.2	61.9 ± 4.4
5-SRTT	Level 6 % Omissions*	71.1 ± 1.8	70.4 ± 3.0	62.5 ± 3.9	65.9 ± 2.2	64.3 ± 4.4	69.1 ± 5.1
						65.4 ± 8.1	50.4 ± 5.2

Note. SA = saline, MA = methamphetamine, ApoE = apolipoprotein E, PPI = pre-pulse inhibition, 5-SRTT = 5-choice serial reaction time task, Loc = location, Rec = recognition.

All Measures shown as group means ± SEM.

* $p < .05$ main effect of genotype, apoE3 higher than apoE4 mice.

Table 3
Hippocampal and cortical M₁ and M₂ muscarinic receptor binding

Receptor	Males			Females		
	ApoE3			ApoE3		
	SA	MA	ApoE4	SA	MA	ApoE4
M ₂ Hipp						
B _{max}	1162.23 ± 84	1262.4 ± 113	1405.53 ± 154	1350.7 ± 38	1454.9 ± 114	1390.1 ± 90
M ₁ Cortex						
B _{max}	851.6 ± 67	882.1 ± 26	790.1 ± 115	830.9 ± 74	887.7 ± 46	926.3 ± 64
K _d	6606.0 ± 523	6958.8 ± 1754	6022.2 ± 568	10,363.4 ± 2904	6094.6 ± 743	7449.5 ± 1337
M ₂ Cortex						
B _{max}	1565.5 ± 225	1257.3 ± 29	1176.1 ± 74	1445.6 ± 258	1297.7 ± 162	1400.5 ± 88
K _d	12670.8 ± 5243	4993.3 ± 638	6226.8 ± 374	12322.6 ± 4449	6979.2 ± 1399	8495.5 ± 1394

Note. SA = saline, MA = methamphetamine, Hipp = hippocampus.

All Measures shown as group means ±

SEM.

Table 4
Summary of significant effects when litter is used as the experimental unit

Outcome Measure	Significant Factor	Without Litter	Changes With Litter	Experimental Unit
Weight Gain	Treatment		Effect of treatment no longer significant	
Novel Location Rec.	Treatment × Sex	Interaction	No change	
Novel Object Rec.	Treatment		No change in effect of treatment, effect of sex now significant	
Hipp M ₁ B _{max}	Treatment		No change	

Note. Rec = recognition, Hipp = hippocampus.