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The interactive effects of nicotinic and muscarinic cholinergic receptor inhibition on fear conditioning in young and aged C57BL/6 mice

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Abstract

Both normal aging and age-related disease, such as Alzheimer's disease, have diverse effects on forebrain-dependent cognitive tasks as well as the underlying neurobiological substrates. The purpose of the current study was to investigate if age-related alterations in the function of the cholinergic system are associated with memory impairments in auditory-cued and contextual fear conditioning. Young (2–3 months) and aged (19–20 months) C57BL/6 mice were administered scopolamine (0.1, 0.3, 0.5, or 1.0 mg/kg), a muscarinic cholinergic receptor antagonist, mecamylamine (1.0 and 2.0 mg/kg), a nicotinic cholinergic receptor antagonist, both scopolamine and mecamylamine (0.1 and 1.0 mg/kg, respectively), or saline prior to training. Training consisted of two white-noise CS (85 dB, 30 s)-footshock US (0.57 mA, 2 s) presentations. Testing occurred 48 h post-training. Scopolamine administration impaired contextual and cued fear conditioning in young and aged mice, although the aged mice were less sensitive to disruption by scopolamine. Mecamylamine did not disrupt conditioned fear in the young or aged mice. Scopolamine and mecamylamine co-administration, at doses sub-threshold for disrupting fear conditioning with separate administration, disrupted contextual and auditory-cued fear conditioning in the young mice, indicating that in the young mice the muscarinic and nicotinic cholinergic processes interact in the formation and maintenance of long-term memories for conditioned fear. Co-administration of both antagonists did not disrupt fear conditioning in the aged mice, indicating that age-related alterations in the cholinergic receptor subtypes may occur.

Keywords: Aging; Hippocampus; Amygdala; Learning; Memory; Mouse; Scopolamine; Mecamylamine; nAChRs; mAChRs; Acetylcholine

1. Introduction

An increasing body of evidence suggests that declines in cholinergic function underlie cognitive deficits and memory impairments associated with both normal aging, as well as diseases such as Alzheimer's disease (AD) (for review, see Bartus et al., 1982; Gold, 2003; Muir, 1997; Picciotto and Zoli, 2002; Woodruff-Pak and Gould, 2002). The involvement of the cholinergic system in memory can be investigated by targeting one or both of the two cholinergic receptor subtypes: nicotinic acetylcholinergic receptors (nAChRs) and muscarinic acetylcholinergic receptors (mAChRs). In humans, the nAChR agonist nicotine increases attention and facilitates memory (Warburton et al., 1992). In rodents, administration of nicotine or other nAChR agonists, such as GTS-21, enhances numerous forms of learning and memory, including aversive conditioning tasks (Gould, 2003; Gould et al., 2004; Gould and Higgens, 2003; Gould and Lommock, 2003; Gould and Wehner, 1999; Eidi et al., 2003; Zarrindast et al., 1996) as well as spatial learning tasks (Arendash et al., 1995; Brown et al., 2000; Levin and Rose, 1990; Levin et al., 1990a,b, 1993; Socci et al., 1995a,b). On the other hand, the effect of nAChR antagonism on learning and memory appears to be task specific. Administration of mecamylamine, a nAChR antagonist, impairs learning the 8-arm radial maze (Levin et al., 2002). However, in contextual fear conditioning mecamylamine blocks the enhancement of learning by nicotine but has no effect on learning when administered alone (Gould and Wehner, 1999). Thus, for some learning

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tasks nAChRs may be critically involved in the formation of memories, but in other tasks ligand-mediated effects at nAChRs may only modulate learning-related processes.

An involvement of mAChRs in learning and memory has also been demonstrated (for a review, see Power et al., 2003). Administration of mAChR agonists, such as oxotremorine, enhances inhibitory avoidance tasks in rodents (Baratti et al., 1979; Izquierdo et al., 1992). In addition, several tasks are disrupted by antagonism of mAChRs. Administration of scopolamine, a mAChR antagonist, is associated with deficits in contextual and cued fear conditioning in rats and mice (Anagnostaras et al., 1995, 1999a,b; Gale et al., 2001; Rudy, 1996; Rogers and Kesner, 2004). In addition, other forms of aversive conditioning, such as inhibitory avoidance, are also disrupted by scopolamine administration (Izquierdo et al., 1992). It has also been demonstrated that spatial learning tasks are susceptible to disruption by mAChR antagonism (Leblond et al., 2002; Maviel and Durkin, 2003). Therefore, the effect of manipulating mAChR or nAChR neurotransmission on the acquisition of numerous learning and memory tasks has established an important role of these cholinergic receptor subtypes in normal learning and memory processes.

The role of cholinergic neurotransmission in cognition has been further defined through the study of diseases, such as AD, in which cholinergic function is deficient. Alzheimer's disease is associated with a decrease in central nervous system (CNS) concentrations of choline acetyltransferase (ChAT), the enzyme involved in the production of acetycholine (Perry et al., 1977). In addition, many studies have linked deficits in cholinergic transmission with the cognitive impairments and dementia that is associated with AD (as reviewed in Bartus et al., 1982; Bartus, 2000; Gold, 2003; Muir, 1997; Woodruff-Pak and Gould, 2002). Because both normal aging (Court et al., 1997; Marutle et al., 1998; Picciotto and Zoli, 2002; Uchida et al., 1997) and AD result in decreased overall CNS cholinergic neurotransmission, the effect of age on not just one, but both subtypes of cholinergic should be examined to determine if aging processes and age-related diseases differentially alter the function of nAChRs and/or mAChRs.

A complete blockade of cholinergic transmission via concurrent antagonism of both nAChRs and mAChRs has been demonstrated previously to impair learning of aversive conditioning and spatial learning tasks. Co-antagonism of cholinergic receptors via co-administration of scopolamine and mecamylamine has been demonstrated to disrupt several spatial learning tasks, such as the radial arm maze (Leblond et al., 2002; Levin et al., 1990a,b; Levin and Rose, 1991; Maviel and Durkin, 2003) as well as the Morris Water Maze (Cozzolino et al., 1994; Riekkinen et al., 1990). In addition, scopolamine and mecamylamine co-administration had a synergistic effect (i.e., greater than additive effect in comparison to either antagonist administered alone) on disrupting passive avoidance (Riekkinen et al., 1990). Because the co-antagonism of both cholinergic receptor subtypes produces a greater than additive effect in both spatial and aversive conditioning tasks, it appears that the contributions of both cholinergic receptor subtypes are not separate but instead may interact during the formation of memories. Even though the effect of co-administration of the cholinergic antagonists scopolamine and mecamylamine has been demonstrated to be synergistic for the aforementioned learning tasks, the effect of cholinergic receptor co-antagonism has not been examined on auditory-cued or contextual fear conditioning in young or aged animals.

Of importance in the study of the contribution of changes in cholinergic function to aging-related and AD-related memory impairments is the availability of learning tasks which are sensitive to both aging and manipulation of cholinergic function. The Pavlovian fear conditioning paradigm is well suited for studying the effects of aging and the involvement of the cholinergic processes on learning for multiple reasons. First, in a previous study examining the effect of aging on fear conditioning, we found that aged C57BL/6 mice were impaired in the maintenance of memories for auditory-cued fear conditioning but were unimpaired in the initial acquisition of auditory-cued fear conditioning or contextual fear conditioning (Feiro and Gould, under review). Second, ligand-mediated effects at both nAChRs and mAChRs influence fear conditioning (Anagnostaras et al., 1995, 1999a,b; Gale et al., 2001; Gould, 2003; Gould et al., 2004; Gould and Higgens, 2003; Gould and Wehner, 1999; Rudy, 1996; Rogers and Kesner, 2004). Third, fear conditioning assesses both hippocampus-dependent and hippocampus-independent learning in the same animals, which allows for a within subject examination of two different types of learning that differ in hippocampal involvement (Anagnostaras et al., 1999a,b; Kim and Faneslow, 1992; Logue et al., 1997; Phillips and LeDoux, 1992). Therefore, the aim of the present study was to investigate the interactive effects of nAChR and mAChR antagonism on fear conditioning in young and aged mice to obtain a better understanding of how the cholinergic receptor subtypes contribute to the formation and maintenance of long-term memories for conditioned fear and how aging affects these systems.

2. Methods

2.1. Subjects

Young, 2–3 month old, male C57BL/6J mice (Jackson Labs, Bar Harbor, ME) and aged, 19–20 month old, male C57BL/6 mice (NIA/NIH) were maintained at Temple University. C57BL/6 mice were chosen over other strains because this strain has demonstrated sensitivity and proficiency for fear conditioning (Gould et al., 2001; Gould and Wehner, 1999; Paylor et al., 1994) and because NIH maintains a colony of aged C57BL/6 mice for extramural research. All mice were group housed and given ad libitum

access to food and water. The light/dark cycle of the colony was 12 h/12 h with the lights on at 7:00 a.m., and all testing occurred between 9:00 a.m. and 5:00 p.m. All procedures were approved by the Temple University Institutional Animal Care and Use Committee.

2.2. Apparatus

Training and subsequent testing of contextual learning occurred in four identical conditioning chambers (17.8×19.1×38.1 cm) housed in sound attenuating boxes (Med Associates, St. Albans, VT). The conditioning chambers consisted of clear Plexiglas panels in the front and back and stainless steel panels on both sides. Stainless steel grid floors of the chambers connected to shock scramblers and generators delivered the foot-shock US. The auditory CS was administered via speakers mounted to the side of each chamber. An IBM-PC compatible computer running MED-PC software was interfaced with the four conditioning chambers to control stimuli presentation.

Testing of the cued association, or CS, occurred in a different room in four altered chambers (20.3×22.9×20.1 cm). The altered chambers were equipped with solid plastic flooring and wall mountings that differed in shape, size, and color from the training chambers. Vanilla extract underneath each chamber provided a novel olfactory cue. Speakers mounted to the left side wall of each chamber delivered the auditory cue CS. All chambers were cleaned with 95% ethanol before and after each use.

2.3. Fear conditioning

2.3.1. Training

Twenty-four hours before training, mice were weighed and given identifying tail marks. On training day, mice were administered scopolamine, mecamylamine, both scopolamine and mecamylamine, or saline before being placed in a conditioning chamber. All groups received the same fear conditioning training protocol. Freezing was scored for 120 s prior to stimulus presentation to assess baseline levels of activity. Two trials were presented during training, each consisting of a 30-s 85 dB white noise CS followed by and co-terminating with a 2-s 0.57 mA footshock US. The two CS-US pairings were separated by a 120-s inter-trial-interval (ITI). Freezing was scored during the ITI in order to assess immediate fear associated with the training session. All mice remained in the conditioning chambers for an additional 30 s following the second CS-US pairing.

2.3.2. Testing

Testing occurred 48 h after training. This train-test interval was selected because we previously demonstrated that a 48-h interval significantly impaired fear to the CS but not the context in aged mice (Feiro and Gould, under review). Contextual fear was tested by placing mice back into the conditioning chambers and scoring for freezing in the absence of stimuli presentation for 5 min. One hour later, mice were placed in altered chambers to measure freezing to the white noise CS in the absence of conditioned contextual cues. A 3-min preCS period with no stimuli presentation was scored to assess generalized freezing in the altered environment. A 3-min CS period followed in which the white noise CS was presented and freezing was scored.

2.4. Experiment 1: Scopolamine dose–response curve

In order to determine if antagonism of mAChRs differentially disrupts cued and contextual fear conditioning in young and aged mice, scopolamine or saline was administered to young and aged C57BL/6 mice prior to training in the fear conditioning paradigm. Four different doses of scopolamine were used (0.1, 0.3, 0.5 and 1.0 mg/kg).

2.5. Experiment 2: Mecamylamine dose–response curve

In order to determine if antagonism of nAChRs prior to training in the fear conditioning paradigm differentially disrupts fear conditioning in young and mice, mecamylamine, a nicotinic receptor antagonist, or saline was administered to young and aged C57BL/6 mice. Two different doses were administered (1.0 and 2.0 mg/kg).

2.6. Experiment 3: Scopolamine and mecamylamine co-administration

In order to determine if nAChRs and mAChRs interact for acquisition and maintenance of conditioned fear, sub-threshold doses of scopolamine and mecamylamine were co-administered prior to training. Sub-threshold doses of each drug (0.1 mg/kg for scopolamine and 1.0 mg/kg for mecamylamine) were determined from Experiments 1 and 2. A scopolamine and mecamylamine co-administration group and a saline control group were tested in both young and aged mice.

2.7. Drugs

Scopolamine-HBr (Sigma, St. Louis, MO) was dissolved in physiological saline and administered via intraperitoneal injection (i.p.) 25 min prior to training. Scopolamine was administered in a series of doses (0.1, 0.3, 0.5 and 1.0 mg/kg) that produced reliable dose–response curves in previous studies (Anagnostaras et al., 1995, 1999a,b; Rudy, 1996). Mecamylamine HCl (Sigma) was also dissolved in physiological saline and administered via i.p. injection 15 min prior to training. Mecamylamine was administered in two different doses (1.0 and 2.0 mg/kg) based upon previous studies (Gould and Higgens, 2003; Gould et al., 2001; Gould and Wehner, 1999). Intraperitoneal injections of physiological saline (0.9%) were administered to the control groups either 25 or 15 min prior to training to match the drug groups. All injection volumes were 0.01 ml/g body weight.

2.8. Scoring

Levels of freezing were measured by manually scoring movement in a time-sampling procedure. Every 10 s each mouse was observed for 1 s and determined to be either freezing or moving. Freezing was defined as a total lack of movement aside from respiration.

2.9. Statistical testing

Separate two-factor (age×dose) analyses of variance (ANOVA) were performed on each behavioral measure (SPSS, version 12.0). Significant interactions were analyzed post-hoc using Bonferroni corrected MANOVA for tests on the marginal means and Tukey corrected contrasts were utilized to make pair-wise comparisons. Significant main effects were analyzed post-hoc using Tukey HSD, Dunnett, or Tukey corrected contrasts. All significant main effects of age are reported at α=0.05 level.

3. Results

3.1. Experiment 1: Scopolamine dose–response curve

3.1.1. Training

Baseline activity recorded on training day before the first CS presentation is presented in Fig. 1 as the mean percent freezing in young (A) and aged (B) mice. A 2-way (drug dose×age) ANOVA on the baseline data revealed a significant dose by age interaction [$F(4, 70)=4.04$, $p<0.05$]. Further analyses of the data revealed that overall the young mice had higher baseline activity than the aged mice: the young mice only froze 0.42% of the time before the first CS-US presentation, whereas the aged mice froze 3.33% of the time. In addition, increasing doses of scopolamine decreased baseline freezing in the aged mice but had no effect in the young mice. Post-hoc Tukey corrected contrasts revealed that within aged mice, 0.3, 0.5 and 1.0 mg/kg of scopolamine significantly lowered baseline freezing compared to saline controls [$t(70)=4.06$, $p<0.05$, $t(70)=3.55$, $p<0.05$, and $t(70)=3.55$, $p<0.05$, respectively]. Therefore, it appears that control aged mice show significantly less baseline activity than control young mice, however the age-related increase in baseline freezing is reversed with increasing doses of scopolamine. Immediate freezing, or post-shock freezing during the training session, was also analyzed using a two-way (dose×age) ANOVA, which revealed a significant dose by age interaction [$F(4, 70)=8.35$, $p<0.05$]. The significant interaction within the immediate freezing data closely resembles the baseline activity data; the aged mice demonstrated significantly higher immediate freezing within the control (saline) group compared to the young mice. In addition, as the dose of scopolamine increased within the aged mice the immediate freezing decreased, in that all four doses of scopolamine (0.1, 0.3, 0.5 and 1.0 mg/kg) were associated with significantly lower levels of immediate freezing compared to aged saline controls [$t(70)=3.64$, $p<0.05$, $t(70)=7.29$, $p<0.05$, $t(70)=8.75$, $p<0.05$ and $t(70)=8.26$, $p<0.05$, respectively]. Thus, the interactions found in both the baseline data and the immediate freezing data can be attributed to a decline in freezing with increasing doses of scopolamine in the aged mice and consistent low levels of baseline and immediate freezing in the young mice.

3.1.2. Contextual fear conditioning

Data representing freezing during the context test are presented in Fig. 1. These data are presented as mean percent freezing in young (A) and aged (B) mice administered saline or one of four doses of scopolamine. A two-way (dose×age) ANOVA was used to analyze the data and revealed a significant main effect of scopolamine dose [$F(4, 70)=8.80$, $p<0.001$] but no significant main effect of age [$F(1,70)=2.68$, $p>0.05$]. Tukey HSD tests, performed post-hoc to compare doses of scopolamine, indicated that across age freezing in the 0.3, 0.5 and 1.0 mg/kg scopolamine

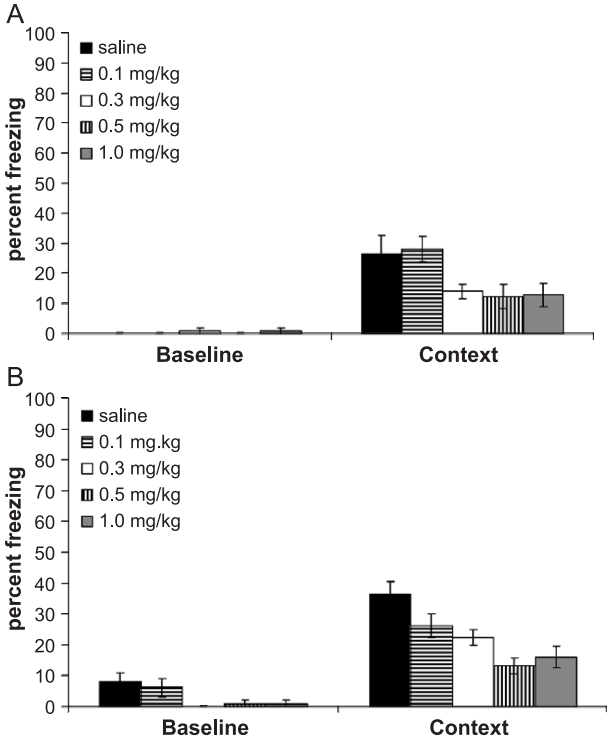

Fig. 1. The effect of scopolamine administration on contextual fear conditioning. Data are represented as mean percent freezing (±S.E.M.) in young (A) and aged (B) C57BL/6 mice during training (Baseline) and testing of contextual fear (Context). (A) The three highest doses of scopolamine (0.3, 0.5 and 1.0 mg/kg) when administered to the young mice before training significantly disrupted freezing to the context 48 h later compared to saline controls. (B) The three highest doses of scopolamine (0.3, 0.5 and 1.0 mg/kg) also disrupted freezing to the context in the aged mice compared to saline controls, demonstrating that contextual fear conditioning is uniformly disrupted by scopolamine in the young and aged C57BL/6 mice.

groups was significantly lower than freezing in the saline control or 0.1 mg/kg scopolamine groups. Therefore, it appears that the three highest doses of scopolamine (0.3, 0.5 and 1.0 mg/kg) effectively disrupt conditioned fear to the context in both young and aged C57BL/6 mice.

3.1.3. Auditory-cued fear conditioning

Data representing freezing during testing of auditory-cued fear conditioning are presented in Fig. 2 as mean percent freezing in young (A) and aged (B) mice. Freezing during the preCS period, a 3-min period without CS presentation in the altered context used to assess generalized freezing, was analyzed using a two-way (dose×age) ANOVA. The ANOVA revealed a significant main effect of age [$F(1, 70)=17.75$, $p<0.001$] but no significant main effect of scopolamine dose [$F(4, 70)=28.36$, $p>0.05$]. The main effect of age can be attributed to the fact that higher preCS period freezing was observed in the age mice (7.50%) compared to the young mice (3.06%). Thus, aged mice demonstrate more generalized freezing in an altered context than young mice.

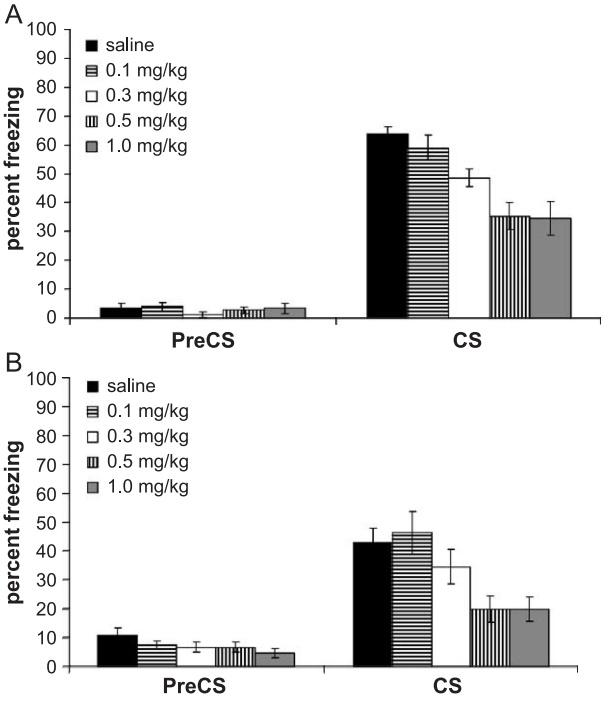

Fig. 2. The effect of scopolamine administration on auditory-cued fear conditioning. Data are represented as mean percent freezing (±S.E.M.) in young (A) and aged (B) C57BL/6 mice during testing of cued fear conditioning in an alternate context (PreCS and CS). (A) The three highest doses of scopolamine (0.3, 0.5 and 1.0 mg/kg) when administered before training, significantly disrupted freezing to the CS 48 hours later in the young mice. (B) Overall, the aged mice froze significantly less to the CS compared to the young mice. In addition, only the two highest doses of scopolamine (0.5 and 1.0 mg/kg) significantly disrupted freezing to the CS in the aged mice, demonstrating that cued fear conditioning in the young and aged C57BL/6 mice is differentially disrupted by scopolamine administration.

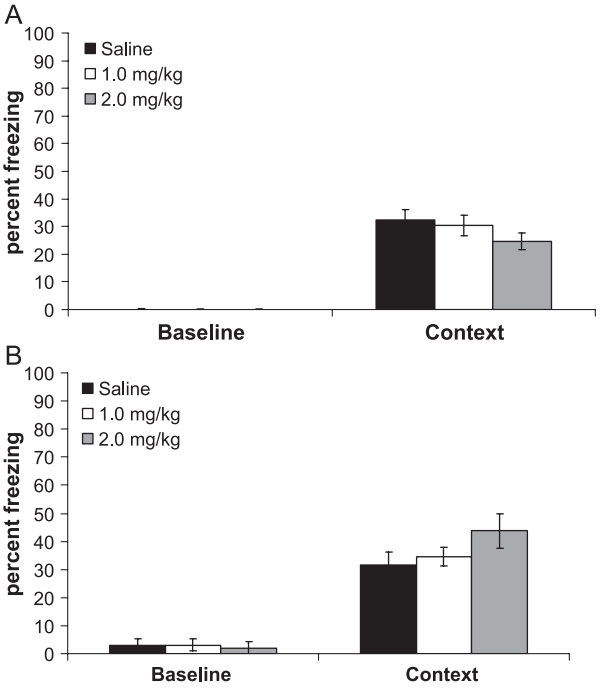

Fig. 3. The effect of mecamylamine administration on contextual fear conditioning. Data are presented as mean percent freezing (±S.E.M.) in young (A) and aged (B) C57BL/6 mice during training (Baseline) and testing of contextual fear (Context). Administration of mecamylamine (1.0 and 2.0 mg/kg) before training did not disrupt freezing to the context 48 h later compared to saline controls in the young or the aged C57BL/6 mice.

Data representing freezing during the CS presentation in the altered context is also presented in Fig. 2. A two-way (dose×age) ANOVA on freezing to the CS revealed a significant main effect of scopolamine dose [$F(4, 70)=13.54$, $p<0.001$] as well as a significant main effect of age [$F(1, 70)=24.65$, $p<0.001$]. The main effect of age is attributed to significantly higher freezing to the CS in the young mice compared to the aged mice across doses of scopolamine. Post-hoc comparisons (Tukey HSD) of scopolamine doses revealed that across age, the 0.5 and 1.0 mg/kg doses of scopolamine produced significantly lower freezing to the CS compared to saline controls. However, because there was a main effect of age, in that overall the aged mice froze less than the young mice, additional post-hoc tests were conducted to compare doses of scopolamine within age. Tukey corrected post-hoc contrasts revealed that within young mice, the 0.3, 0.5 and 1.0 mg/kg doses of scopolamine produced significantly lower freezing to the CS compared to the saline group [$t(70)=2.20$, $p<0.05$, $t(70)=4.10$, $p<0.05$ and $t(70)=4.20$, $p<0.05$, respectively]. The same post-hoc contrasts were performed within the aged mice, and the effect of scopolamine was maintained, but the dose response was shifted to the right. Only the 0.5 and the 1.0 mg/kg doses of scopolamine produced significantly lower freezing to the CS [$t(70)=3.30$, $p<0.05$, $t(70)=3.30$, $p<0.05$]. Because the 0.3 mg/kg dose significantly disrupted freezing in the young but not aged mice,

this suggests that the sensitivity of the muscarinic receptors is decreased in the aged mice.

As reported above, the aged mice demonstrated significantly increased freezing during the preCS period compared to young mice. Therefore, CS period-preCS period difference scores were calculated and analyzed to rule out the possibility that generalized freezing was contributing to the freezing measured during the CS presentation in the aged mice. Analysis of the CS period-preCS period difference scores produced results that mirrored what was found above in the analysis of the CS test data. A two-way ANOVA revealed a significant main effect of scopolamine dose [$F(4, 70)=10.78$, $p<0.001$] as well as a significant main effect of age [$F(1, 70)=38.28$, $p<0.001$]. Thus, the factoring in generalized freezing does not alter the age-related deficit in freezing to the CS.

3.2. Experiment 2: Mecamylamine dose–response curve

3.2.1. Training

Baseline activity is presented in Fig. 3 as mean percent freezing in young (A) and aged (B) mice. A two-way (dose×age) ANOVA performed on the data revealed a significant main effect of age [$F(1, 42)=4.82$, $p<0.05$] but no significant effect of mecamylamine dose [$F(2, 42)=0.09$, $p>0.05$]. The main effect of age is attributed to higher average freezing in the age mice (2.78%) compared to the young mice (0.04%). Thus for the aged mice, even though freezing of 2.78% is characterized as low for baseline activity, it was still significantly higher than the baseline activity of the young mice.

The immediate freezing data for the mecamylamine dose response was also analyzed using a two-way ANOVA. Analyses revealed a significant main effect of age [$F(1, 42)=29.01$, $p<0.001$] but no main effect of dose [$F(2, 42)=2.92$, $p>0.05$]. The main effect of age is attributable to significantly increased immediate freezing in the aged mice compared to the young mice. Thus, both baseline activity and immediate freezing are unaffected by mecamylamine administration but show significant increases in aged mice.

3.2.2. Contextual fear conditioning

Freezing measured during the test of conditioned fear to the context is presented in Fig. 3 as mean percent freezing in young (A) and aged (B) mice. A two-way (dose×age) ANOVA revealed no main effect of mecamylamine dose [$F(2, 42)=0.15$, $p>0.05$] but a significant main effect of age [$F(1, 42)=4.89$, $p<0.05$] due to higher freezing to the context in aged mice compared to young mice. Thus, mecamylamine administration did not impair freezing to the context in young or aged mice.

3.2.3. Auditory-cued fear conditioning

Data representing freezing during the preCS and CS test periods are presented in Fig. 4 as mean percent freezing in young (A) and aged (B) mice. A two-way (dose×age) ANOVA on freezing during the preCS period revealed a significant main effect of age [$F(1, 42)=19.18$, $p<0.05$] but no main effect of mecamylamine [$F(2, 42)=1.18$, $p>0.05$]. The main effect of age is due to higher freezing in the aged mice compared to the young mice, indicating age-related increases in generalized freezing in the altered context.

Freezing during the CS presentation was also analyzed using a two-way ANOVA. Analyses revealed no effect of mecamylamine dose on freezing to the CS [$F(2, 42)=0.02$, $p>0.05$] but a main effect of age [$F(1, 42)=0.98$, $p>0.05$] caused by lower overall freezing in the aged mice compared to the young mice. Thus, similar to the findings of Experiment 1, the aged mice have deficits in freezing to the CS when tested 48 h post-training regardless of mecamylamine administration.

Due to the significant main effect of age in freezing during the preCS period reported above, CS period-preCS period difference scores were calculated and analyzed. A two-way ANOVA revealed a significant main effect of age [$F(1, 42)=108.10$, $p<0.05$] but no effect of mecamylamine [$F(2, 42)=0.24$, $p>0.05$]. The main effect of age is due to lower freezing in the aged mice. These findings mirror what was found in the CS period analyses, indicating that the age-related difference in freezing

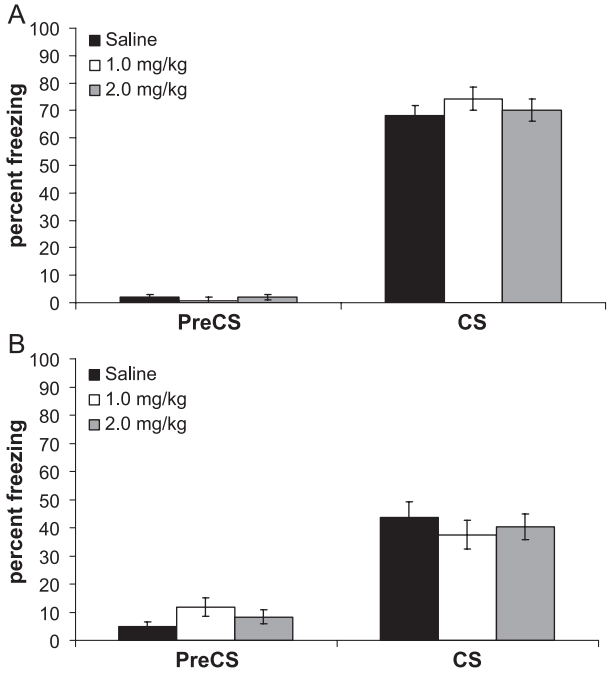

Fig. 4. The effect of mecamylamine administration on auditory-cued fear conditioning. Data are presented as mean percent freezing (±S.E.M.) in young (A) and aged (B) C57BL/6 mice during the testing of cued fear conditioning in an alternate context (PreCS and CS). Administration of mecamylamine (1.0 and 2.0 mg/kg) before training had no effect on freezing to the CS when tested 48 h later in the young or the aged C57BL/6 mice compared to saline controls.

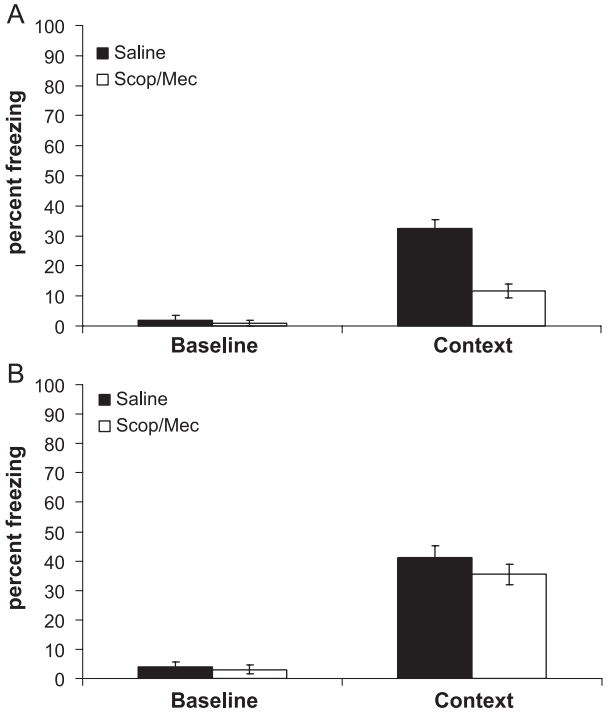

Fig. 5. The effect of scopolamine and mecamylamine co-administration on contextual fear conditioning. Data are presented as the mean percent freezing (±S.E.M.) in young (A) and aged (B) C57BL/6 mice during training (Baseline) and testing of contextual fear (Context). (A) Co-antagonism of mAChRs and nAChRs with doses of scopolamine and mecamylamine sub-threshold for disrupting learning when administered alone, significantly disrupted freezing to the context in the young mice compared to saline controls. (B) The co-administration of scopolamine and mecamylamine before training in the aged mice failed to disrupt freezing to the context compared to saline controls, demonstrating that in the young but not the aged C57BL/6 mice, co-antagonism of scopolamine and mecamylamine disrupts contextual fear conditioning.

during the CS period was not attributable to changes in generalized freezing.

3.3. Experiment 3: Scopolamine and mecamylamine co-administration

3.3.1. Training

Data representing baseline activity are presented in Fig. 5 as mean percent freezing in the young (A) and aged (B) mice. A two-way (drug×age) ANOVA conducted on the data revealed no main effect of age [$F(1, 28)=2.23$, $p>0.05$] or drug [$F(1, 28)=0.55$, $p>0.05$]. In contrast to the findings of Experiments 1 and 2, the aged mice did not demonstrate higher baseline activity compared to the young mice. In addition, the scopolamine and mecamylamine co-administration did not alter baseline activity compared to saline controls in young or aged mice.

Immediate freezing measured after the first CS-US presentation was also analyzed using a two-way (dose×age) ANOVA. Analyses revealed no effect of drug [$F(1, 28)=1.22$, $p>0.05$] but a main effect of age [$F(1, 28)=25.81$, $p<0.05$] caused by higher immediate freezing in the aged mice compared to the young mice. Thus, both baseline activity and post-shock immediate freezing were unaffected by scopolamine and mecamylamine co-administration, but immediate freezing was affected by age.

3.3.2. Contextual fear conditioning

Freezing to the context is presented in Fig. 5 as mean percent freezing in young (A) and aged (B) mice. A 2-way (drug×age) ANOVA conducted on the data revealed a significant drug by age interaction [$F(1, 28)=5.47$, $p<0.05$]. Further analyses of the data indicated that scopolamine and mecamylamine co-administration impaired freezing to the context in the young mice but did not impair freezing to the context in the aged mice. Thus, the drug by age interaction is explained by the cholinergic antagonist co-administration differentially affecting the two age groups.

3.3.3. Auditory-cued fear conditioning

Freezing during the preCS period and CS period is presented in Fig. 6 as mean percent freezing in the young (A) and aged (B) mice. A two-way (dose×age) ANOVA was conducted on freezing during the preCS period and revealed a significant main effect of age [$F(1, 28)=9.88$,

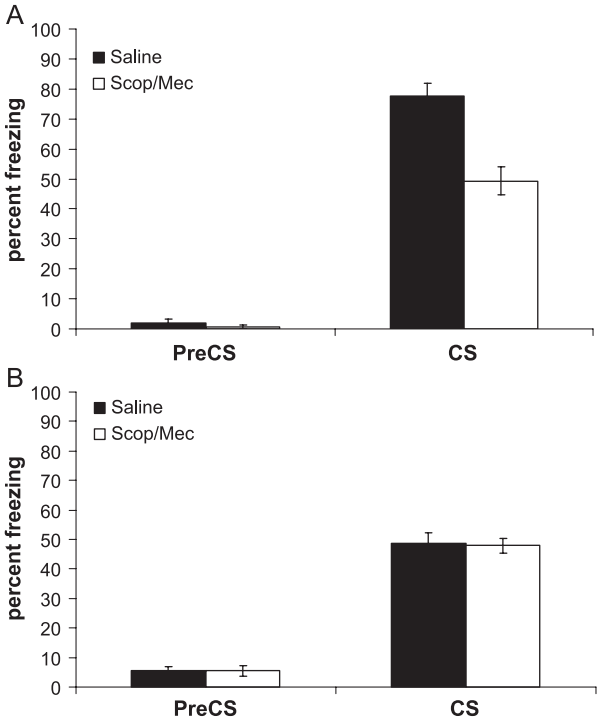

Fig. 6. The effect of scopolamine and mecamylamine co-administration on auditory-cued fear conditioning. Data are presented as mean percent freezing in young (A) and aged (B) C57BL/6 mice during testing of cued fear conditioning (PreCS and CS). (A) Co-antagonism of mAChRs and nAChRs with sub-threshold doses of scopolamine and mecamylamine significantly disrupted freezing to the CS in the young mice. (B) Co-antagonism of mAChRs and nAChRs with scopolamine and mecamylamine failed to produce a significant deficit in the aged mice compared to saline controls, demonstrating that the co-administration of sub-threshold doses of scopolamine and mecamylamine disrupts cued fear conditioning in the young but not the aged C57BL/6 mice.

$p<0.01$] but no main effect of drug [$F(1, 28)=0.28$, $p>0.05$]. Higher preCS period freezing was observed in the aged mice compared to the young mice, which is similar to the data found in Experiments 1 and 2.

Freezing data from CS presentation was also analyzed using a two-way ANOVA. Analyses revealed a significant drug by age interaction [$F(1, 28)=13.18$, $p<0.01$]. Similar to the context freezing data, scopolamine and mecamylamine co-administration impaired freezing to the CS in the young mice but had no effect in the aged mice. Thus, subthreshold doses of cholinergic antagonists impair learning in the young mice but have no effect in aged mice.

As in Experiments 1 and 2, CS period-preCS period difference scores were calculated to determine if generalized freezing was affecting freezing in the aged mice measured during the CS presentation. A two-way ANOVA conducted on the difference scores revealed a significant drug×age interaction [$F(1, 28)=10.29$, $p<0.05$] attributed to cholinergic antagonist co-administration disrupting learning in the young mice but not in the aged mice. Thus, scopolamine and mecamylamine co-administration impairs both contextual and cued fear conditioning in young but not aged mice. In addition, the difference scores replicate the CS presentation data and confirm that factoring in generalized freezing does alter the age-related differences seen.

4. Discussion

The goal of the present study was to determine if cholinergic antagonism via systemic administration of scopolamine and mecamylamine differentially affects auditory-cued and contextual fear conditioning in young and aged C57BL/6 mice. The main findings are summarized as follows: (1) Antagonism of mAChRs by scopolamine uniformly disrupts contextual fear conditioning across age but differentially disrupts auditory-cued fear conditioning in that young mice are more sensitive to the disruption of cued associations by scopolamine than aged mice. (2) Antagonism of nAChRs by mecamylamine has no effect on contextual or auditory-cued conditioned fear in young and aged mice. (3) Co-antagonism of mAChRs and nAChRs by scopolamine and mecamylamine at doses subthreshold when administered alone disrupts conditioned fear in young but not aged mice demonstrating that the effects of cholinergic antagonism on conditioned fear are differentially affected by age. In addition, the disruption of contextual and auditory-cued fear conditioning in young mice by co-antagonism with subthreshold doses of scopolamine and mecamylamine suggests that the mAChRs and nAChRs either influence similar processes or interact at the receptor level.

4.1. The effect of scopolamine on fear conditioning

The data from the scopolamine dose–response experiment demonstrate that both contextual and auditory-cued fear conditioning are disrupted by scopolamine in young and aged C57BL/6 mice. In both the young and the aged mice, scopolamine administration prior to training at the doses of 0.3, 0.5 and 1.0 mg/kg significantly reduced freezing to the context when mice were placed back into the original chamber following a 48-h train-test interval. These findings are in agreement with previous research investigating the effect of scopolamine on contextual fear conditioning in the rat (Anagnostaras et al., 1995, 1999a,b; Gale et al., 2001; Rogers and Kesner, 2004; Rudy, 1996). Anagnostaras et al. (1995, 1999a,b) found that systemic administration of scopolamine to rats prior to training dose-dependently disrupts conditioned fear to the context, with significant deficits occurring with 1.0, 10.0 and 100.0 mg/kg doses. Rudy (1996) has also demonstrated that 1.0 mg/kg of scopolamine disrupts contextual fear conditioning when administered prior to or up to 3 h post training. In addition, a recent study tested the effect of scopolamine on contextual fear conditioning by directly infusing scopolamine into the hippocampus (Gale et al., 2001). Gale et al. demonstrated that when directly infused into the hippocampus, mAChR antagonism disrupts contextual fear conditioning. These findings provide additional evidence that contextual learning is dependent on the hippocampus as well as muscarinic cholinergic neurotransmission.

Data from the present scopolamine dose–response experiment also demonstrate that scopolamine administration prior to training disrupts memories for conditioned fear to the auditory CS. In both the young and aged mice, the two highest doses of scopolamine (0.5 and 1.0 mg/kg) disrupted cued fear conditioning. Disruption of auditory-cued fear conditioning by systemic administration of scopolamine has been demonstrated previously (Anagnostaras et al., 1999a,b; Rudy, 1996; Young et al., 1995). Rudy (1996) found that 1.0 mg/kg of scopolamine administered prior training disrupted auditory-cued fear conditioning in young rats. Anagnostaras et al. (1999a,b) also found that scopolamine disrupted auditory-cued fear conditioning, but the dose required to significantly reduce freezing was much higher: 100 mg/kg. These discrepancies may be attributed to paradigm and species differences. For example, Anagnostaras et al. (1999a,b) used female rats and tested fear to the CS 1 week after training. In the present study, male C57BL/6 mice were used and testing of fear to the CS occurred 48 h after training. In addition to cued fear conditioning, scopolamine disrupts other forms of aversive conditioning, such as inhibitory avoidance. Direct infusion of scopolamine into the amygdala impairs learning of an inhibitory avoidance task (Izquierdo et al., 1992). Furthermore, direct infusion of telenzipine, a selective M1 antagonist, into the amygdala also impairs inhibitory avoidance (Power et al., 2003). Thus, mAChRs are involved in multiple amygdala-dependent aversive learning tasks.

Overall the aged mice froze significantly less during the CS test compared to the young mice. In addition, only the two highest doses of scopolamine (0.5 mg/kg and 1.0 mg/kg)

significantly disrupted freezing to the CS in the aged mice compared to young mice in which a 0.3 mg/kg dose of scopolamine also disrupted cued fear conditioning. Thus, even though systemic antagonism of mAChRs disrupts cued memories for conditioned fear in both the young and aged mice, it appears that learning in the young mice is more susceptible to disruption by mAChR antagonism with scopolamine. Thus, the dose response curve for the effects of scopolamine on fear conditioning is shifted to the right in the aged mice. This suggests that the sensitivity of the mAChRs is decreased in the aged mice. Decreased sensitivity to scopolamine antagonism in the aged mice suggests that either the function of mAChRs or the density of mAChRs within the CNS is altered by the aging process.

Studies in rodents and postmortem examination of the brains from AD patients indicate that mAChR numbers are not significantly depleted with age (Pearce and Potter, 1991; Russell, 1996). However, the function of mAChRs may be altered with age. Ayyagari et al. (1998) investigated the function of mAChRs in aged rats and found deficits in the G-protein coupled phospholipase C signal transduction cascade. In addition, Flynn et al. (1995) examined the function of specific mAChR subtypes in AD patients and found altered binding but normal receptor number in the M1 subtype of mAChRs. Thus, it appears that age-related alterations in the functioning of mAChRs could contribute to alterations in sensitivity to mAChR antagonism demonstrated in the present study.

4.2. The effect of mecamylamine on fear conditioning

Antagonism of nAChRs with systemic administration of mecamylamine did not disrupt contextual or auditory-cued fear conditioning in young or aged mice. Thus, it appears that cholinergic neurotransmission at nAChRs is not essentially involved in fear conditioning. These findings are in agreement with previous studies investigating the effects of systemic mecamylamine administration on fear conditioning. Mecamylamine administration in C57BL/6 mice blocked the enhancement of contextual fear conditioning by nicotine, but did not disrupt contextual or cued fear conditioning when administered alone (Gould and Higgens, 2003; Gould and Wehner, 1999). However, it has been demonstrated in previous studies that mecamylamine disrupts spatial learning. For example, Levin et al. (2002) found that mecamylamine administration disrupted learning in the 8-arm radial maze spatial learning task. Thus, the findings of the present study provide further support that contextual learning and spatial learning may differentially activate neural areas and involve different molecular processes (Burwell et al., 2004; Owen et al., 1997). Finally, the data from the mecamylamine experiment demonstrate that across conditions, the aged mice are impaired in freezing to the CS. This replicates the age-related deficits observed in Experiment 1, as well as in a previous study examining the effect of age on cued fear conditioning with multiple train-test intervals (Feiro and Gould, submitted for publication).

4.3. The effect of scopolamine and mecamylamine co-administration on fear conditioning

The co-administration of scopolamine and mecamylamine, at doses subthreshold for altering contextual or cued conditioned fear, disrupted fear conditioning in the young but not in the aged mice. This suggests that an interaction between both subtypes of cholinergic receptors contributes to the synaptic changes necessary to support long-term memories in conditioned fear. These findings are in agreement with several previous studies that examined co-administration of scopolamine and mecamylamine in aversive and spatial learning tasks (Cozzolino et al., 1994; Levin et al., 1989; Levin and Rose, 1991; Levin et al., 1990a,b; Little et al., 1998; Maviel and Durkin, 2003; Ragozzino et al., 1994; Riekkinen et al., 1990) Riekkinen et al. (1990) found that scopolamine and mecamylamine co-administration had a greater than additive effect on the disruption of the passive avoidance task. In addition, additive or greater than additive effects of co-antagonism of nAChRs and mAChRs have been demonstrated in the radial arm (5-arm) maze (Leblond et al., 2002; Levin et al., 1990a,b; Levin and Rose, 1991; Maviel and Durkin, 2003) as well as the Morris Water Maze (Cozzolino et al., 1994; Riekkinen et al., 1990). Thus, similar to the findings in the present study, learning of a cholinergic-dependent task is disrupted by the co-antagonism of both cholinergic receptor subtypes. Because the effect of co-antagonism of cholinergic receptors on conditioned fear in C57BL/6 mice is greater than additive, this suggests that the contributions of mAChRs and nAChRs to long-term memory are not separate processes. If the contributions of each cholinergic receptor subtype were separate, their combined effect could only be less than or equal to additive. However, because the effect of co-administration of mecamylamine and scopolamine on contextual and auditory-cued fear conditioning was greater than the combination of each antagonist's effect alone, there must be a synergism between the two receptor subtypes. In contrast to the disruption of conditioned fear by the co-administration of nAChR and mAChR antagonists in the young mice, the same treatment produced no effect on conditioned fear in the aged mice. The inability of co-antagonism of mAChRs and nAChRs to disrupt fear conditioning may indicate that either the function of the mAChRs and/or the nAChRs is affected by aging. As mentioned previously, aging does affect the function of mAChRs (Ayyagari et al., 1998; Flynn et al., 1995; Pearce and Potter, 1991; Russell, 1996). For example, aging is associated with decreased mAChR binding in response to muscarinic agonist administration, indicating decreased receptor activation in response to an agonist with age (Flynn et al., 1995; Sherman and Friedman, 1990). In addition, nAChRs are also affected in normal aging and in

AD (for a review, see Picciotto and Zoli, 2002; Woodruff-Pak and Gould, 2002). Similar to the effects of age on mAChRs, nAChRs also have decreased sensitivity to activation by a nicotinic agonist with increased age (Marutle et al., 1998; Uchida et al., 1997). Aside from the effects of normal aging on nAChR function, AD is also associated with alterations in nAChR function and transmission. Brain imaging through PET scans has revealed a loss of nicotine binding sites in the brain tissue of patients with AD, and research has demonstrated that these nicotine binding deficits in AD may be associated with decreased nAChR subunit mRNA expression (Kasa et al., 1997; Picciotto and Zoli, 2002; Terzano et al., 1998). Therefore, in normal aging and in Alzheimer's disease the function of cholinergic receptors is compromised with age. Thus, these age-associated functional alterations in mAChRs and nAChRs may disable them from acting together in an additive or synergistic fashion during learning.

5. Summary

This is the first study to demonstrate that co-antagonism of mAChRs and nAChRs with doses of scopolamine and mecamylamine subthreshold for disrupting fear conditioning when administered alone disrupt both contextual and cued fear conditioning in young mice when administered together. Previous studies have reported similar results for working memory and spatial memory (Cozzolino et al., 1994; Leblond et al., 2002; Levin et al., 1990a,b; Levin and Rose, 1991; Maviel and Durkin, 2003; Riekkinen et al., 1990). However, as these learning processes involve different neural areas (Bannerman et al., 2003; Burwell et al., 2004; Good and Honey, 1997; Logue et al., 1997; Phillips and LeDoux, 1992), different neurotransmitter receptor subtypes (El Ghundi et al., 1999; Roberts et al., 2004; Voikar et al., 2004), different cellular substrates (Graves et al., 2002; Van Dam et al., 2000; Peters et al., 2003), and different genes (Owen et al., 1997), it is important to establish what neural processes are common between different learning tasks and which are not in order to better understand the neural basis of learning and memory.

In addition, this study also found that aging alters nAChR and mAChR function; co-antagonism of mAChRs and nAChRs by scopolamine and mecamylamine disrupted fear conditioning in young but not aged mice. Previous studies have shown age-related changes in nAChR subunit mRNA expression for the $\alpha3$, $\alpha4$, $\alpha7$, $\beta2$ and $\beta3$ subunits. Thus, follow-up studies can investigate if age-related changes in specific nAChR subunits contribute to the behavior effects observed in the present study. Pharmacological inhibition is one means to examine nAChR subtype involvement. Dihydro-beta-erythroidine (DHβE) is identified as an $\alpha4\beta2$ nAChR antagonist because the nAChR subtype is highly sensitive to DHBE (Khiroug et al., 2004) but the antagonist also blocks other neuronal nAChRs that bind nicotine with high affinity including the $\alpha4\beta4$, $\alpha3\beta2$, $\alpha2\beta2$ and $\alpha2\beta4$ nAChR subtypes (Harvey et al., 1996; Williams and Robinson, 1984). The antagonist Methyllycaconitine (MLA) antagonizes $\alpha7$ nAChRs but also has effects at other nAChRs (Klink et al., 2001; Salminen et al., 2004). Thus, another approach to studying the effects of aging on specific nAChR subunits is to use nAChR subunit null mutant mice. These mice have been successfully used to assess the involvement of nAChR subunits in physiological and behavioral responses (Caldarone et al., 2000; Paylor et al., 1998; Orr-Urtreger et al., 1995, 1997; Shoaib et al., 2002; Xu et al., 1999; Salas et al., 2003; Ross et al., 2000). Currently, we are breeding nAChR subunit null mutant mice for such experiments.

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