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Nicotinic Acetylcholine Receptors and Modulation of Learning in 4- and 27-Month-Old Rabbits

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Using drugs acting on nicotinic acetylcholine receptors (nAChRs), we examined temporal-parietal and frontal cortex, hippocampus, and cerebellum to identify sites of cognition enhancement in 4- and 27-month rabbits. First, we compared radioligand receptor binding for neuronal $\alpha\beta$ heteromeric nAChRs ([^{3}H]epibatidine) and α7 homomeric nAChRs ([^{3}H]methyllycaconitine) in rabbits and rats. In cerebellum, nAChR levels of both species are low, about at the detection limit of the radioligand binding assays. Next, we compared nAChRs in 4- and 27-month vehicle-treated rabbits trained in delay eyeblink conditioning. Older rabbits conditioned more poorly and had lower $\alpha\beta$ heteromeric nAChR binding in hippocampus than young rabbits. For cognition enhancement, galantamine (mild cholinesterase inhibitor and allosteric modulator of nAChRs) or MEM-3389 (α7nAChR agonist formerly identified as AR-R 17779) was injected before conditioning. Drugs improved learning in both age groups. In 27-month rabbits, drugs increased expression of frontal and temporal-parietal $\alpha\beta$ heteromeric nAChRs and hippocampal $\alpha\beta$ and α7nAChRs. In 4-month rabbits, drugs increased expression of α7 homomeric nAChRs in frontal and temporal-parietal cortex and hippocampus, but increased expression of $\alpha\beta$ heteromeric nAChRs only occurred in temporal-parietal cortex. Increased expression of $\alpha\beta$ nAChRs was more extensive in older drug-treated rabbits, whereas increased expression of α7nAChRs was more prevalent in younger drug-treated rabbits, suggesting different substrates for amelioration (27-month rabbits) *vs* facilitation (4-month rabbits) of learning. Results provide evidence for cortical as well as hippocampal nAChR modulation of delay eyeblink conditioning and demonstrate that more sensitive binding assays are required to assess nAChR effects in cerebellum.

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INTRODUCTION

Nicotinic acetylcholine receptors (nAChRs) play a significant role in learning and memory (Changeux *et al*, 1998). During normal aging, there is a reduction in some nAChR subunits (eg, Court *et al*, 1997). A form of learning that has age-associated deficits and activates nAChRs is eyeblink classical conditioning (Woodruff-Pak *et al*, 2001). Eyeblink conditioning reveals age-related deficits in all mammalian species that have been tested, including humans. Moreover, delay eyeblink conditioning is impaired profoundly in patients with AD (Solomon *et al*, 1991; Woodruff-Pak *et al*, 1990), making the paradigm relevant for preclinical studies of cognition-enhancing drugs. In addition to parallels with human behavior and neurobiology, the model system of eyeblink classical conditioning possesses a considerable advantage over the behavioral models commonly used preclinically: the essential neural circuitry in the cerebellum has been identified (Christian and Thompson, 2003) along with circuits in hippocampus and cortex (Weible *et al*, 2007). On the basis of the documented neural circuitry for eyeblink conditioning, we focused on nAChR binding in frontal and temporal-parietal cortex, hippocampus, and cerebellum.

Much of the research on the neurobiology of classical conditioning and aging has been carried out in rabbits, yet the effects of aging on nAChRs in rabbits have not been reported in the literature. Central nervous system nAChRs are composed of five subunits (called α and β subunits) arranged around a ligand-gated excitatory ion channel. The two main categories of neuronal nAChRs are heteropentamers, constructed from combinations of α- and β-subunits (Conroy *et al*, 1992), and homopentamers, constructed from one subunit type, α7 in mammals (Couturier *et al*, 1990; Schoepfer *et al*, 1990). The most abundant nAChR subtypes in the brain appear to be: (a) those that participate in high-affinity agonist binding associated with α4 and β2 subunits and (b) those sensitive to blockade by α-bungarotoxin and containing α7 subunits. It is these two nAChR subtypes that are the focus of our investigation.

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During normal aging, there is a diminution in the expression of high-affinity binding associated with $\alpha\beta$ heterodimers that has been reported in human (eg, Giacobini, 1992) and in rodent brain (Araujo *et al*, 1990; Zhang *et al*, 1990). In human brains, there are age-related declines in α7 homomeric nAChRs in the entorhinal cortex and thalamus, but the levels in the hippocampus (Court *et al*, 1997), frontal cortex, and cerebellum (Falk *et al*, 2003) remain stable. Comparisons of CBA and C57BL/6 mice revealed that loss of α7 homomeric nAChRs in the hippocampus is strain dependent (Gahring *et al*, 2005).

A role for acetylcholine in the model system of eyeblink conditioning has been demonstrated. We focused on nAChRs because of their loss in normal aging and tested cognition-enhancing drugs targeting nAChRs. AR-R-17779 (now called MEM-3389) is an agonist shown to be selective for α7nAChRs in frog oocytes (Papke *et al*, 2004). MEM-3389 had not been tested in eyeblink conditioning in rabbits, making it necessary for us to carry out dose–response testing. We anticipated that MEM-3389 would have efficacy in this model as a partial α7 agonist improved eyeblink conditioning in older rabbits (Woodruff-Pak *et al*, 1994). Galantamine is among the most effective cognition enhancers tested in the eyeblink classical conditioning model (Weible *et al*, 2004; Simon *et al*, 2004; Woodruff-Pak *et al*, 2001, 2003). Galantamine has mechanisms of action that include both mild acetylcholinesterase inhibition and allosteric potentiating effects at nAChRs (Popa *et al*, 2006). A dose–response study on the effects of galantamine in eyeblink conditioning indicated that 3.0 mg/kg facilitated learning (Woodruff-Pak and Santos, 2000).

The initial step in this project was to identify dose(s) of MEM-3389, an agonist selective for α7nAChRs, which enhanced acquisition of conditioned eyeblink responses. Using this dose of MEM-3389 (1.0 mg/kg), 3.0 mg/kg galantamine, and vehicle and radioligand receptor binding assays for neuronal $\alpha\beta$ heteromeric nAChRs ([^{3}H]epibatidine ([^{3}H]Epi)) and α7 homomeric nAChRs ([^{3}H]methyllycaconitine ([^{3}H]MLA)), we aimed to: (a) determine binding affinity and levels of $\alpha\beta$ heteromeric and α7 homomeric nAChRs in four brain regions in young vehicle-treated rabbits and compare them to nAChR subunits at these brain sites in young rats; (b) compare binding affinity and levels of $\alpha\beta$ heteromeric and α7 homomeric nAChRs in four brain regions in 4- and 27-month-old vehicle-treated rabbits; and (c) evaluate the effects of MEM-3389 and galantamine on learning and on binding affinity and levels of $\alpha\beta$ heteromeric and α7 homomeric nAChRs in brain sites of demonstrated involvement in eyeblink conditioning in 4- and 27-month-old rabbits. Our ultimate goal was to identify sites of action of cognition-enhancing drugs.

MATERIALS AND METHODS

Study Population

Rats. Tissue from a total of 12 male Sprague–Dawley rats was used for initial nAChR binding assays to demonstrate that our techniques produced results consistent with the published literature on rodents, as there were no published studies on langomorphs. These rats were 7–8 months old.

Rabbits. A total of 112 female New Zealand white specific pathogen-free rabbits were tested. Fifty-six rabbits were retired breeders of a mean age of 26.9 (SD = 1.7) months and a mean weight of 4.1 (SD = 0.4) kg and 56 rabbits were young adults of a mean age of 4.0 (SD = 0.0) months and a mean weight of 2.9 (SD = 0.3) kg. All rabbits were purchased from Covance (Denver, PA). They were individually housed in stainless steel cages in temperature-and-humidity controlled rooms in an Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC)-approved animal facility. They had *ad lib* access to food and water. The light/dark cycle was 12/12 h. The Institutional Animal Care and Use Committee (IACUC) at Temple University approved research procedures used in this study. This research was carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health.

Behavioral Testing

Over the course of 7 days prior to training, the rabbits were gradually familiarized and adapted to Plexiglas restrainers for 30 min per day. Familiarization training took place in rabbits' individual cages during the first 5 days. At the end of each familiarization session, rabbits were rewarded with a treat formulated for rabbits (Kaytee Yogurt Dips). The last 2 days of familiarization took place outside the individual cages, and rabbits were fully restrained. On the seventh day, a local ophthalmic anesthetic (proparacaine hydrochloride) was applied to the left eye so that a 6–0 nylon suture loop could be placed in the temporal margin of the nictitating membrane (NM). Forty-eight rabbits (twenty-four 4-month, twenty-four 27-month) were tested in a dose–response study of MEM-3389 and were randomly assigned to groups with six 4-month-old and six 27-month-old rabbits at each dose (0 (vehicle), 0.3, 1.0, 3.0 mg/kg). There were 64 rabbits in the study on the effects of galantamine, MEM-3389, and vehicle on learning and nAChRs, with ten 4-month-old and ten 27-month-old rabbits in each drug treatment group and 12 rabbits in each age category in the vehicle group.

The conditioning apparatus consisted of eight separate sound-attenuating chambers, permitting up to eight rabbits to be trained simultaneously. A speaker mounted to the wall of each chamber delivered a pure tone that was used as the conditioned stimulus (CS). The headpiece, affixed behind the rabbit's ears and under its muzzle, held a plastic tube to deliver 3 psi corneal-directed air puff unconditioned stimulus (US) and a minitorque potentiometer (San Diego Instruments, San Diego, CA) to measure the rabbit's NM/eyeblink response. Elastic eyelid retractors kept the rabbit's eye open. The potentiometer was secured to the NM via a lever and the nylon suture loop. Analog output from the potentiometer was digitized, stored, and analyzed using a PC (Chen and Steinmetz, 1998). This system also controlled the timing and presentation of the stimuli. For all experiments, the intertrial interval was randomized and ranged between 20 and 30 s. A single session lasted approximately 45 min and consisted of 90 paired CS–US trials.

The paradigm was 750 ms delay eyeblink classical conditioning. We use this non-optimal interstimulus interval to provide a window of opportunity to demonstrate cognition-enhancing

drug effects in young as well as older rabbits. A 1 kHz, 85 dB SPL tone CS sounded for 850 ms followed by a 100-ms corneal air puff US that commenced 750 ms after CS onset. The rabbits received 10 training sessions (5 days per week for 2 weeks).

Changes in the position of the NM detected by the potentiometer were processed and stored in 3-ms bins by the computer. The program recorded a response when the NM moved a minimum of 0.5 mm. A conditioned response (CR) was recorded if the response occurred between 25 and 750 ms after the onset of the CS. An unconditioned response alone was recorded if the response took place more than 750 ms after the onset of the CS. A trial was eliminated if NM activity crossed the response threshold within 100 ms prior to the onset of the CS.

Drugs and Drug Administration

The drug initially called AR-R-17779 was synthesized and supplied by Memory Pharmaceuticals as MEM-3389. Galantamine hydrobromide was supplied by Janssen Pharmaceutica, NV and Ortho-McNeil Neurologics. A previous dose–response study in our laboratory identified 3.0 mg/kg as optimal for improving eyeblink conditioning in rabbits (Woodruff-Pak and Santos, 2000). Drugs were dissolved in sterile saline and administered subcutaneously (s.c.) at 1 ml/kg 30 min before behavioral testing began. There were a total of 10 injections before the 10 training sessions, and an 11th injection was administered 30 min before euthanasia so that receptor binding assays would be carried out on tissue at drug levels comparable to training.

nAChR Binding Assays

Rats. Rats did not receive any behavioral training. They were euthanized by carbon dioxide overdose, and the brains were rapidly removed and dissected into temporal-parietal and frontal cortex, hippocampus, and cerebellum. Tissue was stored at −80°C. Membrane preparation and binding assays were identical for rats and rabbits.

Rabbits. All assays on rabbit brains were from rabbits that had received behavioral testing in 750 ms delay eyeblink classical conditioning for 10 daily sessions. Three days after behavioral testing, each rabbit was injected with the drug it had received for the previous 2 weeks: 3.0 mg/kg galantamine, 1.0 mg/kg MEM-3389, or 1.0 ml/kg sterile saline. Thirty minutes after injection, the rabbit was euthanized with an overdose of pentobarbital and decapitated. The brain was rapidly removed and dissected. Frontal cortex, temporal-parietal cortex, hippocampus, and cerebellum were stored at −80°C. For binding assays, brain tissues from each of the four sites were combined for the 10 rabbits in each drug and age group.

Membrane preparation for radioligand binding assays. Membrane preparations were performed according to Davies *et al* (1999). Ice cold (10% w/v) sucrose buffer (0.32 M sucrose, 1 mM EDTA, 0.1 mM phenylmethyl sulfonyl fluoride (PMSF), 0.01% (w/v) sodium azide, pH 7.4) was added to weighed crude tissue. The tissue was homogenized in the sucrose buffer using a glass-Teflon homogenizer (10 strokes at 600 r.p.m.). The tissue homogenate was centrifuged at 20 000 g for 30 min at 4°C. The pellet was washed twice by resuspension in 10% (w/v) original weight phosphate buffer (50 mM phosphate, 1 mM EDTA, 0.1 mM PMSF, 0.01% (w/v) sodium azide, pH 7.4). The tissue was homogenized with a glass-Teflon homogenizer and centrifuged again at 20 000 g for 30 min at 4°C. The final pellet was resuspended in phosphate buffer to a concentration of ~6 ml/g original weight and homogenized with a glass-Teflon homogenizer. Protein content was determined using Pierce BCA Protein Assay Kit (Pierce Chemical Company, Rockford, IL). The prepared membrane was stored in 1.0 ml aliquots at −80°C.

Radioligand binding assay. The binding of ^{3}H-labeled ligand to membranes of rat and rabbit temporal-parietal cortex, hippocampus, cerebellum, and frontal cortex was investigated using [^{3}H]Epi and [^{3}H]MLA. The assay was performed according to Davies *et al* (1999) and Wickramaratna *et al* (2004). For [^{3}H]Epi, membranes were diluted in phosphate buffer to give a protein content of 0.5 mg in a final assay volume of 0.5 ml. Total binding of [^{3}H]Epi was determined using six concentrations from 0.03 to 1.0 nM. Radioligand concentrations were prepared by serial dilution. Nonspecific binding of [^{3}H]Epi was defined by the addition of 100 μM nicotine. For [^{3}H]MLA, membranes were diluted in phosphate buffer supplemented with 0.1% (w/v) BSA to a protein content of 0.5 mg in a final assay volume of 0.5 ml. Total binding of [^{3}H]MLA was determined using six concentrations from 0.3 to 10 nM. Nonspecific binding of [^{3}H]MLA was obtained by the addition of 300 μM nicotine or 100 μM MLA (similar nonspecific binding).

Total binding assay volumes were 0.5 ml, consisting of 0.3 ml of diluted membrane, 0.1 ml phosphate buffer for total binding or 0.1 ml of cold ligand for nonspecific binding, and 0.1 ml of radioligand. Samples were run in duplicates for both total binding and nonspecific binding. After 90 min incubation at 0°C, bound and free radioligands were separated by filtration with GF/B filters that had been soaked in a solution of 0.2% polyethylenimine, 0.1 mg BSA/ml, and 50 mM Tris for 60 min. Filters were washed three times with ice cold 1 × PBS. Radioactivity on filters was determined by liquid scintillation counting.

Specific binding was determined by the difference between nonspecific binding and total binding. Maximum binding (B_{max}) and dissociation constant (K_d), expressed in femtomole per milligram protein (fmol/mg protein) and nanomolar (nM), respectively, were determined by nonlinear regression fitting to a single-site ligand binding model found in the software Prism 3.0 (GraphPad Software Inc., San Diego, CA).

Statistical Analyses

Statistical Package for Social Sciences (SPSS) version 14 was used to carry out univariate and repeated measures analysis of variance (ANOVA) of conditioning data. There were three independent repeats for each receptor binding assay. Age and drug comparisons of B_{max} and K_d were made with one-way ANOVA for each brain site for [^{3}H]Epi and [^{3}H]MLA using Prism 3.0 (GraphPad Software Inc., San Diego, CA).

RESULTS

Dose–Response of MEM-3389

The number of trials to a criterion of eight CRs in nine consecutive trials was one of the dependent measures used to evaluate efficacy of the various doses of MEM-3389. A 2 (age) by 4 (drug dose) ANOVA indicated a statistically significant effect of drug dose, $F(3,40)=3.32$, $p=0.029$ (Figure 1). The effect of age was also significant, $F(1,40)=7.24$, $p=0.010$, but the age by drug interaction effect was not significant. A 2 (age) by 4 (drug) by 10 (training sessions) repeated measures ANOVA with percentage of CRs as the dependent measure indicated a statistically significant effect of drug dose, $F(3,40)=3.21$, $p=0.033$. The effect of age was significant, $F(1,40)=14.73$, $p<0.001$, as was the effect of training sessions, $F(9,360)=82.93$, $p<0.001$. The age by training sessions interaction effect was also significant, $F(9,360)=5.04$, $p<0.001$, but none of the other interaction effects attained statistical significance. *Post hoc* analyses of the significant drug effect indicated that a dose of 1.0 mg/kg MEM-3389 was significantly better than vehicle.

Galantamine, MEM-3389, and Learning

To compare the effect of age and cognition-enhancing drugs on eyeblink conditioning, a 2 (age) by 3 (drug) by 10 (training sessions) repeated measures ANOVA was carried out using the dependent measure of percentage of CRs. The main effects of age, drug, and training sessions were all statistically significant, $F(1,58)=25.53$, $p<0.001$; $F(2,58)=3.33$, $p=0.043$; $F(9,522)=94.13$, $p<0.001$, respectively (Figure 2a and b). Four-month-old rabbits acquired CRs at a significantly higher magnitude than 27-month-old rabbits, animals treated with galantamine or MEM-3389 acquired CRs at a significantly higher magnitude than vehicle-treated animals, and over training sessions a significant increase in CRs occurred. The interaction between age and training sessions was statistically significant, $F(9,522)=3.27$, $p<0.001$, with 4-month-old rabbits acquiring CRs over training sessions at a faster rate than 27-month-old rabbits. The three-way interaction between age, drug, and training sessions was statistically significant, $F(18,522)=2.02$, $p<0.008$. *Post hoc* analyses of the significant interaction indicated that 4- and 27-month-old rabbits treated with galantamine or MEM-3389 acquired CRs at a faster rate than vehicle-treated 4- or 27-month-old rabbits, and 4-month-old rabbits treated with galantamine and MEM-3389 performed better than 27-month-old rabbits treated with those drugs (Figure 2b). The age by drug interaction was not statistically significant.

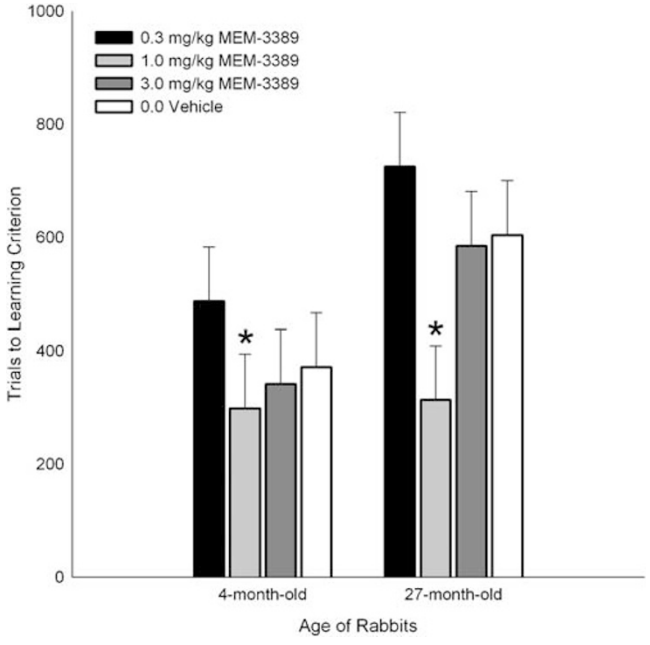

Figure 1 Trials to learning criterion in 4- and 27-month-old rabbits treated with four doses of MEM-3389. Number of trials to produce eight CRs in nine consecutive trials in rabbits treated with 0 (vehicle), 0.3, 1.0, or 3.0 mg/kg MEM-3389. There were six rabbits in each age group at each dose. A dose of 1.0 mg/kg facilitated learning significantly. Error bars are SEM. $^{*}p<0.05$.

Nicotinic Receptor Binding

αβ heteromeric and α7 homomeric nAChR binding in four brain regions in young rats and rabbits. Since [^{3}H]Epi and [^{3}H]MLA binding to nAChRs have not been performed in rabbit brains, we first performed binding in the rat brain to validate binding conditions. To determine the levels of nAChRs in young rat and young rabbit brains, we determined the B_{max} and K_d values of [^{3}H]Epi (for $\alpha\beta$ heteromeric nAChRs) and [^{3}H]MLA (for α7 homomeric nAChRs) binding to the membrane preparations of four brain regions (frontal cortex, temporal-parietal cortex, hippocampus, and cerebellum). The young vehicle-treated rabbits had been tested in delay eyeblink conditioning, whereas the rats were not tested behaviorally.

Rats: In the rat brain regions, the K_d values of [^{3}H]Epi binding for $\alpha\beta$ heteromeric nAChRs were $\sim$0.06 nM and the B_{max} values were in the range of 30–70 fmol/mg protein (Table 1). In addition, the K_d values of [^{3}H]MLA were 1.4–1.7 nM for the four rat brain regions and the B_{max} values ranged from 4 to 32 fmol/mg protein. These results are similar to published reports (Davies *et al*, 1999), and thus validated our binding conditions (Table 1).

Rabbits: In the rabbit brain regions, the K_d values of [^{3}H]Epi binding were 0.05–0.07 nM, consistent with those in the rat. The B_{max} values [^{3}H]Epi binding in rabbit are in the order of frontal cortex > temporal-parietal cortex > hippocampus > cerebellum (Table 1). The rank order in the rabbit was similar to that in the rat, but the levels were lower. In addition, the affinity of [^{3}H]MLA binding in the rabbit brain regions was similar to that in the rat brain and the B_{max} values were lower than those in the rat brain, in the order of temporal-parietal cortex > frontal cortex > hippocampus > cerebellum (Table 1). Whereas the cerebellum was a structure of interest due to its essential involvement in eyeblink classical conditioning, the expression levels of the receptors in the cerebellum were very low (about 5 fmol/mg protein), which was barely at the detection limit of the binding assays with [^{3}H]-labeled ligands. This low expression level limited our ability to detect differences between species or effects of age or drug on cerebellar nAChRs.

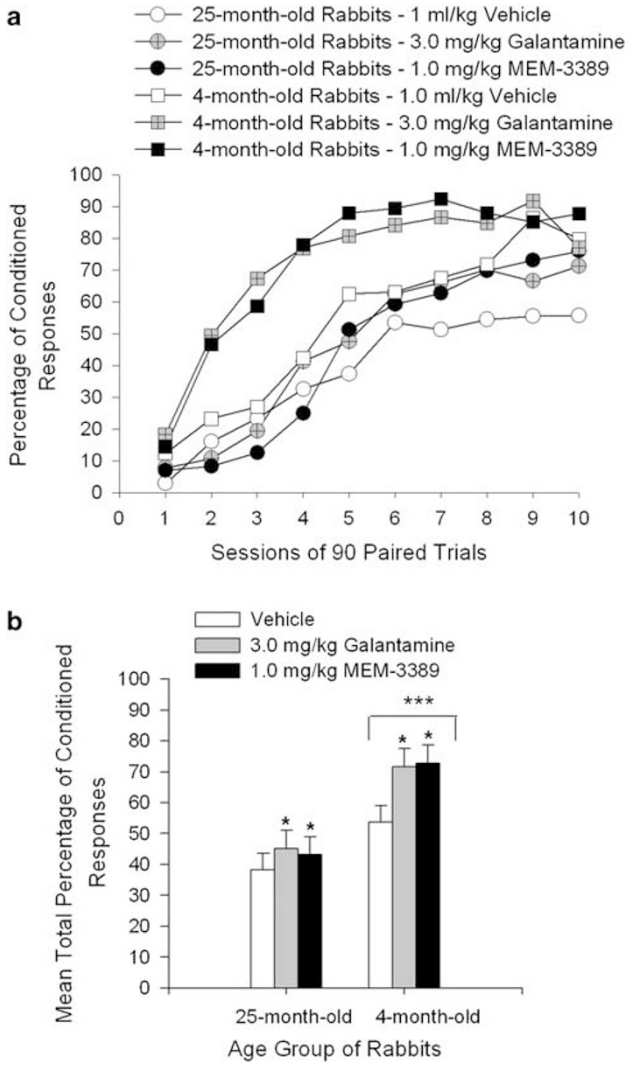

Figure 2 Eyeblink classical conditioning (750 ms delay paradigm) in 4- and 27-month-old rabbits treated with vehicle, 3.0 mg/kg galantamine, or 1.0 mg/kg MEM-3389. (a) Learning as measured with increases in CRs over 10 training sessions (90 trials/session). There were 10 rabbits in each age and drug-treatment group and 12 rabbits in the two vehicle groups. There were statistically significant age, drug, and training sessions effects on learning. (b) Mean total percentage of CRs for the data shown in (a) to illustrate the significant age and drug-treatment effects. Asterisks indicate that 4- and 27-month-old rabbits treated with galantamine or MEM-3389 acquired significantly more CRs than did vehicle-treated rabbits. Asterisks over the bar indicate that for all three treatment groups, 4-month-old rabbits acquired significantly more CRs than did 27-month-old rabbits. Error bars are SEM. $*p<0.05$; $***p<0.001$.

Levels of αβ heteromeric and α7 homomeric nAChR binding in four brain regions of vehicle-treated 4- and 27-month-old rabbits. To examine age differences in $\alpha\beta$ heteromeric nAChR binding, a one-way ANOVA was carried out using three independent repeats for each age group and brain site on B_{max} values of [^{3}H]Epi binding in four brain regions of vehicle-treated 4- and 27-month-old rabbits. There were statistically significant group differences in B_{max} values ($F(7,23)=250.3$; $p<0.0001$). *Post hoc* comparisons using the Tukey Multiple Comparison test indicated that the numerically lower values of 27-month-old rabbits in frontal cortex, temporal-parietal cortex, and cerebellum did not attain significance at the 0.05 level of confidence. In hippocampus, age differences in $\alpha\beta$ heteromeric nAChR binding assessed with [^{3}H]Epi were significant ($p<0.001$) (Figure 3a). K_d values for [^{3}H]Epi binding in each of four brain regions of vehicle-treated 4- and 27-month-old rabbits ranged from 0.03 to 0.10 nM. A one-way ANOVA indicated no differences in K_d values.

To examine age differences in α7 homomeric nAChR binding, a one-way ANOVA was carried out using three independent repeats for each age group and brain site on B_{max} values of [^{3}H]MLA binding in each of four brain regions of vehicle-treated 4- and 27-month-old rabbits. There were statistically significant group differences in B_{max} values ($F(7,23)=49.35$; $p<0.0001$). *Post hoc* comparisons using the Tukey Multiple Comparison test indicated that α7 homomeric nAChR binding as assessed with [^{3}H]MLA was significantly higher in 27-month-old rabbits in temporal-parietal cortex ($p<0.05$) and frontal cortex ($p<0.01$; Figure 3b). K_d values of [^{3}H]MLA binding to α7nAChR subunits in each of four brain regions of vehicle-treated 4- and 27-month-old rabbits ranged from 0.63 to 1.62 nM. A one-way ANOVA indicated no differences in K_d values.

Effects of chronic treatment of galantamine and MEM-3389 on αβ heteromeric nAChR binding in four brain regions of 4- and 27-month-old rabbits. Rabbits were treated with a total of 11 s.c. injections over a 2-week period with 1.0 ml/kg vehicle, 3.0 mg/kg galantamine, or 1.0 mg/kg MEM-3389. A one-way ANOVA using three independent repeats for each age group and brain site comparing drug treatment on B_{max} values of [^{3}H]Epi binding was carried out. The effect of drug treatment was statistically significant in the frontal cortex ($F(5,17)=12.15$; $p<0.0005$), temporal-parietal cortex ($F(5,17)=44.76$; $p<0.0001$), hippocampus ($F(5,17)=30.29$; $p<0.0001$), and cerebellum ($F(5,17)=3.65$; $p=0.0386$). *Post hoc* tests of the significant effects using Tukey's Multiple Comparison Test indicated that galantamine treatment significantly increased the B_{max} value of [^{3}H]Epi binding in the frontal cortex ($p<0.01$), temporal-parietal cortex ($p<0.001$) and hippocampus ($p<0.001$) of 27-month-old rabbits (Figure 4a), and the temporal-parietal cortex of 4-month-old rabbits ($p<0.05$; Figure 4b). Unanticipated effects of MEM-3389 on $\alpha\beta$ heteromeric nAChR binding assessed with [^{3}H]Epi binding were also observed. MEM-3389 has been reported to be selective for α7nAChR subunits in frog oocytes (Papke *et al*, 2004). However, in rabbits, *post hoc* tests indicated that MEM-3389 significantly increased $\alpha\beta$ heteromeric nAChR binding in temporal-parietal cortex ($p<0.001$) and hippocampus ($p<0.001$) of 27-month-old rabbits (Figure 4a), and in temporal-parietal cortex of 4-month-old rabbits ($p<0.05$; Figure 4b). Although there was a statistically significant difference in B_{max} values of [^{3}H]Epi binding in cerebellum among age and drug treatment groups, none of the *post hoc* comparisons between galantamine or MEM-3389 and vehicle achieved statistical significance. Again, the low expression level of receptors in the cerebellum, which was barely at the detection limit of the binding assays with [^{3}H]-labeled ligands, limited our ability to detect drug effects on cerebellar nAChRs.

K_d values of [^{3}H]Epi binding in each of four brain regions of 4- and 27-month-old rabbits treated with galantamine or

Table 1 Mean ± SE of the Mean of B_{max} for Three Independent Experiments Using [^{3}H]Epibatidine and [^{3}H]Methyllycaconitine Binding in Four Brain Regions in Young Naïve Rats and Young Rabbits Trained in Eyeblink Classical Conditioning

		Rats		4-month-old rabbits	
[^{3}H]ligands	**Brain regions**	**B_{max} (fmol/mg protein)**	**K_d (nM)**	**B_{max}[a] (fmol/mg protein)**	**K_d (nM)**
[^{3}H]Epi	Frontal cortex	68.1 ± 4.0	0.06 ± 0.0	56.6 ± 2.2	0.05 ± 0.0
	Temporal-parietal cortex	62.4 ± 1.8	0.06 ± 0.0	51.9 ± 0.9	0.06 ± 0.0
	Hippocampus	49.7 ± 2.9	0.05 ± 0.0	31.5 ± 0.9	0.06 ± 0.0
	Cerebellum	29.6 ± 0.7	0.06 ± 0.0	12.5 ± 1.3	0.07 ± 0.0
[^{3}H]MLA	Frontal cortex	31.83 ± 9.0	1.69 ± 1.0	16.37 ± 1.2	0.87 ± 0.2
	Temporal-parietal cortex	23.83 ± 1.9	1.38 ± 0.6	21.83 ± 0.8	0.63 ± 0.1
	Hippocampus	26.2 ± 2.8	1.48 ± 0.4	11.46 ± 1.0	1.05 ± 0.4
	Cerebellum	4.9[b]	1.5[b]	5.47 ± 2.0	1.62 ± 1.0

[a]Data also shown as young controls in Figures 3–5.
[b]One experiment.

MEM-3389 ranged from 0.03 to 0.12 nM. A one-way ANOVA indicated that K_d values of [^{3}H]Epi binding to $\alpha\beta$ heteromeric nAChR were not changed by drug treatment (data not shown). These results indicate that galantamine and MEM-3389 increase receptor number of $\alpha\beta$ heteromeric nAChRs in some rabbit brain regions without changing binding affinity of the receptors.

Effects of chronic treatment with galantamine and MEM-3389 on α7 homomeric nAChR binding in four brain regions in 4- and 27-month-old rabbits. A one-way ANOVA using three independent repeats for each age group and brain site comparing drug treatment on the B_{max} values of [^{3}H]MLA binding was carried out. The effect of drug treatment was statistically significant in the frontal cortex ($F(5, 17) = 27.69$; $p < 0.0001$), temporal-parietal cortex ($F(5, 17) = 9.87$; $p = 0.0013$), and hippocampus ($F(5, 17) = 10.10$; $p = 0.0012$), but not in cerebellum, possibly due to insensitivity of the assays. *Post hoc* tests of the significant effects using Tukey's Multiple Comparison Test indicated that B_{max} values of [^{3}H]MLA binding for 27-month-old rabbits with galantamine treatment increased only in hippocampus ($p < 0.05$; Figure 5a). Similarly, *post hoc* tests of the significant effects of [^{3}H]MLA binding in 27-month-old rabbits indicated that MEM-3389 treatment increased B_{max} values only in hippocampus ($p < 0.05$; Figure 5a). In 4-month-old rabbits, galantamine treatment was associated with increased B_{max} values of [^{3}H]MLA binding in frontal cortex ($p < 0.05$), temporal-parietal cortex ($p < 0.05$), and hippocampus ($p < 0.05$; Figure 5b). MEM-3389 treatment also increased B_{max} values of [^{3}H]MLA binding in young rabbits in frontal cortex ($p < 0.001$), temporal-parietal cortex ($p < 0.01$), and hippocampus ($p < 0.05$; Figure 5b).

K_d values of [^{3}H]MLA binding in each of four brain regions of 4- and 27-month-old rabbits treated with galantamine or MEM-3389 ranged from 0.67 to 1.45 nM. A one-way ANOVA indicated that K_d values of [^{3}H]MLA binding were not changed by these two drugs.

These results demonstrate that galantamine and MEM-3389 increase receptor level of α7 homomeric nAChRs, but not receptor binding affinity.

Learning and Nicotinic Receptor Binding Relationships

To reduce variability due to individual rabbit differences in nAChR levels, we pooled tissues from frontal cortex, temporal-parietal cortex, hippocampus, and cerebellum for each age and drug treatment. Pooling of tissue at various brain sites precluded our ability to relate individual learning performance with individual binding data. To relate learning to binding data, performance as assessed by mean total percentage of CRs for each age and drug treatment group (Figure 2b) was compared to nAChR binding in the four brain regions. Statistically significant improvement in learning in 4-month-old rabbits occurred along with significantly increased $\alpha\beta$ heteromeric nAChR binding in temporal-parietal cortex and increased α7 homomeric nAChR binding in frontal and temporal-parietal cortex and hippocampus. Statistically significant improvement in learning in 27-month-old-rabbits occurred along with significantly increased $\alpha\beta$ heteromeric nAChR binding in frontal and temporal-parietal cortex and hippocampus, and in α7 homomeric nAChR binding in hippocampus.

DISCUSSION

Chronic treatment of 4- and 27-month-old rabbits with galantamine or MEM-3389 improved learning in 750 ms delay eyeblink classical conditioning and increased levels of $\alpha\beta$ heteromeric and α7 homomeric nAChRs as assessed with [^{3}H]Epi and [^{3}H]MLA, respectively. Although epibatidine is a non-selective agonist at most neuronal $\alpha\beta$ heteromeric nAChR receptors, $\alpha4\beta2$nAChRs are the predominant type of the high-affinity nAChRs in the brain (Whiting and Lindstrom, 1986), and biological effects of epibatidine appear to be mediated largely by $\alpha4\beta2$nAChRs.

In vehicle-treated 27-month-old rabbits, there were significantly lower levels of $\alpha\beta$ heteromeric nAChRs in hippocampus, in comparison to 4-month-old rabbits, which is consistent with research in several species. Compared to 27-month-old rabbits, 4-month-old rabbits had significantly lower levels of α7 homomeric nAChRs in both frontal and temporal-parietal cortex. To the best of our knowledge,

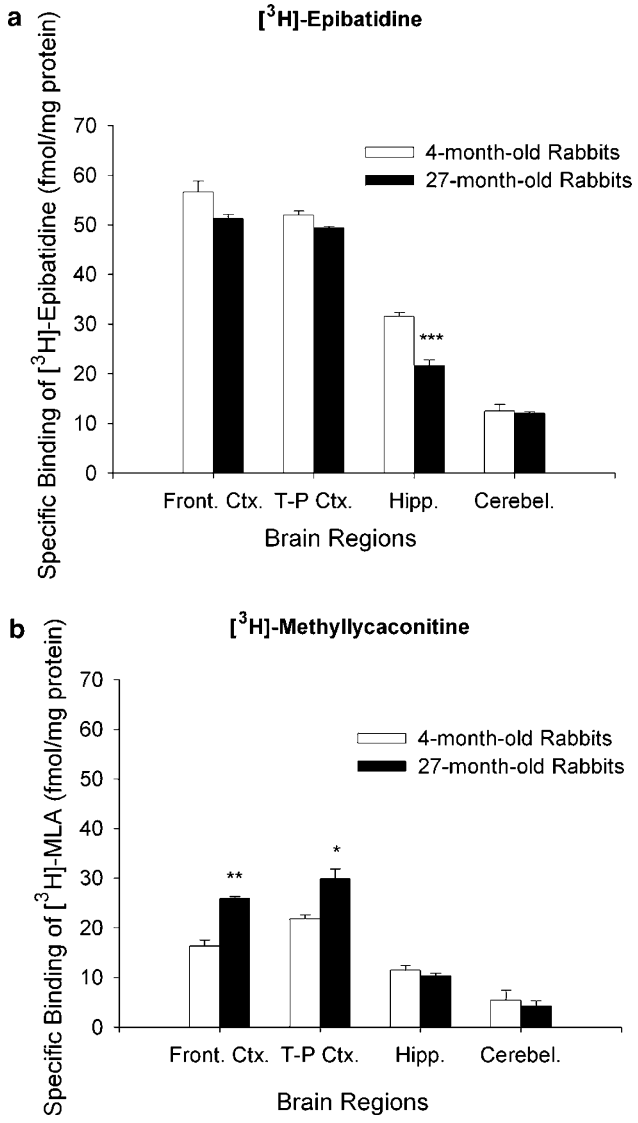

Figure 3 Comparison of [^{3}H]Epi and [^{3}H]MLA binding in four brain regions of 4- and 27-month-old rabbits. B_{max} values for (a) [^{3}H]Epi binding for $\alpha4\beta2$nAChRs and (b) [^{3}H]MLA binding for $\alpha7$nAChRs in four brain regions (frontal cortex, temporal-parietal cortex (T-P cortex), hippocampus, and cerebellum) in 4- and 27-month-old rabbits. Each bar represents the mean B_{max} value for three independent experiments. Error bars are SEM. *$p<0.05$; **$p<0.01$; ***$p<0.001$.

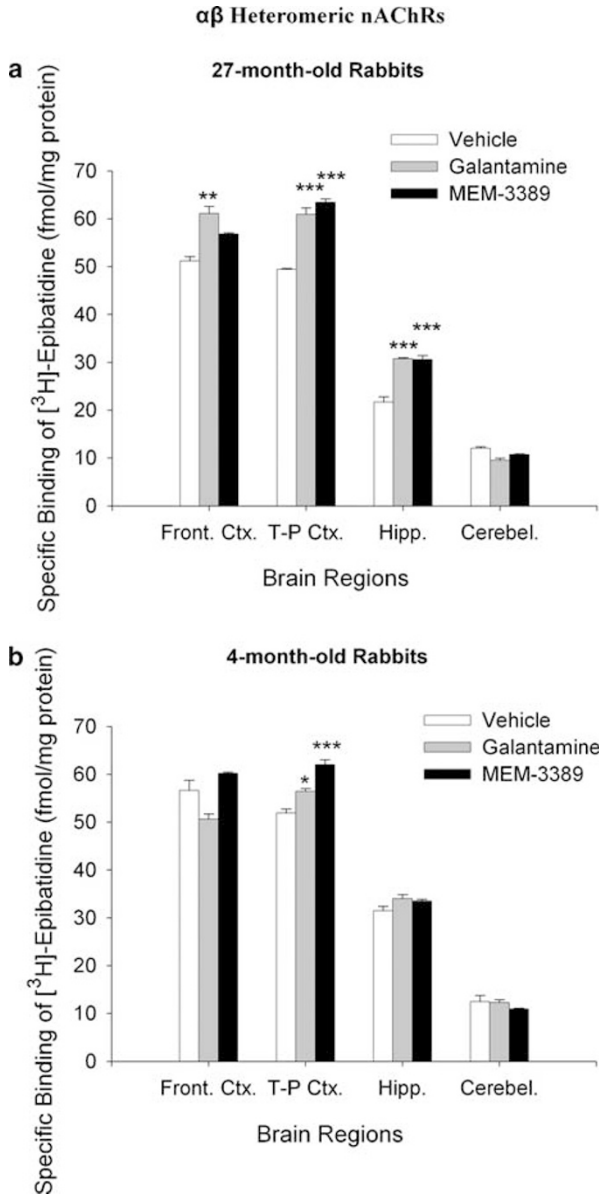

Figure 4 Effect of galantamine and MEM-3389 on expression of $\alpha4\beta2$nAChRs in four brain regions of (a) 27- and (b) 4-month-old rabbits. B_{max} values for [^{3}H]Epi binding for $\alpha4\beta2$nAChRs were determined for frontal cortex, temporal-parietal cortex (T-P cortex), hippocampus, and cerebellum. Each bar represents the mean B_{max} value from three independent experiments for galantamine, MEM-3389, and vehicle. Vehicle data are also shown in Figure 2. Error bars are SEM. *$p<0.05$; **$p<0.01$; ***$p<0.001$.

this is the first report on age differences in levels of $\alpha\beta$ heteromeric and $\alpha7$ homomeric nAChRs in rabbit brains.

Treatment with galantamine and MEM-3389 ameliorated learning impairment in 27-month-old rabbits. Significantly increased levels of $\alpha\beta$ heteromeric nAChRs and $\alpha7$ homomeric nAChRs were consistently found in the hippocampus in 27-month-old rabbits treated with galantamine and MEM-3389. In addition, galantamine treatment was associated with upregulation of $\alpha\beta$ heteromeric nAChRs in frontal and temporal-parietal cortex. MEM-3389 treatment was also associated with upregulation of $\alpha\beta$ heteromeric nAChRs in temporal-parietal cortex. Amelioration of learning impairment in older rabbits was associated with upregulation of $\alpha\beta$ heteromeric nAChRs in three brain sites along with upregulation of $\alpha7$ homomeric nAChRs in the hippocampus. In 4-month-old rabbits that are already performing at high levels, it was primarily upregulation of $\alpha7$ homomeric nAChRs that was associated with significant facilitation of learning by galantamine or MEM-3389.

Comparability of nAChR Levels in Young Rats and Rabbits

In a review of brain nAChRs, Gotti *et al* (2006) summarized the composition, localization, and number of native nAChR subunits in different brain regions of five species: humans, monkeys, rats, mice, and chickens. Species differences in cortical levels of $\alpha\beta$ heteromeric and $\alpha7$ homomeric nAChRs were substantial (approximately 10-fold). Humans and

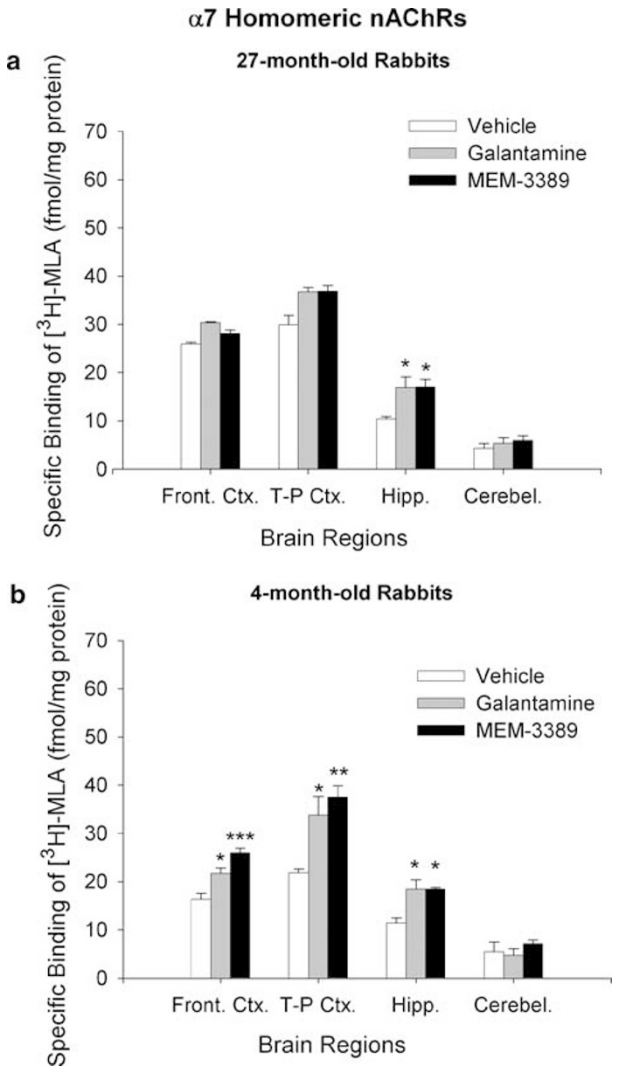

Figure 5 Effect of galantamine and MEM-3389 on levels of α7nAChRs in four brain regions of (a) 27- and (b) 4-month-old rabbits. B_{max} values of [^{3}H]MLA binding for α7nAChRs were determined for frontal cortex, temporal-parietal cortex (T-P cortex), hippocampus, and cerebellum. Each bar represents the mean B_{max} value from three independent experiments galantamine, MEM-3389, and vehicle. Vehicle data are also shown in Figures 2 and 3. Error bars are SEM. *$p<0.05$; **$p<0.01$; ***$p<0.001$.

monkeys had the lowest B_{max} values for $\alpha\beta$ heteromeric and α7 homomeric nAChRs, rodents had intermediate values, and chicks had the highest values. On this continuum, rabbits fall between monkeys and rats, but the values for rabbits are closer to those of rats. The levels of $\alpha\beta$ heteromeric nAChRs labeled with [^{3}H]Epi and α7 homomeric nAChRs labeled with [^{125}I]α-bungarotoxin or [^{3}H]MLA in rat brain regions have slight variations in different reports (Davies *et al*, 1999; Gotti *et al*, 2006). Our data show that the levels of [^{3}H]Epi and [^{3}H]MLA binding sites did not differ much between rats (rodent species) and rabbits (langomorph species; Table 1). Thus, although we compared levels of $\alpha\beta$ heteromeric and α7 homomeric nAChRs in rabbits that had been tested behaviorally to rats that were not behaviorally tested, the values were similar. It seems unlikely that eyeblink classical conditioning affected levels of $\alpha\beta$ heteromeric and α7 homomeric nAChRs in rabbits, but we did not assay the brains of untrained rabbits to determine the effect of training.

Age Differences in $\alpha\beta$ Heteromeric and α7 Homomeric nAChRs

Compared with 4-month-old rabbits, 27-month-old rabbits have significantly lower $\alpha\beta$ heteromeric nAChR binding in the hippocampus, and they have numerically lower $\alpha\beta$ heteromeric nAChR binding in frontal and temporal-parietal cortex. Our observations for $\alpha\beta$ heteromeric nAChRs are consistent with studies in a number of species. During normal aging, there is a diminution in the expression of high-affinity binding sites in the brain that has been reported in humans (eg, Giacobini, 1992), monkeys (Wagster *et al*, 1990), and rodents (Araujo *et al*, 1990; Birtsch *et al*, 1997; Zhang *et al*, 1990). In mice, in the CBA strain, there was a dramatic loss of $\alpha\beta$ heteromeric nAChRs in the 28-month-old hippocampus (Gahring *et al*, 2005; Rogers *et al*, 1998).

The 27-month-old rabbits in this study had significantly higher α7 homomeric nAChR binding in frontal and temporal-parietal cortex in contrast to numerically lower α7nAChR binding in hippocampus and cerebellum. Most studies of α7nAChRs report age-related stability in these receptors in the four brain regions examined in our research. Human α7nAChRs showed stability from the fetal period to late old age in frontal cortex and cerebellum (Falk *et al*, 2003), and in hippocampus (Court *et al*, 1997; Nordberg and Winblad, 1986). Comparisons of CBA and C57BL/6 mice revealed that loss of hippocampal α7 homomeric nAChRs is strain dependent, with C57BL/6 mice showing stability at 28 months and with CBA mice showing dramatic loss (Gahring *et al*, 2005). We found no reports of age-related increases in α7nAChRs in any species. Our observation of statistically significant higher B_{max} values of [^{3}H]MLA binding in frontal and temporal-parietal cortex in 27-month-old rabbits was reliable over three independent repeated experiments (Figure 3). However, the results are based on 10 female 27-month-old rabbits. Data on male and female rabbits, younger and older than 27 months, are required to determine whether there is stability in α7 homomeric nAChRs in New Zealand white rabbits or age-related increment and later-life decline in relation to 27-month-old values.

Galantamine and nAChRs

Galantamine increased receptor levels of both $\alpha\beta$ heteromeric and α7 homomeric nAChRs in at least one age group of rabbits in frontal and temporal-parietal cortex and hippocampus (Figures 4 and 5). These results are consistent with previous results in 29-month-old rabbits treated with 16 s.c. injections of 3.0 mg/kg galantamine that showed increased expression of cortical $\alpha\beta$ heteromeric nAChRs labeled by [^{3}H]Epi (Woodruff-Pak *et al*, 2001). Other investigators have reported associations between galantamine administration and nAChR upregulation. Chronic treatment of old rats with galantamine increased B_{max} for [^{3}H]nicotine binding in frontal cortex and hippocampus (Barnes *et al*, 2000). In rat cortical culture, galantamine increased the number of cells expressing $\alpha\beta$ heteromeric and α7 homomeric nAChR subunits (Kume *et al*, 2005). Using the Morris water maze and receptor autoradiography,

Hernandez *et al* (2006) demonstrated that galantamine enhanced spatial learning in rats and increased $\alpha\beta$ heteromeric nAChR binding.

MEM-3389 also Affected $\alpha\beta$ Heteromeric nAChRs

MEM-3389 significantly upregulated [^{3}H]Epi binding sites in cortex and hippocampus in 27-month-old rabbits and in temporal-parietal cortex in 4-month-old rabbits (Figure 4), in addition to upregulating [^{3}H]MLA binding sites in hippocampus and cortex (Figure 5). These results indicate that MEM-3389 may affect not only α7nAChRs directly but also $\alpha\beta$ heteromeric nAChRs in either a direct or indirect manner. Curiously, the α7nAChR agonist DMAC also increased [^{3}H]Epi binding sites in SH-SY5Y cells following 4 days of treatment (Ridley *et al*, 2001).

Aging, Eyeblink Conditioning, and the Cerebellum

Normal aging affects mammalian eyeblink conditioning through age-related deficits in the cerebellum (eg, Woodruff-Pak, 2006). It has been assumed that drugs that ameliorate impaired acquisition of CRs do so through basal forebrain cholinergic mechanisms via the hippocampus to the cerebellum. Additional evidence for the role of cortical structures in ameliorating impaired conditioning is provided with data from the present study. Older rabbits had poorer learning and lower $\alpha\beta$ heteromeric nAChR binding in hippocampus than 4-month rabbits. Both galantamine and MEM-3389 ameliorated impaired learning in 27-month rabbits and increased expression of temporal-parietal and hippocampal $\alpha\beta$ heteromeric nAChRs and hippocampal homomeric α7nAChRs. The major basal forebrain acetylcholine projections are to cortex, including medial temporal lobes and hippocampus (via the medial septum). However, there is also a second source of acetylcholine to the brain, the pedunculopontine, and laterodorsal tegmental nuclei that have projections to the cerebellum.

Anterograde and retrograde labeling of choline acetyltransferase-positive cerebellar mossy fiber rosettes in several species including the rabbit indicate that these cells originate from the pedunculopontine nucleus (Barmack *et al*, 1992; Jaarsma *et al*, 1997). [^{3}H]nicotine and [^{3}H]cytisine autoradiography was used to analyze the distribution of rabbit cerebellar nAChRs. As reported in the present study, the relative density of labeling in the cerebellum was low compared to cerebral cortex. Jaarsma *et al* (1997) found that nAChR labeling was concentrated in the cerebellar cortical granular layer and in the deep cerebellar nuclei.

The essential site for eyeblink conditioning is in the deep cerebellar nuclei. The techniques we used in the present study, radioligand receptor binding for neuronal $\alpha\beta$ heteromeric nAChRs ([^{3}H]Epi) and α7 homomeric nAChRs ([^{3}H]MLA), did not detect differences in cerebellum because expression level in the cerebellum is low and is barely at the detection limit of these binding assays. We cannot rule out the possibility that drugs affecting nAChRs and acquisition of conditioned eyeblink responses are acting in the cerebellum as well as in cortical brain sites and will aim to evaluate this possibility with more sensitive assays in future research.

Relationships Between Age, Learning, and nAChRs

Nicotinic acetylcholine systems have been found to be important for learning and memory functions. An age-related decrease in the number of $\alpha\beta$ heteromeric nAChRs in brain regions such as cortex and hippocampus is thought to contribute to cognitive impairment. We found an association between age differences in binding of $\alpha\beta$ heteromeric nAChRs, drug treatment-related increases in nAChR binding, and learning. At 27 months of age, vehicle-treated rabbits had significantly lower receptor levels of $\alpha\beta$ heteromeric nAChRs in the hippocampus and significantly poorer learning than young vehicle-treated rabbits. Chronic treatment with galantamine or MEM-3389 ameliorated learning impairment in these older rabbits as well as increased receptor levels of $\alpha\beta$ heteromeric nAChRs in cortex and hippocampus. Treatment with these two drugs elevated the performance of 27-month-old rabbits to the level of vehicle-treated 4-month-old rabbits (Figure 2). Both drugs significantly increased binding of $\alpha\beta$ heteromeric nAChRs to the levels comparable to those of young vehicle-treated rabbits (Figure 4).

Cognition-enhancing drugs also affected homomeric α7nAChRs, and significant increases in receptor binding were especially evident in young rabbits (Figure 5). These results are consistent with studies in rats. Hernandez *et al* (2006) demonstrated that chronic treatment with nicotine improved memory performance in the Morris water maze and increased [^{3}H]Epi and [^{125}I]α-bungarotoxin autoradiographic densities in several brain regions. The knockdown of rat α7nAChR subunits with antisense resulted in a decrease in [^{3}H]MLA binding sites by 42% in hippocampus and 25% in cortex and impaired acquisition on the Morris water maze (Curzon *et al*, 2006).

Drug-treated younger rabbits showed significantly improved learning over vehicle-treated young rabbits. In the 750 ms delay classical conditioning paradigm, young vehicle-treated rabbits are close to ceiling levels of performance. In our experience with a number of cognition-enhancing drugs, very few (galantamine (Woodruff-Pak *et al*, 2001) and now MEM-3389) facilitate learning in this paradigm in young rabbits. Drug treatment in young rabbits also improved learning to levels that were significantly better than learning in drug-treated 27-month-old rabbits. Receptor binding of $\alpha\beta$ heteromeric nAChRs in the hippocampus of young rabbits did not increase with drug treatment. The only significant increase in receptor levels of $\alpha\beta$ heteromeric nAChRs in young rabbits was in temporal-parietal cortex. The receptors in young rabbit brains showing the greatest plasticity in response to galantamine and MEM-3389 were homomeric α7nAChRs. Associated with the superior learning of drug-treated young rabbits were significant increases in receptor binding of homomeric α7nAChRs in frontal cortex, temporal-parietal cortex, and hippocampus. Only hippocampal homomeric α7nAChRs had significant increases in receptor binding in 27-month-old rabbits.

The role of the hippocampus in eyeblink classical conditioning is called 'modulatory', because manipulations of the hippocampus can impair or enhance the rate of acquisition (Berger *et al*, 1986). The memory trace itself is not in the hippocampus, but the hippocampus can markedly influence the storage process (reviewed in Thompson,

2005). In rabbits, age-related deficits in hippocampal electrophysiology have been associated with impairments in eyeblink conditioning (Power *et al*, 2002). Our data, showing increased binding of $\alpha\beta$ heteromeric and $\alpha7$ homomeric nAChRs in the hippocampus of 27-month-old rabbits treated with galantamine or MEM-3389, indicate that these drugs ameliorate learning impairment at least in part by potentiating hippocampal $\alpha\beta$ and $\alpha7$nAChRs.

What is new in these results is the association of highly optimized performance of young rabbits with significant increases in receptor binding of $\alpha7$ homomeric nAChRs in cortical brain sites as well as in the hippocampus. Both galantamine (Popa *et al*, 2006) and MEM-3389 (Papke *et al*, 2004) increase binding of $\alpha7$ homomeric nAChRs. Frontal and temporal-parietal cortex of drug-treated young rabbits as well as hippocampus showed significant increases in receptor binding of $\alpha7$ homomeric nAChRs, whereas $\alpha7$ binding in cortical sites in drug-treated older rabbits was not different from vehicle-treated older rabbits. Electrophysiological (Powell *et al*, 1996), pharmacological (Takehara-Nishiuchi *et al*, 2006), and lesion (Simon *et al*, 2005; Weible *et al*, 2000) studies of the role of frontal cortex in eyeblink conditioning have demonstrated involvement of prefrontal cortex. Our data indicate that the frontal cortex in addition to the hippocampus may be a site of modulation of CRs. Treatments that optimize functioning in prefrontal cortex, such as drugs that increase receptor binding of nAChRs, may improve learning.

To date, our results indicate that 27-month-old rabbits retain plasticity to increase receptor binding of $\alpha\beta$ heteromeric nAChRs in the hippocampus where they had significant loss of $\alpha\beta$ nAChRs, compared with 4-month-old rabbits. In particular, 3.0 mg/kg of galantamine or 1.0 mg/kg MEM-3389 administered chronically to older rabbits returned binding of $\alpha\beta$ heteromeric nAChRs in the hippocampus to levels observed in young vehicle-treated rabbits. In older rabbit brains, there was also increased receptor binding of $\alpha\beta$ heteromeric nAChRs in frontal and temporal-parietal cortex—sites that showed no age-related deficits in $\alpha\beta$ heteromeric nAChRs. Whereas increased receptor binding of $\alpha\beta$ heteromeric nAChRs was associated with significant amelioration of learning impairment in drug-treated older rabbits, their learning proficiency was still below that of drug-treated young rabbits who showed increased receptor binding of $\alpha7$ homomeric nAChRs. Plasticity in $\alpha7$ homomeric nAChRs caused by chronic administration of galantamine or MEM-3389 was associated with highly optimized learning in young rabbits. Cortical as well as hippocampal upregulation of nAChRs in drug-treated rabbits was associated with improvement in learning over vehicle-treated rabbits. Increases in expression of $\alpha\beta$ heteromeric nAChRs occurred at the most brain sites in the case of amelioration of learning impairment in 27-month-old rabbits. Increases in expression of $\alpha7$ homomeric nAChRs occurred at the most brain sites in the case of facilitation or optimization of learning in 4-month-old rabbits. These results indicate that substrates underlying plasticity at different ages may vary. In the present study, $\alpha\beta$ heteromeric nAChRs were most consistently associated with amelioration of learning impairment in older rabbits, whereas $\alpha7$ homomeric nAChRs were most associated with optimization of learning in young rabbits.

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DISCLOSURE/CONFLICT OF INTEREST

None of the authors of this paper have any potential conflicts of interest that would influence the objectivity of this report.

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