Preclinical Investigation of the Functional Effects of Memantine and Memantine Combined with Galantamine or Donepezil

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Combinations of drugs approved to treat Alzheimer's disease (AD) were tested in older rabbits with delay eyeblink classical conditioning, a form of associative learning severely impaired in AD. In Experiment 1 ($n = 49$ rabbits), low doses (0.1, 0.5, 1.0, and 0.0 (vehicle) mg/kg) of memantine (Namenda™) were tested. These three doses neither improved nor impaired acquisition at a statistically significant level. The 0.5 mg/kg dose had the greatest effect numerically and did not cause sensitization or habituation in explicitly unpaired controls. In Experiment 2 ($n = 56$), doses of galantamine (Razadyne™; 3.0 mg/kg) and donepezil (Aricept™; 0.75 mg/kg) that had comparable magnitudes of cholinesterase inhibition were tested alone and in combination with 0.5 mg/kg memantine. Older rabbits treated with galantamine and with galantamine + memantine learned significantly better than vehicle-treated rabbits, but adding memantine did not improve learning over galantamine alone. Older rabbits treated with donepezil or a combination of memantine and donepezil did not learn significantly better than rabbits treated with vehicle. Galantamine has two mechanisms of action: mild cholinesterase inhibition and allosteric modulation of nicotinic acetylcholine receptors (nAChRs). When equated for cholinesterase inhibition, galantamine had significant efficacy in the eyeblink conditioning model system, but donepezil did not, indicating that modulation of nAChRs may be the mechanism that significantly ameliorates learning deficits in this model. In the absence of AD neuropathology in older rabbits, memantine had no efficacy alone or in combination with the other drugs.
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INTRODUCTION

Acetylcholine neurotransmission plays a crucial role in learning and memory and has been the focus of pharmacological therapy for Alzheimer's disease (AD). The first four drugs approved by the Food and Drug Administration to treat memory impairment in mild to moderate AD were acetylcholinesterase (AChE) inhibitors that promoted acetylcholine's action at the synapse. Glutamate neurotransmission and its activation of alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and *N*-methyl-D-aspartate (NMDA) receptors contribute to long-term memory storage, but glutamatergic hyperactivity is neurotoxic and plays a role in neurodegeneration. Drugs targeting NMDA receptors to block excitotoxicity are an additional focus of pharmacological therapy for AD. A drug approved by the FDA to treat symptoms in severe AD is memantine, an uncompetitive NMDA-receptor antagonist. The fact that drugs with different mechanisms of action are available to treat AD introduces the prospect of prescribing drug combinations to amplify drug efficacy. Evidence on combination therapy for AD is limited (Schmitt *et al*, 2004), although negative drug–drug interactions have not yet been observed in clinical studies (Dantoine *et al*, 2006; Enz and Gentsch, 2004). Cholinesterase inhibitors are approved for treatment during the early stages of disease onset and often continued as the disease progresses, whereas memantine is approved for treatment at later stages when dementia is present. In severely demented patients with AD (Mini-Mental State Examination scores of 5–14), who received stable doses of donepezil, the addition of memantine resulted in significantly better outcomes than placebo (Cummings *et al*, 2006; Tariot *et al*, 2004). The major aim of this study was to evaluate further combinations of drugs targeting AD with an animal model and a task, delay eyeblink classical conditioning, of demonstrated impairment in human patients diagnosed with AD (Solomon *et al*, 1991; Woodruff-Pak *et al*, 1990).

Disruption of the brain cholinergic system in AD links this dementing disease to the model system of eyeblink conditioning in mammals, including humans. Eyeblink

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conditioning impairment in AD may reflect medial–temporal lobe atrophy and associated central nervous system cholinergic dysfunction that occurs early in the disease progression. Disruption of acetylcholine neurotransmission in the septohippocampal system impairs learning of the conditioned eyeblink response in young (Harvey *et al*, 1983; Solomon *et al*, 1983; Woodruff-Pak *et al*, 1994a) and older rabbits (Pak *et al*, 2002). Data also indicate that NMDA receptor activity is involved in the acquisition of classically conditioned eyeblink responses (Chen and Steinmetz, 2000; Churchill *et al*, 2001; Thompson and Disterhoft, 1997). There are dramatic neurobiological and behavioral parallels between humans and other mammals in this simple form of associative learning called eyeblink classical conditioning, making this model system useful in preclinical research (Woodruff-Pak, 1995).

Acetylcholinesterase Inhibitors and Eyeblink Classical Conditioning

Pharmacologic therapies to preserve the action of a dwindling acetylcholine pool in the AD brain have focused on prolonging its presence at the synapse. The acetylcholine molecule is inactivated in a single step. AChE is an enzyme that breaks down acetylcholine into choline and acetic acid. Inhibition of AChE is equivalent to increasing the activity of acetylcholine. Drugs approved by the FDA as reversible AChE inhibitors for the treatment of AD include tacrine (a 4-aminopyridine derivative), donepezil (a benzylpiperidine derivative), and galantamine (a phenanthrene tertiary alkaloid). Rivastigmine (a carbamate derivative) is a 'pseudoirreversible' inhibitor. Of these compounds, galantamine is the only molecule approved in Europe with mechanisms of action that include both AChE inhibition and allosteric potentiating effects at the nicotinic acetylcholine receptor (nAChR). In the United States, galantamine is approved as having one mechanism of action, AChE inhibition. Galantamine is a relatively mild AChE inhibitor. For an equivalent magnitude of AChE inhibition to donepezil, 3–15 times as much galantamine must be administered (Geerts *et al*, 2005). Galantamine and donepezil have been tested previously in preclinical studies using eyeblink classical conditioning.

Four doses of galantamine were tested in the 750 ms delay eyeblink classical conditioning procedure, and a dose of 3.0 mg/kg enabled older rabbits to achieve learning criterion rapidly and to produce a very high percentage of conditioned responses (CRs) (Woodruff-Pak and Santos, 2000). In a second experiment, the effects of galantamine were examined in young and older rabbits (Woodruff-Pak *et al*, 2001). A dose of 3.0 mg/kg galantamine improved learning significantly in young and older rabbits. Not only did galantamine-treated younger rabbits achieve learning criterion in significantly fewer trials, the 3.0 mg/kg dose of galantamine enabled older rabbits to learn at the same rate as young vehicle-treated rabbits. Two other laboratories have also demonstrated the efficacy of galantamine in eyeblink classical conditioning using the trace conditioning procedure (Simon *et al*, 2004; Weible *et al*, 2004).

Donepezil was tested at doses of 0.3 and 3.0 mg/kg in retired breeder rabbits in the 750 ms delay procedure. A dose of 3.0 mg/kg donepezil improved acquisition of CRs

significantly (Woodruff-Pak, 2001). Donepezil did not facilitate conditioning in the 750 ms delay procedure in young rabbits (Woodruff-Pak *et al*, 2003). The demonstrated efficacy of donepezil and galantamine each at a dose of 3.0 mg/kg suggests that the mechanism by which galantamine produces its therapeutic effect is something other than AChE inhibition, as its potency as a AChE inhibitor may be as much as 15 times weaker than that of donepezil.

NMDA Receptor Antagonism and Eyeblink Classical Conditioning

NMDA receptors are ligand gated and voltage sensitive to the most common excitatory neurotransmitter, glutamate. NMDA receptors are found throughout the brain, including the structures normally engaged in eyeblink classical conditioning. In the case of NMDA receptor affinity for memantine, there is relatively higher affinity in the cerebellum, the structure essential for eyeblink conditioning, than in forebrain regions (Bresink *et al*, 1995; Porter and Greenamyre, 1995). Memantine is a clinically well-tolerated uncompetitive NMDA receptor antagonist with strong voltage dependency and rapid blocking/unblocking kinetics (Parsons *et al*, 1999). Mild glutamate toxicity to postsynaptic neurons is blocked by memantine at concentrations significantly lower than those concentrations that impair normal physiological function and synaptic plasticity. Such kinetics may allow memantine to block ongoing pathologic processes that involve chronic NMDA activation, while simultaneously permitting normal neurophysiologic brain functions (Lipton and Chen, 2004). Memantine treatment has been directed primarily toward AD and other senile dementias (Jain, 2000; Tariot *et al*, 2004), although additional therapeutic applications have been suggested for this drug (Parsons *et al*, 1999).

NMDA antagonism was first tested in the eyeblink classical conditioning model system with a potent non-competitive antagonist, MK-801 (Robinson, 1993), and with a competitive antagonist, CGP-39551 (Servatius and Shors, 1996). Both studies used the delay eyeblink classical conditioning procedure, but MK-801 was tested in rabbits whereas CGP-39551 was tested in rats. In both cases, delay eyeblink classical conditioning was impaired. Mutant mice lacking subunits NR2A and NR2C of the NMDA receptor channel were tested in delay eyeblink classical conditioning, and mice lacking the NR2A (but not NR2C) subunits were impaired (Kishimoto *et al*, 1997). Based on the distribution of these NMDA receptor subunits, the investigators concluded that acquisition of the conditioned eyeblink response does not depend on NMDA receptors in the cerebellar cortex, but does depend on these receptors in hippocampus and/or deep cerebellar nuclei. NMDA receptor antagonists also impair trace eyeblink classical conditioning that is hippocampus dependent (Takatsuki *et al*, 2001; Thompson and Disterhoft, 1997).

One study using human subjects, eyeblink conditioning, and memantine was carried out in Germany (Schugens *et al*, 1997) where memantine has been approved for the treatment of neurological disorders since 1982. Sixteen men of a mean age of 34 years were administered one 30 mg dose of memantine or placebo and classically conditioned

4 h later using the delay eyeblink conditioning procedure. There was significant impairment in the acquisition of CRs in the men treated with memantine. Verbal and visuospatial memory were not impaired at this dose, supporting the results of binding studies that indicated increased potency of memantine in the cerebellum (essential for eyeblink conditioning) as compared to the forebrain (essential for verbal and visuospatial memory).

Using doses of 0.01, 0.05, 0.1, and 0.3 mg/kg MK-801 in rats, Jackson *et al* (2004) found that the lowest dose increased firing rate of prefrontal cortical neurons and improved performance on spontaneous alternation performance. The investigators did not record from other regions of the brain, including the hippocampus with its abundant distribution of NMDA receptors. Hippocampal and prefrontal cortical neurons are of demonstrated involvement in eyeblink classical conditioning (McLaughlin *et al*, 2002; Takehara *et al*, 2004; Weible *et al*, 2000). Research by Jackson *et al* (2004) showing that low doses of a potent noncompetitive NMDA antagonist increased neuronal firing rate and improved behavioral performance suggest the potential for facilitation by NMDA antagonists.

The lowest dose of MK-801 administered to rabbits tested in delay eyeblink conditioning was 0.05 mg/kg (Robinson, 1993). A dose of 0.05 mg/kg MK-801 depressed precortical neuronal firing rate and impaired behavior in rats, whereas a dose of 0.01 mg/kg MK-801 was excitatory in rat prefrontal cortical neurons and improved learning (Jackson *et al*, 2004). A dose of 0.01 MK-801 might facilitate eyeblink conditioning as it facilitated behavioral performance. Also, the clinical results with memantine demonstrate cognitive facilitation by NMDA antagonism (Jain, 2000; Tariot *et al*, 2004). Memantine is significantly less potent than MK-801 (Wenk *et al*, 1995). Given that very low doses of the potent NMDA antagonist MK-801 as well as many doses of memantine facilitate learning, one aim of the present study was to identify a dose of memantine that would ameliorate, or at a minimum not impair acquisition in older rabbits in delay eyeblink classical conditioning.

The second aim was to determine the effect of combined doses of memantine and galantamine or donepezil. In rabbit, between four and eight times less donepezil is needed to achieve the same level of brain AChE inhibition (Geerts *et al*, 2005). We chose to use a dose of donepezil four times lower than the dose of galantamine to equate the two drugs for AChE inhibition. The effective (or non-impairing) dose of memantine was combined with a dose of galantamine (3.0 mg/kg) effective in rabbits in eyeblink classical conditioning, and a dose of donepezil (0.75 mg/kg) with a relatively equal magnitude of AChE inhibition to galantamine.

MATERIALS AND METHODS

Study Population: Rabbits

A total of 105 specific pathogen-free (SPF) female retired breeder rabbits were tested in Experiments 1 and 2. Rabbits were purchased from Covance (Denver, PA). The mean age of rabbits was 26.3 months (SD 0.2), and the mean weight was 4.1 kg (SD 0.04). Rabbits were individually housed in stainless steel cages in temperature- and humidity-con-

trolled rooms. They had *ad libitum* access to food and water during the experiment. 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.

Study Procedures

The 750 ms delay eyeblink classical conditioning procedure, used in previous studies of eyeblink classical conditioning testing efficacy of galantamine or donepezil, was used in the present study. Techniques were similar to published reports (eg Woodruff-Pak *et al*, 2001). The rabbits were adapted to Plexiglas restrainers and headpieces for 1 h on each of the two consecutive days following arrival at the animal facility. On the second day of adaptation, 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). A 3-cm^2 patch of fur was also shaved from the rabbit's back in order to expose the skin where subcutaneous injections were administered.

The conditioning apparatus consisted of four separate sound-attenuating chambers, permitting four rabbits to be trained simultaneously. A speaker mounted to the wall of each chamber delivered a 1-kHz, 85-dB 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 an IBM PC-compatible system (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 trials.

In the delay eyeblink conditioning procedure, the duration of the tone CS was 850 ms, followed 750 ms after its onset by a 100-ms US. The CS and US coterminated. The rabbits received 10 training sessions (5 days per week for 2 weeks, excluding weekends). In the explicitly unpaired procedure, rabbits received the same treatment as in the paired procedure, with the exception that the tone and the air puff were never paired. On each unpaired trial, rabbits received either a tone-alone presentation or an air puff-alone presentation. Forty-five trials of each stimulus type were presented in a randomized format during each session.

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 CR was recorded if the response occurred between 25 and 750 ms after the onset of the CS. An unconditioned response (UR) was recorded if the response took place more than 750 ms after the onset of the CS. In both paradigms, CR and UR amplitudes were taken as a measure of response magnitude. A trial was eliminated if NM activity crossed the response threshold

within 100 ms before the onset of the CS. The criterion for learning was defined as eight consecutive CRs in a block of nine trials, with at least 40% of all trials within a training session as CRs.

Drugs

Janssen Pharmacautica supplied galantamine. Memantine was purchased from Tocris Cookson, Inc. (Ellisville, MO, USA), and donepezil was purchased from A&A Pharmachem Inc. (Ottawa, ON, Canada). Drugs were mixed into sterile saline vehicle so that all injections were at volumes of 1.0 ml/kg. Subcutaneous (s.c.) injections were administered 15 min before behavioral training. Animal testers were not blind during drug administration, primarily to insure that the correct dose combinations were given to the appropriate rabbits. Testing equipment was completely automated, making it highly unlikely that animal testers' knowledge of drug type could have affected animals' performance.

Research Design

In Experiment 1, a total of 49 retired breeder rabbits in six groups were tested. Three groups of rabbits ($n = 8$/group) were administered doses of 0.1, 0.5, and 1.0 mg/kg memantine and tested in the paired 750 ms delay eyeblink conditioning procedure. A fourth group of rabbits received sterile saline vehicle ($n = 8$) and was tested in the paired conditioning procedure. Two additional groups of rabbits were treated in the explicitly unpaired condition with 0.5 mg/kg memantine ($n = 7$) or sterile saline vehicle ($n = 10$).

In Experiment 2, a total of 56 additional retired breeder rabbits in eight groups were tested. Two groups of rabbits ($n = 8$/group) were administered doses of 3.0 mg/kg galantamine or 0.75 mg/kg donepezil and tested in the paired 750 ms delay eyeblink conditioning procedure. Two groups of rabbits ($n = 8$/group) were given separate injections of 0.5 mg/kg memantine and 3.0 mg/kg galantamine or 0.5 mg/kg memantine and 0.75 mg/kg donepezil and tested in the paired 750 ms delay eyeblink conditioning procedure. A fifth group of rabbits received sterile saline vehicle ($n = 10$) and was tested in the paired conditioning procedure. Two additional groups of rabbits ($n = 7$/group) were treated in the explicitly unpaired condition with 0.5 mg/kg memantine and 3.0 mg/kg galantamine or 0.5 mg/kg memantine and 0.75 mg/kg donepezil. Data from the control group treated with sterile saline vehicle and tested in the explicitly unpaired condition ($n = 10$) in Experiment 1 were used for the unpaired analyses in Experiment 2. All rabbits received two injections and an equal volume of solution. In the case of the groups with one dose of drug, the second injection was sterile saline vehicle.

Acetylcholinesterase Assays in Plasma and Brain

At 3 days after the tenth training session and drug administration, an 11th s.c. injection of the same drug combination, which the rabbit had been receiving, was administered. After a 30 min interval, the rabbit was secured in a restrainer, 2 ml of blood was drawn from the ear vein, and the rabbit was killed with 1–2 ml of Pentobarbital

injected i.v. The brain was immediately removed, and the cerebral cortex, hippocampus, and cerebellum were dissected and stored in separate containers at -80°C. Blood was centrifuged for 10 min at 3000 r.p.m. in an Eppendorf (Hamburg, Germany) 5702R centrifuge, and plasma was stored at -80°C. Brain and plasma for rabbits treated with 3.0 mg/kg galantamine or 0.75 mg/kg donepezil were shipped on dry ice from Philadelphia, PA to Newark, NJ overnight and analyzed for AChE. After results from these analyses were complete, brain and plasma of rabbits treated with combinations of 0.5 mg/kg memantine and 3.0 mg/kg galantamine or 0.75 mg/kg donepezil were shipped and analyzed for acetylcholinesterase.

Cholinesterase activity was assessed based on established methodology (Luine and McEwen, 1983). Cerebral cortex, hippocampus, and cerebellum sections (0.5 g wet weight per animal per brain region) were homogenized in 1 ml of 50 nM Tris-HCl buffer (pH 8.0) and subsequently diluted for sampling 1:20 (cortex and cerebellum) and 1:50 (hippocampus) in Tris-HCl buffer. Plasma samples were diluted 1:5 in deionized H_2O. Samples (10 μl) were combined with 50 μl of 0.05 M KH_2PO_4 (pH 7.0), 20 μl deionized H_2O, and 20 μl [^{3}H]acetylcholine iodide (5 μCi/ml) in 2.0 mM acetylcholine iodide in KH_2PO_4. The samples are incubated for 30 min at room temperature; afterwards, 100 μl stop solution (1.0 M chloroacetic acid, 2.0 M NaCl, and 0.5 M NaOH) was added. Subsequent scintillation mixture (in 10% isoamyl alcohol) was added and the resulting solution was incubated at 21°C for 30 min at room temperature before the reading of sample for beta radiation (Beckman LS6500). Brain samples were calculated in reference to protein content as determined by the Bradford method (Bradford, 1976).

RESULTS

Experiment I—Identification of a Dose of Memantine that Does Not Impair Learning

For the four groups of rabbits tested in paired 750 ms CS–US delay eyeblink classical conditioning, a 4 (drug dose) by 10 (training sessions) repeated measures analysis of variance (ANOVA) was carried out to compare performance of rabbits at three doses of memantine (0.1, 0.5, and 1.0 mg/kg) and sterile saline vehicle (0.0 mg/kg). There was a significant effect of training sessions, $F(9, 252) = 35.92$, $p < 0.001$, but no significant effect of drug dose ($F(3, 28) = 2.14$, $p = 0.117$) or drug dose by training sessions interaction ($F(27, 252) = 1.20$, $p = 0.237$). These low doses of memantine did not interfere with acquisition, and the dose of 0.5 mg/kg memantine had the numerically greatest effect (Figure 1a).

Comparisons of two other dependent measures in the paired condition, CR amplitude and response latency, in separate 4 (drug dose) by 10 (training sessions) repeated measures ANOVAs yielded similar results. For each dependent measure, there was a significant effect of training sessions, $F(9, 252) = 10.17$, $p < 0.001$ for CR amplitude and $F(9, 252) = 24.61$, $p < 0.001$ for response latency. There was not a significant effect of group or group by training sessions interaction for either variable. A one-way ANOVA comparing the effect of trials to a learning criterion of eight
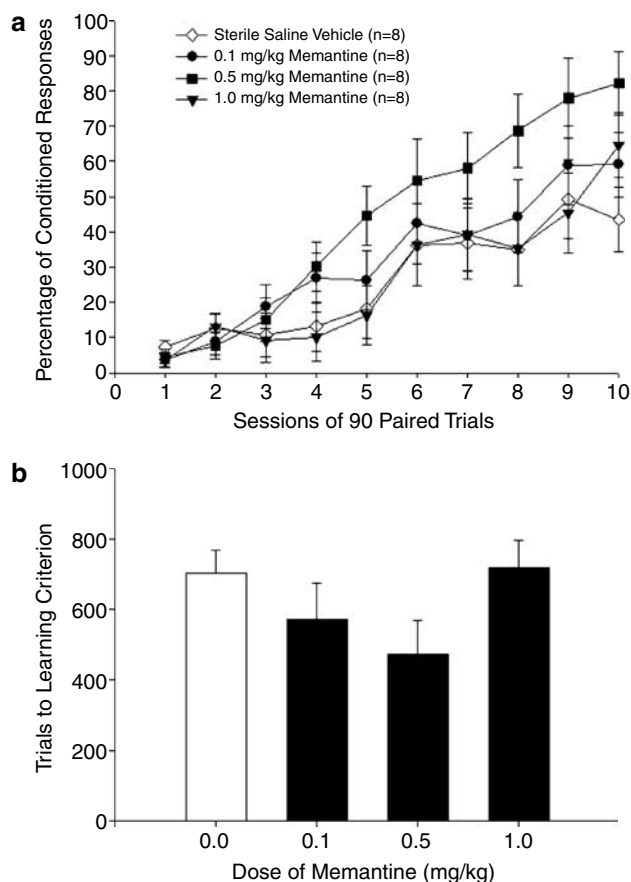
Figure 1 (a) Percentage of conditioned responses in 32 retired breeder rabbits tested with doses of 0.1, 0.5, 1.0 mg/kg memantine and vehicle for 10 sessions in the 750 ms delay eyeblink classical conditioning procedure. The effect of training sessions was statistically significant ($p < 0.001$), but the differences between rabbits treated with different doses of memantine did not attain statistical significance ($p = 0.117$). Error bars are standard error of the mean. (b) Trials to learning criterion in the same 32 retired breeder rabbits shown in (a) above. There was not a significant difference between the groups. Error bars are standard error of the mean.

Figure 2 Unconditioned response (UR) amplitude in: (a) 32 retired breeder rabbits tested with doses of 0.1, 0.5, 1.0 mg/kg memantine and vehicle for 10 sessions in the 750 ms delay eyeblink classical conditioning procedure. The effect of training sessions was statistically significant ($p = 0.037$), but the effect of drug dose and the interaction effect were not significant. (b) Forty-two retired breeder rabbits tested with doses of 3.0 mg/kg galantamine, 3.0 mg/kg galantamine + 0.5 mg/kg memantine, 0.75 mg/kg donepezil, 0.75 mg/kg donepezil + 0.5 mg/kg memantine, and vehicle for 10 sessions in the 750 ms delay eyeblink classical conditioning procedure. There were no significant main effects of drug dose or training sessions, but the interaction between drug dose and training sessions was significant ($p = 0.013$). Error bars are standard error of the mean.

CRs in nine consecutive trials in the paired condition did not attain significance at the 0.05 level of confidence, $F(3, 28) = 1.82$, $p = 0.167$ (Figure 1b).

A measure of the motor response to the air puff US is the magnitude of the UR. For the paired condition, a 4 (drug dose) by 10 (training sessions) repeated measures ANOVA on UR amplitude indicated a significant effect of training sessions, $F(9, 252) = 2.021$, $p = 0.037$ (Figure 2a). The effect of drug dose and the drug dose by training sessions interaction did not approach statistical significance.

For the explicitly unpaired condition, two measures were analyzed: Responses in the CS period (these responses are called CRs when the CS and US are paired, but they are simply random blinks or blinks to the CS itself in the explicitly unpaired condition) and UR amplitude. Responses in the CS period were analyzed in a 2 (drug dose (0.5 mg/kg memantine, sterile saline vehicle)) by 10 (training sessions) repeated measures ANOVA. Neither the two main effects nor the interaction effect approached statistical significance. A similar 2 (drug dose) by 10 (training sessions) repeated measures ANOVA was carried
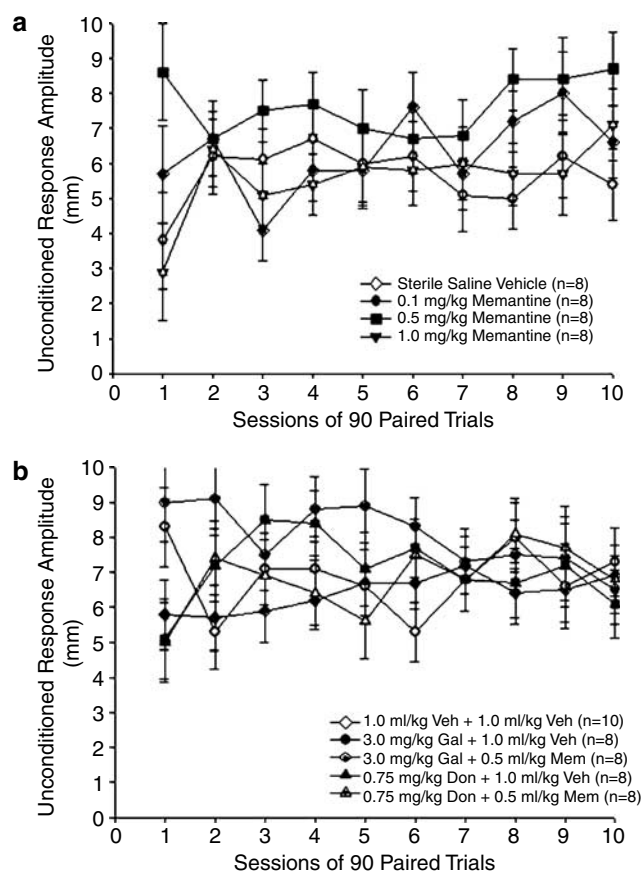
out on UR amplitude. Neither the two main effects nor the interaction effect approached statistical significance.

The goal of Experiment 1 was to identify a dose of memantine that ameliorated learning deficits in older rabbits or, at a minimum, a dose that did not impair learning. A dose of 0.5 mg/kg memantine met the latter criterion. The 0.5 dose of memantine did not cause sensitization or habituation of the eyeblink response in the unpaired control condition, and the motor response (UR) was not affected in the unpaired condition. The 0.5 mg/kg dose of memantine was used to test drug combination effects in Experiment 2.

Experiment 2—Combined Effect of Memantine + Galantamine or Memantine + Donepezil

Acetylcholinesterase levels. Doses of 3.0 mg/kg galantamine and 0.75 mg/kg donepezil were chosen to equate the two drugs for level of cholinesterase inhibition based on

Table 1 Mean Plasma and Brain AChE Levels for Rabbits Treated with 11 Daily Doses of 3.0 mg/kg Galantamine or 0.75 mg/kg Donepezil

Drug	Plasma (nm/min/ml protein)	Cerebral cortex (nm/min/mg protein)	Hippocampus (nm/min/mg protein)	Cerebellum (nm/min/mg protein)
3.0 mg/kg Galantamine	201.05 (63.26)	811.88 (175.84)	3303.21 (322.04)	1156.55 (165.07)
0.75 mg/kg Donepezil	210.77 (35.36)	832.76 (217.50)	3018.03 (500.2)	1075.52 (153.12)

previously reported assays (Geerts *et al*, 2005). Plasma AChE in rabbits treated with 3.0 mg/kg galantamine was a mean of 201.05 nm/min/ml (standard deviation = 63.3), and in rabbits treated with 0.75 mg/kg donepezil was a mean of 210.77 nm/min/ml (standard deviation = 35.4). A *t*-test indicated that the difference in plasma AChE levels did not approach statistical significance. A multivariate one-way ANOVA indicated that brain AChE levels in rabbits treated with 3.0 mg/kg galantamine were similar to AChE levels in rabbits treated with 0.75 mg/kg donepezil in cerebral cortex, hippocampus, and cerebellum (Table 1).

A multivariate one-way ANOVA was used to compare plasma and brain AChE levels for rabbits treated with 0.5 mg/kg memantine plus 3.0 mg/kg galantamine or 0.75 mg/kg donepezil. There was not a significant difference between the groups either in plasma AChE or brain AChE levels in cerebral cortex, hippocampus, or cerebellum.

Eyeblink classical conditioning. For the five groups of rabbits tested in paired 750 ms CS–US delay eyeblink classical conditioning, a 5 (drug dose) by 10 (training sessions) repeated measures ANOVA was carried out to assess the effect of various drug combinations on acquisition of CRs. The main effects of drug dose and training sessions were statistically significant, $F_{(4, 37)} = 4.66$, $p < 0.001$ and $F_{(9, 333)} = 68.82$, $p < 0.001$, respectively. The interaction between drug dose and training sessions was also significant, $F_{(36, 333)} = 1.59$, $p = 0.020$ (Figure 3a). *Post hoc* analysis of the significant drug dose effect using the Tukey Honestly Significant Difference (HSD) test indicated that older rabbits treated with 3.0 mg/kg galantamine or with 3.0 mg/kg galantamine + 0.5 mg/kg memantine produced a higher percentage of CRs than vehicle-treated rabbits. These were the only two groups with a performance that was significantly better than rabbits treated with vehicle.

Comparisons of two other dependent measures in the paired condition, CR amplitude and response latency, in separate 5 (drug dose) by 10 (training sessions) repeated measures ANOVAs yielded a similar pattern of results. In the case of response latency, the main effects and interaction were significant. The effect of drug dose was $F_{(4, 37)} = 4.09$, $p = 0.008$, the effect of training sessions was $F_{(9, 333)} = 59.57$, $p < 0.001$, and the interaction between drug dose and training sessions was $F_{(36, 333)} = 1.74$, $p = 0.007$. *Post hoc* analysis of the significant drug dose effect using the Tukey HSD test indicated that older rabbits treated with 3.0 mg/kg galantamine or with 3.0 mg/kg galantamine + 0.5 mg/kg memantine produced a shorter response latency than vehicle-treated rabbits. These were the only two groups with a performance that was significantly better than rabbits treated with vehicle.
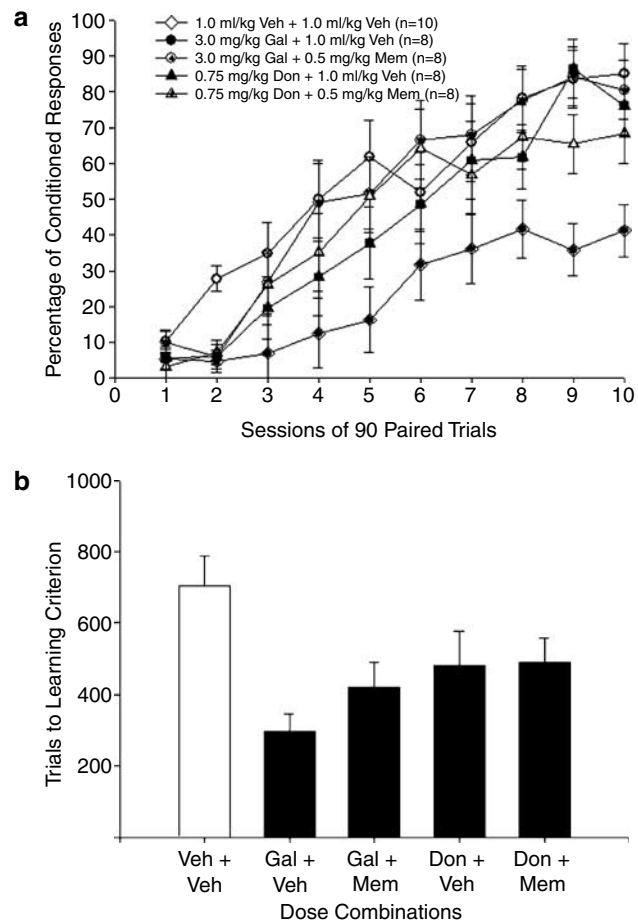

Figure 3 (a) Percentage of conditioned responses in 42 retired breeder rabbits tested with doses of 3.0 mg/kg galantamine, 3.0 mg/kg galantamine + 0.5 mg/kg memantine, 0.75 mg/kg donepezil, 0.75 mg/kg donepezil + 0.5 mg/kg memantine, and vehicle for 10 sessions in the 750 ms delay eyeblink classical conditioning procedure. The effect of drug dose, the effect of training sessions, and the interaction effect were statistically significant. *Post hoc* analysis of the significant drug dose effect indicated that doses of 3.0 mg/kg galantamine and 3.0 mg/kg galantamine + 0.5 mg/kg memantine produced significantly better learning than did vehicle. (b) Trials to learning criterion in the 42 retired breeder rabbits shown above. There was a significant effect of drug dose, and *post hoc* analysis indicated that 3.0 mg/kg galantamine resulted in significantly faster learning (fewer trials to criterion). Error bars are standard error of the mean.

In the case of CR amplitude, the drug dose effect did not attain significance at the 0.05 level of confidence, but the effect of training sessions and the drug dose by training sessions interaction effect were significant. The effect of drug dose was $F_{(4, 37)} = 2.45$, $p = 0.063$, the effect of training sessions was $F_{(9, 333)} = 23.59$, $p < 0.001$, and the

interaction between drug dose and training sessions was $F_{(36, 333)} = 1.51$, $p = 0.034$.

A one-way ANOVA comparing the effect of trials to a learning criterion in the paired condition was significant, $F_{(4, 41)} = 3.98$, $p = 0.009$ (Figure 3b). *Post hoc* Tukey HSD tests indicated that rabbits treated with 3.0 mg/kg galantamine had significantly fewer trials to learning criterion than vehicle-treated rabbits. None of the other groups were significantly different from the vehicle group in this *post hoc* test.

A measure of the motor response to the air puff unconditioned stimulus is the UR. In the paired condition, a 5 (drug dose) by 10 (training sessions) repeated measures ANOVA with the dependent measure of UR amplitude, there were no significant main effects, but the interaction between drug dose and training sessions was significant, $F_{(36, 333)} = 1.65$, $p = 0.013$ (Figure 2b).

For the explicitly unpaired condition, responses in the CS period were analyzed in a 3 (drug dose (3.0 mg/kg galantamine + 0.5 mg/kg memantine, 0.75 mg/kg donepezil + 0.5 mg/kg memantine, sterile saline vehicle)) by 10 (training sessions) repeated measures ANOVA. The effect of training sessions was significant, $F_{(9, 189)} = 2.79$, $p = 0.004$ (Figure 4). *Post hoc* comparisons in each of the 10 sessions indicated that none of the individual sessions were significantly different. The effect of drug dose and the interaction effect were not significant. For the explicitly unpaired condition, a similar 3 (drug dose) by 10 (training sessions) repeated measures ANOVA was carried out on UR amplitude, and neither of the two main effects nor the interaction effect approached statistical significance.
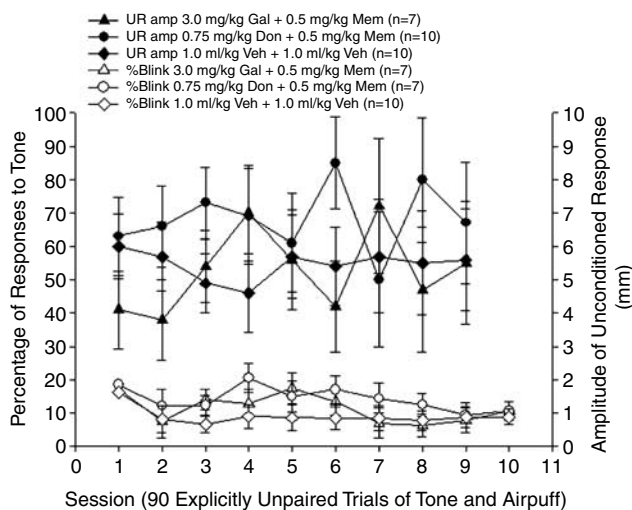
Combined Experiments 1 and 2: Drug Efficacy on Learning

Percentage of CRs from paired CS–US 750 ms eyeblink classical conditioning in Experiment 1 on the 0.5 mg/kg dose of memantine was compared to percentage of CRs in Experiment 2 on 3.0 mg/kg galantamine or 0.75 mg/kg donepezil in a 3 (drug dose) by 10 (training sessions) repeated measures ANOVA. The main effects of drug dose and training sessions were significant, $F_{(3, 30)} = 5.76$, $p = 0.003$ for drug dose, $F_{(9, 270)} = 48.92$, $p < 0.001$ for training sessions. The interaction between drug dose and training sessions was also significant, $F_{(27, 270)} = 1.53$, $p = 0.050$. *Post hoc* analysis of the significant drug dose effect using the Tukey HSD test indicated that older rabbits treated with 3.0 mg/kg galantamine produced a higher percentage of CRs than vehicle-treated rabbits (Figure 5). This was the only group with a performance that was significantly better than rabbits treated with vehicle. Similar results occurred in a comparison of trials to learning criterion in a one-way ANOVA. The effect of drug dose was statistically significant, $F_{(3, 30)} = 4.10$, $p = 0.015$, and *post hoc* the Tukey HSD test indicated that the only drug dose resulting in significantly fewer trials to learning criterion was 3.0 mg/kg galantamine.

DISCUSSION

To evaluate the efficacy of combinations of FDA-approved drugs for the treatment of AD, the effect of the NMDA antagonist, memantine on eyeblink classical conditioning was compared to and combined with two AChE inhibitors: galantamine and donepezil. In Experiment 1, three doses of memantine were tested in the 750 ms delay eyeblink conditioning procedure to identify a dose of memantine that improved or did not impair acquisition of CRs. A dose of 0.5 mg/kg memantine met those criteria and did not cause sensitization, habituation, or alterations in the motor
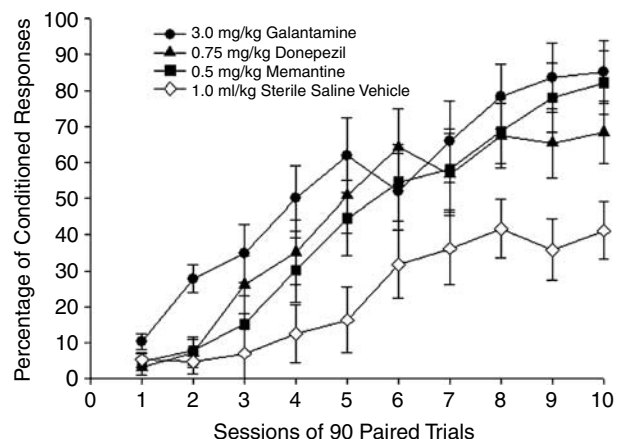

Figure 4 Responses to the tone conditioned stimulus (CS) in the period between 25 and 750 ms after tone CS onset (left ordinate) and unconditioned response (UR) amplitude (right ordinate) in 24 rabbits treated with 3.0 mg/kg galantamine + 0.5 mg/kg memantine, 0.75 mg/kg donepezil + 0.5 mg/kg memantine, and vehicle for 10 sessions tested in the explicitly unpaired condition. The effect of training sessions on responses to the tone CS was significant ($p = 0.004$). The effect of drug dose and the drug dose by training sessions interaction effect were not significant. There were no significant effects in the analysis of UR amplitude. Error bars are standard error of the mean.

Figure 5 Mean total percentage of conditioned responses (CRs) over 10 training sessions in rabbits treated with 0.5 mg/kg memantine ($n = 8$), 3.0 mg/kg galantamine ($n = 8$), 0.75 mg/kg donepezil ($n = 8$), or sterile saline vehicle ($n = 10$). Rabbits treated with galantamine had significantly higher percentages of CRs in comparison to vehicle, and no other group was significantly different from vehicle. Error bars are standard error of the mean.

response (UR). In Experiment 2, 0.5 mg/kg memantine was combined with doses of galantamine (3.0 mg/kg) and donepezil (0.75 mg/kg) equated for AChE inhibition. Galantamine and galantamine + memantine ameliorated impairment of acquisition of CRs in older rabbits above vehicle-treated levels. Donepezil and donepezil + memantine did not ameliorate impairment of acquisition of CRs in older rabbits above vehicle-treated levels. Adding memantine to galantamine or donepezil did not improve conditioning over acquisition with galantamine or donepezil alone. Comparisons of acquisition with a 0.5 mg/kg dose of memantine and 3.0 mg/kg galantamine, 0.75 mg/kg donepezil, and vehicle indicated that galantamine was the only drug that significantly improved acquisition of CRs and reduced the number of trials required for attainment of learning criterion in comparison to vehicle.

Using groups of eight to 10 rabbits yields high power in the eyeblink classical conditioning procedure. For the dependent measure of percentage of CRs, the observed power for the effect of training sessions was 1.0 for every analysis. The observed power for the interaction effects was above 0.9 in all cases, and the observed power for the group effect was 0.49 in Experiment 1, 0.92 in Experiment 2, and 0.92 for the comparison of galantamine, donepezil, and memantine. Thus, it was only in the comparison of groups treated with low and relatively similar doses of 0.1, 0.5, and 1.0 mg/kg memantine that the power was below 0.9. The sample sizes used in these experiments have ample power to support the conclusions.

Our goal was to take advantage of the fact that drugs with different mechanisms of action are available to treat AD by examining in a preclinical model the prospect of prescribing drug combinations to amplify drug efficacy. Previously we demonstrated that some drug combinations do act synergistically (Woodruff-Pak et al, 2004). However, the nootropic (nefiracetam) and cholinesterase inhibitor/nicotinic allosteric modulating (physostigmine) drugs we combined have not been approved for treatment in human patients with AD. Using FDA-approved drugs for the treatment of AD in the model system of eyeblink classical conditioning in rabbits, we observed no synergy with combinations of memantine and galantamine or donepezil. Nevertheless, these combinations merit additional exploration. Rationales for combinations of drugs affecting the cholinergic and glutamatergic systems are persuasive (Geerts and Grossberg, 2006; Grossberg et al, 2006). Clinical trial comparisons of the addition of memantine or placebo to donepezil-treated patients with moderate to severe AD demonstrated a reduction in behavioral symptoms with memantine (Cummings et al, 2006; Tariot et al, 2004). Clearly, prospects for effective combinations of drugs that are available for the treatment of AD require additional exploration.

Experiment I—Identification of a Dose of Memantine that Does Not Impair Learning

Whereas NMDA receptor antagonism typically impairs learning, in some conditions learning can be improved. Using extremely low doses of an NMDA antagonist is one method that can be used to improve learning. For example, a very low dose of MK-801 (0.01 mg/kg) increased firing rate of prefrontal cortical neurons in rats and improved

performance on a spontaneous alternation task (Jackson et al, 2004). It has also been shown that in animals with extremely poor learning ability, low doses of NMDA receptor antagonists can enhance learning (Mondadori et al, 1989). Introducing impairments in animals is another means to show improved learning with NMDA antagonists. Animals showing deficits in learning as a result of entorhinal cortex lesions showed improvement in spatial learning when administered memantine (Zajaczkowski et al, 1996).

In the present study, although older rabbits with moderately impaired eyeblink conditioning were used, no dose of memantine significantly improved acquisition of CRs over levels achieved by vehicle-treated older rabbits. Rabbits of a mean age of 26 months have demonstrated age-related neuropathology in the hippocampus, including a decrease in hippocampal neuronal excitability (Power et al, 2002), and significant loss of hippocampal nAChRs (J-G Li, MA Lehr, L-Y Liu-Chen, DS Woodruff-Pak, unpublished data). This degree of neuropathology may be too limited to show a benefit of memantine. Furthermore, memantine may affect aspects of cognition such as attention and processing speed that are not assessed in delay eyeblink classical conditioning. In dual-task studies in young adult humans, manipulations of attention did not cause differences in acquisition of CRs in delay eyeblink classical conditioning (Papka et al, 1997).

The model system of eyeblink classical conditioning in older rabbits has been used as a preclinical test for cognition-enhancing drugs in part because of the parallels in older rabbits and humans in delay eyeblink classical conditioning. An additional rationale for the use of delay eyeblink conditioning in older rabbits for preclinical studies is the fact that delay eyeblink conditioning is severely impaired in AD (Solomon et al, 1991; Woodruff-Pak et al, 1990). Memantine is approved for use in the late stages of AD, whereas eyeblink classical conditioning in older rabbits has proved useful in assessing drugs targeting the cholinergic system in mild to moderate AD. In this regard, the AD model rabbit created by increasing dietary cholesterol may be a better model for assessment of memantine's effects on learning. AD neuropathology (Ghribi et al, 2006; Sparks et al, 1994) as well as impaired eyeblink conditioning have been observed in this model (Sparks and Schreurs, 2003).

In pharmacological studies, to have a valid conclusion about effects on learning as assessed by the CR, the motor response, the UR, should not be affected by the drug. Drug dose did not affect UR amplitude in the paired conditioning data, but there was a significant difference in UR amplitude over training sessions (Figure 2a). In Session 1, rabbits in the 0.1 and 1.0 mg/kg memantine groups had significantly lower UR amplitudes, introducing large variability in UR amplitude. Mean UR amplitude varied from 2.9 mm at the 1.0 mg/kg dose to 8.6 mm at the 0.5 mg/kg dose. As shown in Figure 1, CR percentage for the same rabbits was extremely low and not variable in Session 1. Mean CR amplitude for the four groups also had a narrow range, from 0.19 mm at the 0.1 mg/kg dose to 0.31 mm for vehicle-treated rabbits. In the explicitly unpaired condition, the effect of 0.5 mg/kg memantine on UR amplitude was no different from the effect of vehicle. We attribute the significant difference in

UR amplitude over training sessions to high variability of the rabbits in Experiment 1 on the first day of training. This variability was reduced over subsequent training sessions. Variability in UR amplitude in Session 1 that resulted in the significant effect of UR amplitude over training sessions cannot account for the acquisition curves of percentage of CRs (shown in Figure 1a) and CR amplitude over the 10 training sessions.

Experiment 2—Combined Effect of Memantine + Galantamine or Memantine + Donepezil

As mentioned in regard to UR amplitude data in Experiment 1, to have a valid conclusion about effects on learning, as assessed by the CR, the motor response, the UR, should not be affected by the drug. In Experiment 2, the analysis of UR amplitude in the paired condition yielded a significant interaction effect of drug dose and training sessions. There were no significant main effects of drug dose or training sessions in this analysis of UR amplitude. Like UR amplitude in Session 1 of Experiment 1, there was large variability of UR amplitude in Session 1 of Experiment 2. UR amplitude in Session 1 ranged from 5.0 mm in the 0.75 mg/kg donepezil + 0.5 mg/kg memantine group to 9.0 mm in the 3.0 mg/kg galantamine + 0.5 mg/kg memantine groups. As can be seen in Figure 2b, over training sessions the groups with higher UR amplitude lowered their mean UR amplitude, and the groups with lower UR amplitude increased mean UR amplitude so that by Session 10 the values were quite similar. Whereas there was a wide range in UR amplitude in Session 1, the range of percentage of CRs was narrow in Session 1 (Figure 3a). Mean CR amplitude had a range of 0.19 mm—ranging from 0.14 in the vehicle group to 0.33 in both the 3.0 mg/kg galantamine and 0.75 mg/kg donepezil + 0.5 mg/kg memantine groups. As with Experiment 1, we interpret the significant drug dose by training sessions interaction in UR amplitude in Experiment 2 to excessive variability with a UR amplitude range of 4.0 mm in Session 1 that was reduced to a very narrow range of 1.24 mm by Session 10.

In the explicitly unpaired condition, there was a significant effect of training session for responses to the tone CS. However, *post hoc* analyses of the significant effect indicated that none of the group differences were significant on a given session. For all three drug doses, responses to the tone were generally in the 10% eyeblink response range that is the range for spontaneous blinking in rabbits.

The cognition-enhancing efficacy of 3.0 mg/kg galantamine in older rabbits tested with eyeblink classical conditioning has been replicated in this research along with the result that galantamine combined with memantine has an ameliorating effect. Both 3.0 mg/kg galantamine and 3.0 mg/kg galantamine + 0.5 mg/kg memantine yielded superior performance over vehicle-treated rabbits in terms of acquisition of CRs. However, the magnitude of cognition enhancement resulting from 3.0 mg/kg galantamine was not significantly changed by the addition of 0.5 mg/kg memantine. The number of trials to learning criterion (the speed or rate of learning) was reduced significantly by 3.0 mg/kg galantamine, but not by any other drug or drug combination.

A dose of 0.75 mg/kg donepezil did not improve learning significantly over vehicle in older rabbits in this study, although a dose of 3.0 mg/kg donepezil improved learning in older (Woodruff-Pak, 2001) but not younger rabbits (Woodruff-Pak *et al*, 2003). Higher levels of AChE inhibition provided by the 3.0 mg/kg donepezil dose apparently ameliorate learning deficits in older rabbits. The addition of 0.5 mg/kg memantine did not significantly affect the efficacy of 0.75 mg/kg donepezil. A dose of 0.5 mg/kg memantine had a nonsignificant effect on learning in older rabbits, and it did not improve learning over the effect of 3.0 mg/kg galantamine or 0.75 mg/kg donepezil when it was combined with them.

In rabbits, AChE inhibition is four to eight times greater in donepezil than it is in galantamine (Geerts *et al*, 2005), and consequently the 0.75 mg/kg dose of donepezil was selected as it was four times lower than the effective dose of 3.0 mg/kg galantamine. In this regard, we replicated the Geerts *et al* (2005) analyses as plasma and brain AChE levels were similar for rabbits treated with 3.0 mg/kg galantamine or 0.75 mg/kg donepezil. Further, there was no difference in plasma and brain AChE levels in rabbits treated with combinations of 0.5 mg/kg memantine and 3.0 mg/kg galantamine or 0.75 mg/kg donepezil. This is the first study in which we attempted to equate galantamine and donepezil for AChE inhibition, and a lower dose of donepezil appeared to have less efficacy in older rabbits. Previously, we demonstrated that a dose of 3.0 mg/kg donepezil ameliorated learning impairment in older rabbits (Woodruff-Pak, 2001). A dose of 0.75 mg/kg donepezil equated in level of AChE inhibition to 3.0 mg/kg galantamine (Geerts *et al*, 2005) does not improve acquisition of CRs. With an equal level of AChE inhibition to that of donepezil, galantamine significantly improved learning—possibly with galantamine's second mechanism of action: allosteric modulation of nAChRs. Currently we are testing the hypothesis that nAChR activation is associated with cognition enhancement by measuring nAChR binding in various brain sites after drug administration and behavioral testing. Drugs being tested include galantamine, targeted nAChR agonists, and drugs such as donepezil that are not nAChR agonists.

Given that cholinesterase inhibition alone in 0.75 mg/kg donepezil does not ameliorate learning impairment in older rabbits, galantamine's allosteric activation of nAChRs may be the mechanism through which learning is affected in this animal model. Evidence for an allosteric modulation mechanism in galantamine and physostigmine was provided in electrophysiological studies (Pereira *et al*, 1994). Activation of muscle-type nAChRs by galantamine and nicotinic agonists indicated that galantamine interacted with a binding site that was distinct from the site for nicotinic agonists (Akk and Steinbach, 2005). Allosteric potentiation of nAChR currents by galantamine was transduced in downstream cellular responses to nAChR activation, including increases in intracellular Ca^{2+} and [^{3}H]noradrenaline release (Dajas-Bailador *et al*, 2003).

Testing human α7 nAChRs expressed in Xenopus oocytes, it was demonstrated that memantine inhibits the function of these receptors (Maskell *et al*, 2003). In cultured hippocampal neurons, memantine also caused a concentration-dependent reduction of the amplitudes of whole-cell

currents evoked by α7 nAChRs, and in this study memantine was more potent in inhibiting α7 nAChRs than NMDA receptors (Aracava et al, 2005). Blockade of α7 nAChRs by memantine may have decreased its efficacy in the model system of eyeblink classical conditioning. An antagonist to nAChRs, mecamylamine, significantly impaired eyeblink classical conditioning in young rabbits (Woodruff-Pak et al, 1994a), and a partial agonist of α7 nAChRs, GTS-21, improved eyeblink conditioning in older rabbits (Woodruff-Pak et al, 1994b).

The three drugs approved to treat AD tested in this study can be placed on a continuum in terms of their effect on α7 nAChRs. Galantamine, a mild cholinesterase inhibitor, allosterically modulates nAChRs, including α7 nAChRs. Donepezil, a cholinesterase inhibitor with four to eight times the potency of galantamine, has no demonstrated allosteric effect on α7 nAChRs (eg Dajas-Bailador et al, 2003). Memantine, an NMDA antagonist also blocks α7 nAChRs (eg Aracava et al, 2005; Maskell et al, 2003). Galantamine and donepezil were equated for cholinesterase inhibition, and galantamine was the only drug to show a significant amelioration of learning impairment in older rabbits. It is possible that this amelioration occurred as a consequence of galantamine's allosteric modulation of nAChRs.

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