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We investigated the effect of several doses of scopolamine in older rabbits that were trained for 20 days in the 750 ms delay eyeblink classical conditioning procedure. Our aim was to determine if the scopolamine-injected older rabbit would be a useful model for testing drugs for cognition enhancement in Alzheimer’s disease (AD). A total of 39 rabbits with a mean age of 31 months received classical eyeblink conditioning with daily injections of 0.25, 0.75, or 1.5 mg/kg scopolamine hydrobromide or sterile saline vehicle. Doses of 0.75 and 1.5 mg/kg scopolamine significantly impaired acquisition, whereas acquisition was not significantly impaired with 0.25 mg/kg scopolamine. Results exhibit parallels in performance on delay eyeblink classical conditioning between scopolamine-treated older rabbits and human patients diagnosed with AD.

It has long been established that acetylcholine neurotransmission plays a crucial role in learning and memory. The suggestion that impairment in cholinergic neurons in the brains of older adults might be associated with age-related memory impairment was made decades ago (Drachman & Leavitt, 1974). Relatively low doses of the muscarinic cholinergic antagonist scopolamine administered to young adult humans resulted in a pattern of learning and memory deficits that simulated age-related impairment. When scopolamine was administered to older adults, the pattern of drug-induced cognitive deficits mimicked some cognitive deficits in Alzheimer’s disease (AD). The significance of the simulation of AD impairments with scopolamine became more apparent with the demonstration that acetylcholine levels were lower in the brains of AD patients (Davies & Maloney, 1976; Perry, Perry et al., 1977) and that cholinergic deficits were associated with cognitive impairment in AD (Bowen et al., 1976). The cholinergic neurotransmitter system became a central focus for research on AD, and that research focus continues to the present time (Bartus, 2000).

Muscarnic agonists are currently being evaluated in pre-clinical and clinical studies for their potential to reverse the cholinergic deficits in AD (e.g., Fisher et al., 2000; Growdon, 1997). Cholinergic deafferentation may be one factor that can contribute to the deposition of β-amyloid, a major AD-related neuropathology (Beach et al., 2000). There is evidence that β-amyloid is decreased with muscarinic cholinergic agonist treatment (Fisher, 2000). Muscarinic agonist-related reduction of β-amyloid in cerebral spinal fluid was reported in

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human patients with AD (Nitsch et al., 2000) and in rabbits with cholinergic deafferentation (Beach, Walker et al., 2001).

Bartus, Flicker, and Dean (1983) emphasized the critical necessity of animal models for the investigation of learning and memory in normal aging and AD, and they created logical criteria for developing animal models for pharmacological applications. When these criteria are applied to the model system of eyelink classical conditioning in the rabbit, the model system is of demonstrated strength in investigations of associative learning, normal aging, and AD. Although the neural circuitry essential for acquisition and retention of the conditioned eyelink response resides in the cerebellum, the hippocampus is engaged during basic associative learning of the sort represented by delay eyelink classical conditioning.

Disruption or facilitation of the septo-hippocampal system, a system impaired early in AD, affects the rate of acquisition of conditioned eyelink responses. Neural activity recorded in the hippocampus forms a predictive "model" of the amplitude-time course of the learned behavioral response (Berger & Thompson, 1978b). Hippocampal modeling of the behavioral conditioned response (CR) and unconditioned response (UR) is generated largely by hippocampal pyramidal neurons in the CA1 and CA3 fields (Berger et al., 1983; Berger & Thompson, 1978a). CA1 pyramidal neuron excitability in slices from young and older rabbits that had received classical eyelink conditioning had significantly reduced post-burst afterhyperpolarizations and reduced spike frequency adaptation compared with neurons from naïve rabbits and older rabbits that failed to learn (Disterhoft & McEchron, 2000). It is our working hypothesis that selective loss of hippocampal pyramidal cells (West et al., 1994) and disruption of the septo-hippocampal cholinergic system in AD (Coyle, Price & DeLong, 1983) impair acquisition of delay eyelink classical conditioning in AD beyond the impairment observed in normal aging. The hypothesis was supported (Ewers, Braitman & Woodruff-Pak, 2001; Solomon, Levine et al., 1991; Woodruff-Pak, Finkbiner & Sasse, 1990; Woodruff-Pak, Papka et al., 1996) and extended in adults over the age of 35 with Down’s syndrome and associated AD neuropathology (DS/AD) (Woodruff-Pak, Papka & Simon, 1994).

In rabbits, disruption of muscarinic cholinergic receptors with scopolamine injections impairs acquisition of CRs, and this disruption occurs only when the hippocampus is intact (Solomon, Solomon et al., 1983). Scopolamine injections eliminate hippocampal pyramidal cell activity in conjunction with the CR and UR (Salvatierra & Berry, 1989). Microinjections of scopolamine to the medial septum prolong the rate of acquisition of the classically conditioned eyelink response in rabbits (Solomon & Gottfried, 1981). Scopolamine administration also disrupts eyelink conditioning in humans (Bahro et al., 1995; Solomon et al., 1993).

Because AD-like learning and memory impairment was simulated with the muscarinic cholinergic antagonist, scopolamine, it was initially thought that the cholinergic deficits in AD were predominantly of a muscarinic nature (Davis & Yamamura, 1978). The view that muscarinic cholinergic receptors were impaired in AD was challenged by evidence for nicotinic (but not muscarinic) cholinergic impairment in AD. Audioradiographic and histochemical studies of human brain tissue collected post mortem (Nordberg & Winblad, 1986; Perry et al., 1995; Schroder et al., 1994; Whitehouse et al., 1986) and brain imaging studies in living AD patients (Nordberg et al., 1995) demonstrated specific loss of nicotinic receptors and almost complete sparing of muscarinic receptors in AD. More specifically, the α4 subunit-bearing subtype is selectively lost in AD, whereas the α7 nicotinic cholinergic receptor is retained (Martin-Ruiz et al., 1999; Warman & Nordberg, 1995; Wevers et al., 1999) along with muscarinic receptors including M2 receptors.
Cholinergic afferents to the hippocampal formation are generated from a single source in the medial septal area (McKinney, Coyle & Hendreen, 1983). Both muscarinic cholinergic receptors (Spencer, Horvath & Raber, 1986) and nicotinic cholinergic receptors (Schwartz, 1986) are present in the same target regions of these cholinergic afferents. It is likely that both muscarinic and nicotinic receptor types are activated in parallel. Cholinergic modulation of hippocampal function likely reflects a complex, dynamic combination of muscarinic and nicotinic cholinergic receptor activation, rather than an exclusive action of either type of cholinergic receptor. Thus, cholinergic antagonists that block either muscarinic or nicotinic cholinergic receptors simulate some of the impairment caused by AD. The aim of the present study was to examine an animal model that might simulate AD by using older rabbits injected with various doses of the muscarinic cholinergic antagonist, scopolamine. We hypothesized that older rabbits injected with various doses of scopolamine would simulate some of AD-like disruption and would be impaired beyond normal older rabbits in delay eyeblink classical conditioning.

**Method**

**Subjects**

A total of 39 female retired breeder specific pathogen free (SPF) New Zealand white rabbits were tested. One additional retired breeder rabbit died of causes unrelated to drug administration or training, and data from this rabbit are not included in the analyses. Rabbits' weight ranged between 3.0—6.2 kg with a mean of 4.3 (s.d. = 0.6) kg. Their ages ranged from 24 to 49 months with a mean age of 31.3 (s.d. = 7.8) months. Rabbits were individually housed in stainless steel cages and fed 17 grams of Purina high fiber rabbit chow per day and tap water. They were on a 12/12 hour light/dark cycle.

**Apparatus**

Plexiglas customized rabbit restrainers were used for restraint during behavioral training. Elastic eyelid retractors held the left eye open. A removable platform to hold the headstage was secured behind the ears and under the animal's muzzle. The headstage attached to this platform held a nozzle positioned 1 cm from the cornea through which the airpuff US was presented. Also on the headstage was a minitorque potentiometer for measurement of the nictitating membrane (NM)/eyeblink response. The potentiometer was attached by a lever and a thread to a nylon suture loop in the NM. A computer programmed with Forth and assembly language and interfaced with the stimulus equipment controlled the presentation and duration of stimulus events, collected analog data, and extracted dependent variable measures of conditioning. A minitorque potentiometer on the headstage converted NM movements into electrical signals that were subjected to an analog-to-digital analysis. The digitized values were analyzed from RAM immediately after each training session and also routinely logged on to a floppy disk for permanent storage.

**Procedure**

Rabbits were placed in restrainers for two adaptation sessions on consecutive days before training and drug injections began. In the second adaptation session, rabbits were given a local ophthalmic anesthetic (proparacaine hydrochloride) in the left eye so that a 6-
O nylon suture loop could be placed in the temporal margin of the NM. Sterile saline was used to irrigate the eye, and eye ointment was applied. For training in the paired condition, beginning two days after the second adaptation session, rabbits were injected subcutaneously with drugs or vehicle 15 minutes before training. Next they were placed into the restrainer, fitted with the headstage, and then positioned in a ventilated and sound-attenuated experimental chamber. An 850-ms, 85 dB sound pressure level, 1-kHz tone CS was followed 750 ms after its onset by a 100-ms, 3-psi corneal airpuff US. The CS and US coterminated. The inter-trial-interval was random, ranging from 10–20 seconds, and the session lasted 35–45 min.

Rabbits were trained for a total of 20 90-trial sessions, making the total number of paired trial presentations 1,800. Twenty sessions were required because previous experience with older rabbits indicated that the average vehicle-treated rabbit achieved learning criterion during the 9th session. We expected that prolonging acquisition in older rabbits with scopolamine might require more than the 15 training sessions we normally used for older rabbits. After completion of the 20th training session, animals were deeply anesthetized with Nembutal and decapitated. Brains were removed rapidly and frozen for later analysis.

Behavioral Data Analyses

A CR was automatically scored by the computer system if the NM movement was 0.5 mm or greater and occurred in the period between 25 to 750 ms after onset of the tone CS. Amplitude measurements on this system were calibrated to be accurate to the nearest 0.1 mm, and latency measurements were accurate to the nearest millisecond. The learning criterion was the production of 8 CRs in a sequence of 9 consecutive trials. If a rabbit never attained this criterion in 1800 trials, a score of 1850 was assigned for trials to learning criterion. The UR was the reflexive blink to the corneal airpuff and was scored as any response of 0.5 mm or greater occurring in the interval between 751 to 1041 ms after CS onset. Amplitude of the UR was measured at the point of peak amplitude of the response.

Drugs

For muscarinic cholinergic antagonism, scopolamine hydrobromide (Sigma) was administered at dosage levels of 1.5, 0.75, and 0.25 mg/kg. Scopolamine was prepared in solution as needed, stored in a refrigerated, covered container, and injected 15 min before behavioral testing. Vehicle-injected animals received an equal volume of sterile saline.

Results

A one-way analysis of variance (ANOVA) was used to compare the four groups receiving paired conditioning on the dependent variable of number of trials required to attain a learning criterion of 8 CRs in 9 consecutive trials. There was a significant difference among groups, $F (3, 35) = 3.55, p = 0.024$. Post hoc comparisons using the Tukey HSD test indicated that the difference between trials to criterion in the 1.5 mg/kg scopolamine group and the vehicle group was significant ($p = 0.033$). Differences among the other groups were not significance (Figure 1).
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A 4 (Group) by 20 (Session) repeated measures ANOVA was conducted on the dependent variable percentage of CRs in the 4 groups receiving paired eyelink conditioning over 20 sessions of testing. This analysis revealed a significant effect of Group, $F(3, 36) = 6.46$, $p = .001$, a significant effect of Session, $F(19, 665) = 20.85$, $p < .0001$, and a significant interaction between Group and Session, $F(57, 665) = 2.46$, $p < .0001$ (Figure 2). Post hoc analysis of the significant Group effect indicated that both the 0.75 and the 1.5 mg/kg scopolamine groups had significantly fewer CRs than the vehicle group, $p = 0.003$ and 0.013, respectively. Further analysis of the significant interaction effect using Tukey HSD post hoc tests indicated that rabbits receiving 0.75 or 1.5 mg/kg scopolamine showed significantly fewer CRs than rabbits receiving vehicle alone in Sessions 10, 11, 13, 15, 19, and 20. In addition, the group receiving 0.75 mg/kg scopolamine also had significantly fewer CRs than vehicle-treated rabbits in Sessions 14, 16, and 18. There were significantly fewer CRs in the 0.25 mg/kg group than in the vehicle-treated group in Session 11.

The amplitude of the UR provides a measure of the magnitude of the reflexive NM response to the US. A 4 (Group) by 20 (Sessions) ANOVA was used to compare the four drug treatment groups receiving paired conditioning over 20 training sessions on the dependent variable UR amplitude. The effect of Group did not attain significance, $F(3, 35) =$
Fig. 2. Percentage of conditioned responses in four groups of older rabbits injected with 0, 0.25, 0.75, and 1.5 mg/kg scopolamine. Rabbits were trained in the 750 ms delay eyeblink classical conditioning paradigm for 20 sessions comprised of 90 trials of paired tone and corneal airpuff presentations. A diamond indicates the sessions on which the 1.5 mg/kg scopolamine group differed significantly from the vehicle-treated group. A plus represents a significant difference from vehicle for the 0.75 mg/kg scopolamine group, and a tilde represents a significant difference from vehicle for the 0.25 mg/kg scopolamine group.

2.66, \( p = 0.098 \). The effect of Sessions was significant, \( F(19, 665) = 5.77, p < 0.0001 \) (Figure 3), but the Group by Sessions interaction effect did not attain statistical significance, \( F(57, 665) = 0.69, p = 0.96 \). Scopolamine-treated rabbits had motor responses to the corneal airpuff that were similar to the motor responses of vehicle-treated rabbits. As is typical in acquisition, the UR increased in magnitude during the early portion of training.

**Discussion**

Results demonstrated that disruption of the cholinergic system of older rabbits with scopolamine impaired significantly the acquisition of CRs in the 750 ms delay procedure beyond the impairment associated with normal aging. Vehicle-injected older rabbits attained learning criterion on average in Session 10 (821 trials), whereas older rabbits injected with 1.5 mg/kg scopolamine attained criterion on average in Session 15 (1391 trials).

The magnitude of the impairment was somewhat dependent on the dose of scopolamine. The dose of 1.5 mg/kg scopolamine was the only dose that resulted in significantly more trials to learning criterion over vehicle-treated older rabbits. Doses of 0.75 and 1.5 mg/kg scopolamine caused a lower total CR percentage over the 20 training sessions, and...
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Fig. 3. Unconditioned response amplitude over 20 sessions in the 750 ms delay eyeblink classical conditioning paradigm for four groups treated with 0 (vehicle), 0.25, 0.75, or 1.5 mg/kg scopolamine.

these doses caused a significantly lower percentage of CRs in many of the individual training sessions. Total CR percentage was not different from vehicle in older rabbits treated with 0.25 mg/kg scopolamine, and this group had significantly fewer CRs than the vehicle group only in Session 11. The fact that there were actually more training sessions in which the 0.75 mg/kg scopolamine group had significantly fewer CRs than the vehicle-treated group indicates that an impairment threshold was reached for older rabbits with 0.75 mg/kg scopolamine.

Most studies of scopolamine effects on eyeblink conditioning in young rabbits used a dose of 1.5 mg/kg scopolamine (e.g., Harvey, Gormezano & Cool-Hauser, 1983; Moore, Goodell & Solomon, 1976; Solomon et al., 1983; Woodruff-Pak & Hinchliffe, 1997). Young rabbits in our laboratory treated with 1.5 mg/kg scopolamine were 4-month-old females weighing 1.8 to 2.3 kg with a mean of 2.2 kg (Woodruff-Pak & Hinchliffe, 1997). For the average young rabbit weighing 2.2 kg, the daily scopolamine injection was 3.3 mg. For the older female retired breeder rabbits in Experiment 1, rabbits’ weight ranged between 3.0—6.2 kg with a mean of 4.3 kg. The average daily scopolamine injection was 6.5 mg. The difference in brain size between young and older rabbits is minimal, the metabolism of the older rabbits is slowed, yet the average daily injections for older rabbits contained about twice as much scopolamine due to the older rabbits’ greater body weight.
Although trials to learning criterion in the 0.75 mg/kg scopolamine group approached but did not attain statistical significance ($p = 0.159$; but see Figure 1), total CR percentage was significantly lower in this group. Data suggest that to antagonize muscarinic cholinergic receptors in older rabbits to the degree that behavior is impaired, a dose of 0.75 mg/kg scopolamine need not be exceeded.

Various doses of scopolamine did not affect UR amplitude in groups receiving paired CS-US presentations. We did not test older rabbits treated with scopolamine in the explicitly unpaired condition. Previous studies testing young rabbits injected with scopolamine hydrobromide in the explicitly unpaired condition demonstrated no drug-induced effects on sensitization or habituation (Harvey et al., 1983; Moore et al., 1976; Solomon et al., 1983; Woodruff-Pak & Hinchcliffe, 1997).

Over a decade ago we proposed that patients with probable AD would be impaired on eyeblink conditioning based on behavioral evidence available from older rabbits and scopolamine-injected young rabbits (Woodruff-Pak, Finkbiner & Katz, 1989). Severe impairment in 400 ms delay eyeblink conditioning in probable AD and in DS/AD has repeatedly been demonstrated. Multivariable regression analysis using age, gender, and total percentage of CRs in a session of 400 ms delay eyeblink conditioning distinguished subjects with AD from healthy older adults with a probability of 80 percent (Ewers et al., 2001).

Because rabbits with disrupted hippocampal cholinergic systems have delayed acquisition of CRs but eventually acquire them (e.g., Harvey et al., 1983; Moore et al., 1976; Solomon et al., 1983), we predicted that if probable AD and DS/AD patients were given enough training trials, they would eventually produce CRs. Testing probable AD and DS/AD patients on eyeblink conditioning for five consecutive days, we observed that they eventually acquired CRs (Woodruff-Pak, Romano & Papka, 1996). The majority of probable AD and DS/AD patients attained a learning criterion of 8 CRs in 9 consecutive trials and exceeded production of 25 percent CRs when they were given additional training. Another study testing probable AD patients in paired tone and corneal airpuff presentations in the 400 ms delay paradigm for 4 consecutive 70-trial sessions reported similar results (Solomon et al., 1995). These results indicate that the neural substrate supporting eyeblink conditioning is impaired by probable AD and DS/AD beyond the impairment observed in normal aging, but this neural substrate is not destroyed. These results with human probable AD and DS/AD patients provide further support for the validity of the original scopolamine-injected older rabbit model.

Our data continue to support the hypothesis that the disrupted hippocampus, known to play a modulatory role in eyeblink conditioning, delays acquisition of the conditioned eyeblink response in probable AD and DS/AD. Probable AD and DS/AD patients perform eyeblink conditioning in a comparable fashion to young adult humans treated with scopolamine (Bahro et al., 1995; Solomon et al., 1993). Doses of 0.75 and 1.5 mg/kg scopolamine simulated in older rabbits the additional deficit in delay eyeblink conditioning beyond normal older vehicle-treated rabbits that parallels the delay eyeblink conditioning impairment in AD beyond healthy aging.

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