Research Report

Delay-dependent cholinergic modulation of visual short-term memory in rhesus macaques

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Abstract

Cholinergic neuromodulation is known to play a key role in visual working memory (VWM) – keeping relevant stimulus representations available for cognitive processes up to a few minutes. Despite the growing body of evidence on how the neural and cognitive mechanisms of working memory dynamically change over retention time, studies that measure cholinergic effects as a function of time in non-human primates has been scarce. Using the delayed matching to sample (DMTS) VWM task in rhesus macaques (N=6), we studied how cholinergic neuromodulation influences VWM maintenance across a wide range of delays (1 to 72 s). We aimed at disentangling which delay intervals are most affected by transient amnestic treatments using the muscarinic receptor antagonist scopolamine administered alone and in combination with two doses of the clinically widely used acetylcholinesterase inhibitor (AChEI) donepezil, probing for the reversal of scopolamine-induced impairments. Results indicate that scopolamine-induced impairments of VWM maintenance are delay-dependent and specifically affect the 10-30 seconds time range, suggesting that scopolamine speeds up the normal deterioration of VWM with the passage of time. Donepezil partially rescued the scopolamine-induced impairments of VWM performance. These results are in line with our current knowledge on the role of muscarinic acetylcholine receptors in the maintenance of working memory. Taking delay length into account can be a valuable component of basic and preclinical pharmacological research on the behavioral manifestations of VWM maintenance and can deepen our current understanding of short-term memory and its age-related impairments.

1. Introduction

The importance of a visual stimulus is not necessarily commensurate with whether it is present in the current visual environment, or how much time passed since it has disappeared. To support adaptive behaviors, humans and animals have to maintain relevant stimulus representations available despite the passage of time or changes in the environment. How different memory systems achieve this across various timescales is an important key question in current neuroscience, with particular relevance to the ability of retaining mental representations up to a few minutes in visual working memory (VWM) [1] as being an early indicator of cognitive impairments in healthy and pathological aging [2]. The cholinergic hypothesis of age-related cognitive decline highlights the role of normal cholinergic neuromodulation in VWM and can provide important insights for basic research on memory by the application of cholinergic agents in clinical settings or in basic research [3] [4] [5]. Among others, cholinergic neuromodulation is a currently widely investigated and promising symptomatic therapeutic treatment regime and is targeted for drug development, especially as part of currently evolving mono and combination therapies [6–8].

The delayed matching to sample test (DMTS) is simple and translatable paradigm to study working memory that has been extensively used in both animal [9–12] and human [13–16] research. The basic structure of the DMTS task is to memorize a sample stimulus, maintain it across a delay period, then choose the memorized stimulus among an array of non-matching distractor stimuli. A wealth of studies examined the connections between VWM and the cholinergic system in rats [4,17–19] and non-human primates in behavioral pharmacology experiments. For example, the anticholinergic agent scopolamine decreased performance [20], while nicotine has been shown to improve DMTS performance in older animals [21]. The acetylcholinesterase inhibitor donepezil has been found to cause a dose-dependent improvement in the performance of macaques in the DMTS task [22–24]. The combined

application of the acetylcholinesterase inhibitor physostigmine with the α2 adrenergic receptor agonist clonidine also promoted VWM performance [25,26].

It is well known that longer delay periods ensue lower memory performance, which provides a simple way to measure the decay and dynamics of working memory over time [27,28]. However, interestingly, only very few of the previous behavioral pharmacology studies on the cholinergic modulation of working memory have attempted to explicitly localize pharmacological effects in time in conjunction with the classical DMTS paradigm. Only a single series of experiments have addressed the effects of scopolamine under pre-defined delay periods (up to 10 s) on DMTS performance using three rhesus macaque monkeys [29] in a simple positional discrimination paradigm using a matrix illuminated food boxes, not being able to conclude on the full range temporal dynamics of scopolamine induced deficits. While several later studies have taken into account the length of the delay period, they usually did so by initially titrating the delay length to calibrate the preferred performance level of the individual animal [22–24,28]. This procedure can be very useful to optimize the sensitivity of the tests to increase the main effects of the pharmacological manipulation, however, among other methodological characteristics [30], it certainly precludes the researchers from drawing conclusions about how the effects might interact with the temporal dynamics of memory encoding and maintenance. For example, in [31], nicotine improved cognitive performance, and this effect was stronger in long delays, but setting delay categories based on individual animal performance led to heterogenous delays across animals and poor conclusion on the effects of the delay itself.

Here, using the DMTS task in rhesus macaques, we studied how cholinergic neuromodulation might influence VWM maintenance across a wide range of delays (1 to 72 s) that were fixed across animals. By using identical delays for all animals participating in the study, assuming that despite potentially differing performance levels the temporal dynamics of

memory encoding and maintenance would be similar among animals, we sought to pinpoint in what phases of the delay period the well-established cholinergic modulations [32,33] would most strongly affect working memory performance: we measured the effects of the anticholinergic agent scopolamine administered alone, or in combination with two doses of the acetylcholinesterase inhibitor (AChEI) donepezil to probe for the reversal of scopolamine effects. We hypothesized that, as implied by earlier research, both scopolamine-induced impairment effects and donepezil induced reversal effects would be stronger at longer delays, sparing very short delays that might rely on the lower-level sensory components of VWM [34].

2. Methods

2.1. Subjects

Six male 5-year-old rhesus macaques (*Macaca mulatta*) were included in the study, weighing 4.62 ± 0.25 kg at the beginning of the experiments. Animals were fed once per day, in the afternoons, following the daily testing session. Diet was standard nutritionally complete lab chow specifically designed for non-human-primates (Altromin Spezialfutter GmbH, Lage, Germany) and was daily supplemented with fresh fruit and vegetables. Water was available *ad libitum*. In the home cage and testing rooms, temperature and relative humidity were maintained at 24 ± 1 °C and 55 ± 5%, respectively.

All procedures were conducted in the Grastyán Translational Research Center of the University of Pécs. The study was approved by the Local and National Ethical Committees on Animal Research and the Department of Animal Health and Food Control of the County Government Offices of the Ministry of Agriculture (BA02/2000-11/2012). Measures were taken to minimize pain and discomfort of the animals in accordance with the Directive 40/2013. (II.14.): 'On animal experiments' issued by the Government of Hungary, and the Directive 2010/63/EU 'On the protection of animals used for scientific purposes' issued by the European Parliament and the European Council.

2.2. Delayed matching to sample paradigm

Animals performed a DMTS task (Figure 1A) in one session per day. Each experimental session consisted of 120 trials and lasted for approximately 60 min. Conditioning and experimental (drug) sessions were performed in a computerized operant testing chamber equipped with a touch screen and dry pellet delivery apparatus (Monkey CANTAB Intellistation, Campden Instruments, UK). The paradigm was controlled by the Monkey CANTAB Stimulus Test Battery. In the testing chamber, the animals were free to move and access the CANTAB touch panel. At the beginning of each trial, a short tone was played to

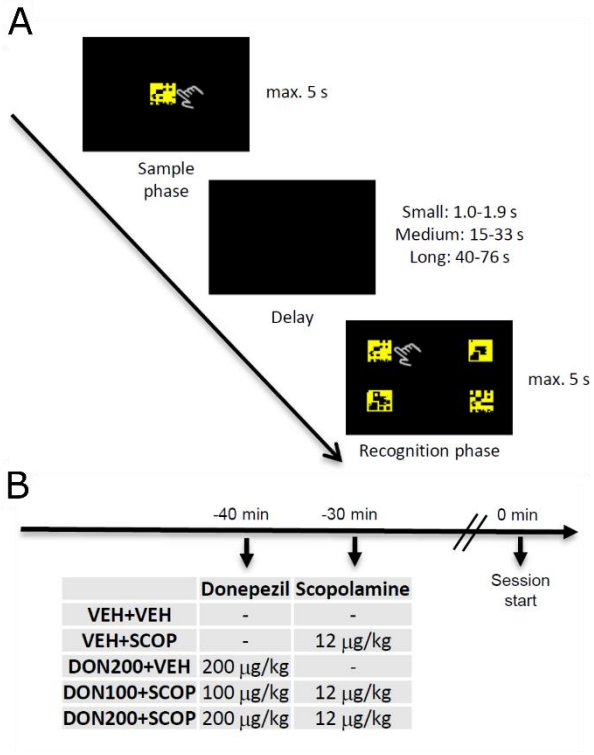
indicate the trial start. Each trial consisted of a sample and a recognition phase. During the sample phase, a stimulus was presented at the center of the screen that the animals had to touch for a maximum of 5 seconds. If the animal did not touch the sample stimulus, the trial was considered unsuccessful and was terminated with no reward delivered. If the subject responded while the sample stimulus was still displayed, the sample phase was followed by a delay with blank screen. The delay time on conditioning days was 5 seconds. On the experimental sessions we applied 3 delay duration categories: short delay between 1.0 and 1.9 s; medium delay between 15 and 33 s; long delay between 40 and 76 s. Experimental sessions included 40 trials in each delay category. There were 10 possible delay durations in each delay category: 1.0; 1.1; 1.2; 1.3; 1.4; 1.5; 1.6; 1.7; 1.8; 1.9 s for short delay, 15; 17; 19; 21; 23; 25; 27; 29; 31; 33 s for medium delay, and 40; 44; 48; 52; 56; 60; 64; 68; 72; 76 s for long delay. After the delay time has passed, the recognition phase stimuli appeared on the corner of the screen. There were 4 stimuli in the recognition phase, one of which was identical to the stimulus presented in the sample phase (target stimulus) and 3 were different (distractor stimuli). During this phase, the animals had 5 seconds to touch the target stimulus. Upon a correct response the stimuli disappeared, and subjects received a reward immediately after the response. The animals were rewarded with 190 mg pellets (Bio-Serv, Fruit Crunchies, 190 mg Pellets, Certified, Contaminant Screened). In the case of wrong choice or if the animal did not respond within 5 seconds, the trial was considered unsuccessful and was terminated with no reward delivered. Each trial was followed by a 6±1 s inter-trial interval.

Animals performed the task at the same time of the day on every weekday. All animals had been previously trained to a criterion of at least 60% performance accuracy.

2.3. Procedures and drug administration

In the present placebo-controlled crossover and repeated measures experimental design all subjects underwent 5 recording sessions with at least 72 hours of washout interval between

the sessions. The five sessions covered all the pharmacological treatment conditions. To achieve stable and high plasma levels at the time of task performance, intramuscular injections of donepezil (Gedeon Richter Plc., Budapest, Hungary) were administered 40 min prior to behavioral testing, followed by scopolamine (Tocris Bioscience, Bristol, UK) or the corresponding vehicle treatment (saline) at 30 min before the behavioral testing session (Figure 1B). Donepezil and scopolamine were both dissolved in saline (0.9% NaCl). Saline was also used for vehicle (sham) treatments. Injection volume was set to 0.05 ml per kg bodyweight. There were five types of treatment: vehicle + vehicle (VEH+VEH); vehicle + 12 μg/kg dose of scopolamine (VEH+SCOP); 100 μg/kg dose of donepezil + 12 μg/kg scopolamine (DON100+SCOP); 200 μg/kg dose of donepezil + 12 μg/kg dose of scopolamine (DON200+SCOP); 200 μg/kg dose of donepezil + vehicle (DON200+VEH). Each solution was freshly prepared before each recording session and was stored for less than two hours. One animal was sensitive to scopolamine, so for him, a lower dose was used throughout the whole experiment (4 μg/kg).



	Donepezil	Scopolamine
VEH+VEH	-	-
VEH+SCOP	-	12 μg/kg
DON200+VEH	200 μg/kg	-
DON100+SCOP	100 μg/kg	12 μg/kg
DON200+SCOP	200 μg/kg	12 μg/kg

Figure 1. (A) Schematic illustration of a single trial in the Delayed Matching to Sample paradigm and (B) combination of treatments. In the sample phase, the sample stimulus appeared in the center of the screen, and the animals had to respond by touching the stimulus within 5 s. This was followed by a blank-screen delay period with 3 duration categories (short: 1 to 1.9 s; medium: 15 to 33 s; long: 40 to 76 s). After the delay had elapsed, 4 recognition stimuli appeared in each quadrant of the screen, one of which was identical to the stimulus presented in the sample phase (target stimulus) and 3 were different (distractor stimuli). During this phase, the animals had to touch the target stimulus within 5 s. **(B) Schematic illustration of the treatment regime and timeline.** In each experimental session animals were administered acetylcholinesterase inhibitor donepezil (DON) and muscarinic receptor antagonist scopolamine (SCOP) alone or combined, or vehicle (VEH). Donepezil (or VEH) was injected at 40 min before the behavioral session, while SCOP (or VEH) was injected at 30 min before the session. We applied five treatment combinations as shown in the table.

2.4. Data analysis

We analyzed performance accuracy (proportion of correct responses; PA) and mean reaction time (time elapsed between the appearance of the stimulus and the response; RT). The data were analyzed by repeated measures analysis of variance (rANOVA). To test the assumption of sphericity we used Mauchly's sphericity test. The results of Mauchly's test showed that the sphericity assumption was not violated in any of the tests, so no correction was applied. We analyzed the main effects of Treatment and Delay and their interaction, and we used Fisher's Least Significant Difference (LSD) post hoc test to examine pairwise comparisons, focusing on the delay effect within treatments and differences between treatments within the delay category. Effects were considered significant at $p<0.05$.

3. Results

3.1. Effect of delay duration in vehicle treatment

Six male young adult rhesus macaques performed DMTS task in each of five treatment combinations (**Figure 1**). We applied a placebo-controlled, crossover, repeated measures design. In the vehicle (VEH+VEH) session the average ratio of performance accuracy (PA) was 71.5 ± 5.2% (Mean ± s.e.m.). In the vehicle treatment the analysis of delay effects showed significant main effect on PA ($F_{2,10}=16.842$, $p<0.001$, $\eta_p^2=0.77$; see **Figure 2**). In the medium and the long delay PA significantly decreased compared to short delay (post hoc for SHORT vs. MEDIUM: $p=0.032$; MEDIUM vs. LONG: $p=0.008$).

In the vehicle (VEH+VEH) session, the average reaction time (RT) was 2363 ± 141 ms. In the vehicle treatment the analysis of delay effects showed significant main effect on RT ($F_{2,10}=18.902$, $p<0.001$, $\eta_p^2=0.79$, see **Figure 5**). Reaction times were significantly larger in medium compared to short delay (SHORT vs. MEDIUM post hoc: $p=0.001$), however there was no significant difference between the long and medium delays (MEDIUM vs. LONG post hoc: $p=0.198$).

3.2. Effect of treatments on rate of performance accuracy

We analyzed the effects of treatments on performance accuracy (PA). The analysis of the main effect of Treatment **(Figure 2)** showed that scopolamine induced a significant decrease in PA compared to vehicle ($F_{1,5}$=18.133, p=0.008, η_p^2=0.78, VEH+VEH vs. VEH+SCOP post hoc p=0.008). We found a significant main effect of Delay ($F_{2,10}$=25.079, p<0.001, η_p^2=0.83), and we also found significant interaction between Treatment and Delay ($F_{2,10}$=5.213, p=0.028, η_p^2=0.51). In the case of vehicle treatment, PA decreased as the delay increased (SHORT vs. MEDIUM post hoc p=0.029; MEDIUM vs. LONG post hoc p=0.007). In contrast, in the case of scopolamine treatment, medium delay led to decreased PA compared to short (SHORT vs. MEDIUM post hoc p<0.001), but long delay had no effect compared to medium (MEDIUM vs. LONG post hoc p=0.438). Scopolamine treatment significantly decreased PA in medium delay (VEH+VEH vs. VEH+SCOP post hoc p<0.001), but not in short (VEH+VEH vs. VEH+SCOP post hoc p=0.136) and long delay (VEH+VEH vs. VEH+SCOP post hoc p=0.269).

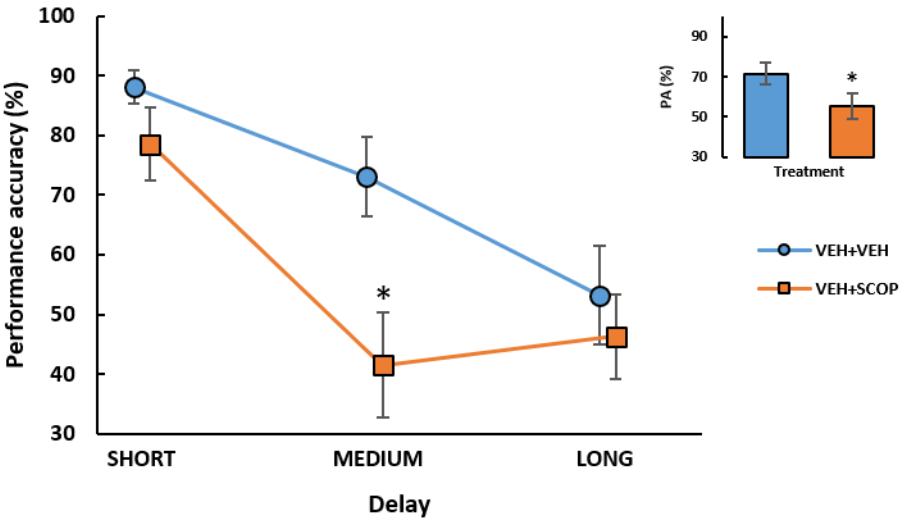


Figure 2. Effect of scopolamine (VEH+SCOP) treatment compared to vehicle (VEH+VEH) on average performance accuracy (PA) in three delay categories (SHORT, MEDIUM, LONG). The inset illustrates that scopolamine induced a significant decrease in average PA compared to vehicle. In the case of scopolamine treatment, the PA reached the floor

in the medium delay, while in the vehicle treatment the PA decreased continuously. Averages across N=6 subjects, error bars show s.e.m. For details on treatments see **Figure 1B**. Asterisks mark significant ($p<0.05$, post hoc) simple Treatment effects within each level of Delay.

The effect of donepezil monotreatment (**Figure 3**) had no significant main effect on PA ($F_{1,5}=0.4$, $p=0.555$, $\eta_p^2=0.07$) compared to vehicle treatment. We found a significant main effect of Delay ($F_{2,10}=29.627$, $p<0.001$, $\eta_p^2=0.86$). No significant interaction between Treatment and Delay was found ($F_{2,10}=0.73$, $p=0.502$, $\eta_p^2=0.13$). In accordance with this, PA significantly decreased with longer delays in both the vehicle (see Section 3.1) and the donepezil monotreatment (SHORT vs. MEDIUM post hoc $p=0.002$; MEDIUM vs. LONG post hoc $p=0.052$).

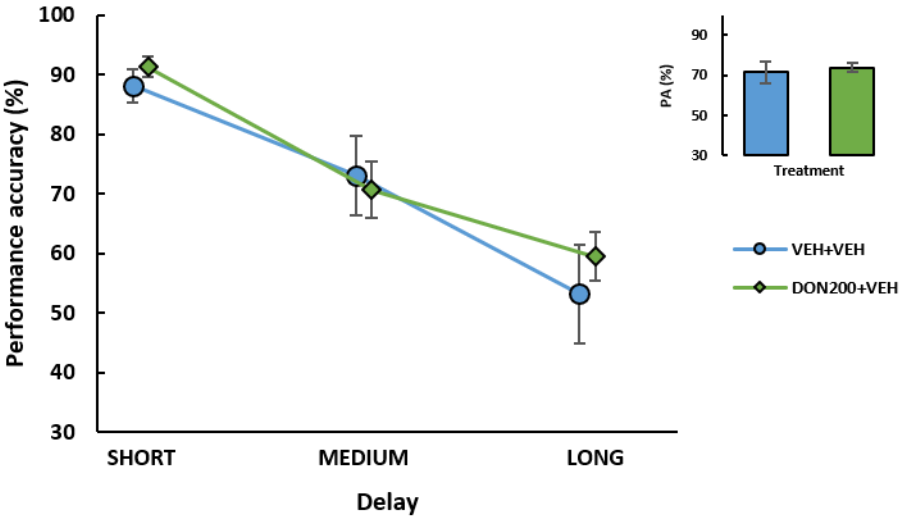


Figure 3. Effect of donepezil (DON200+VEH) monotreatment compared to double vehicle control treatment (VEH+VEH) on average performance accuracy (PA) in the three delay categories (SHORT, MEDIUM, LONG). The inset illustrates that donepezil monotreatment had no significant main effect on average PA compared to vehicle. The graph shows that PA similarly decreased with longer delays after donepezil and vehicle treatments. Data is depicted

in averages across N=6 subjects, error bars indicate s.e.m. For details on treatments see **Figure 1B**.

We analyzed the effect of simultaneous administration of donepezil and scopolamine (DON100+SCOP; DON200+SCOP) compared to scopolamine monotreatment (VEH+SCOP, see **Figure 5**). No significant main effect of Treatment was found on the PA ($F_{2,10}$=1.415, p=0.288, η_p^2=0.22). In contrast, we found a significant main effect of Delay ($F_{2,10}$=31.475, p<0.001, η_p^2=0.86). We also found significant interaction between Treatment and Delay ($F_{4,20}$=2.985, p=0.044, η_p^2=0.37). The way PA decreased with delay differed across treatments: for scopolamine, it reached its floor value already at medium delays (see above), while under DON+SCOP treatments it decreased monotonously across the entire delay interval range (DON100+SCOP: SHORT vs. MEDIUM post hoc p<0.001; MEDIUM vs. LONG post hoc p=0.016; DON200+SCOP: SHORT vs. MEDIUM post hoc p<0.001; MEDIUM vs. LONG post hoc p=0.019; see also **Figure 4**), as in the VEH+VEH condition (**Figure 2**). As a result of this differential pattern of delay sensitivity, both low and high donepezil treatments significantly increased PA compared to scopolamine in medium delay (DON100+SCOP vs. VEH+SCOP post hoc p=0.025; DON200+SCOP vs. VEH+SCOP post hoc p=0.005), but not in short (DON100+SCOP vs. VEH+SCOP post hoc p=0.264; DON200+SCOP vs. VEH+SCOP post hoc p=0.890) and long delay (DON100+SCOP vs. VEH+SCOP post hoc p=0.222; DON200+SCOP vs. VEH+SCOP post hoc p=0.678).

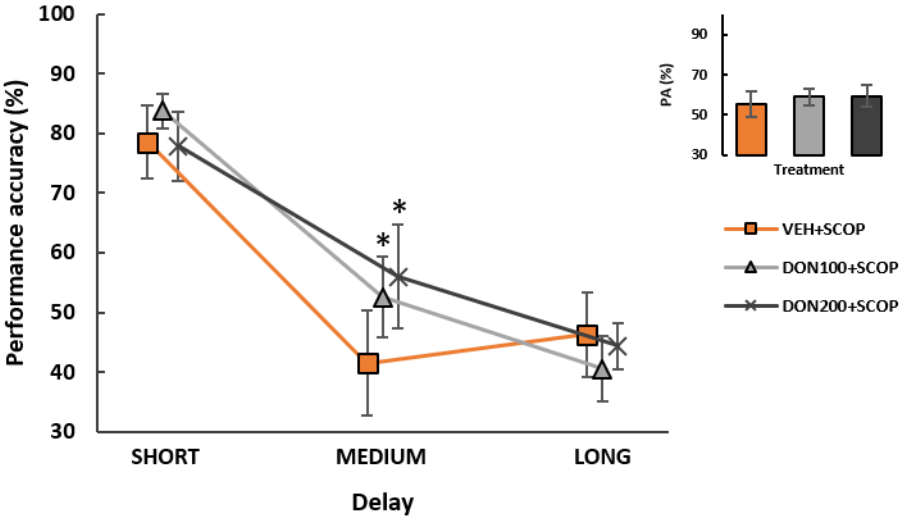


Figure 4. Effect of simultaneous administration of donepezil and scopolamine (DON100+SCOP; DON200+SCOP) treatments compared to scopolamine (VEH+SCOP) on average performance accuracy (PA) in three delay categories (SHORT, MEDIUM, LONG). The inset illustrates that donepezil treatments had no significant main effect on average PA compared to scopolamine. The graph shows that in the case of scopolamine treatment, the PA reached the floor in the medium delay, while in the donepezil treatments the PA is decreased continuously. Averages across N=6 subjects, error bars show s.e.m. For details on treatments see **Figure 1B**. Asterisks mark significant ($p<0.05$, post hoc) differences compared to the VEH+SCOP control condition within each level of Delay.

3.3. Effect of treatments on reaction time

The effect of scopolamine on the average reaction time of the animals was analyzed (Figure 6). ANOVA did not show significant difference between scopolamine and vehicle treatments (main effect of treatment: $F_{1,5}=3.681$, $p=0.113$, $\eta_p^2=0.42$). According to the results, Delay has a significant main effect on RT ($F_{2,10}=66.217$, $p<0.001$, $\eta_p^2=0.93$) of the animals. There was no significant interaction between Treatment and Delay ($F_{2,10}=0.22$, $p=0.804$, $\eta_p^2=0.04$). In the medium delay, RT was significantly slower compared to the short delay for both vehicle and scopolamine treatments (post hoc for VEH+VEH: SHORT vs. MEDIUM

p=0.003; pot hoc for VEH+SCOP: SHORT vs. MEDIUM p=0.004), but no significant difference was found between medium and long (pot hoc for VEH+VEH: MEDIUM vs. LONG p=0.265, post hoc for VEH+SCOP: MEDIUM vs. LONG p=0.654) delay periods.

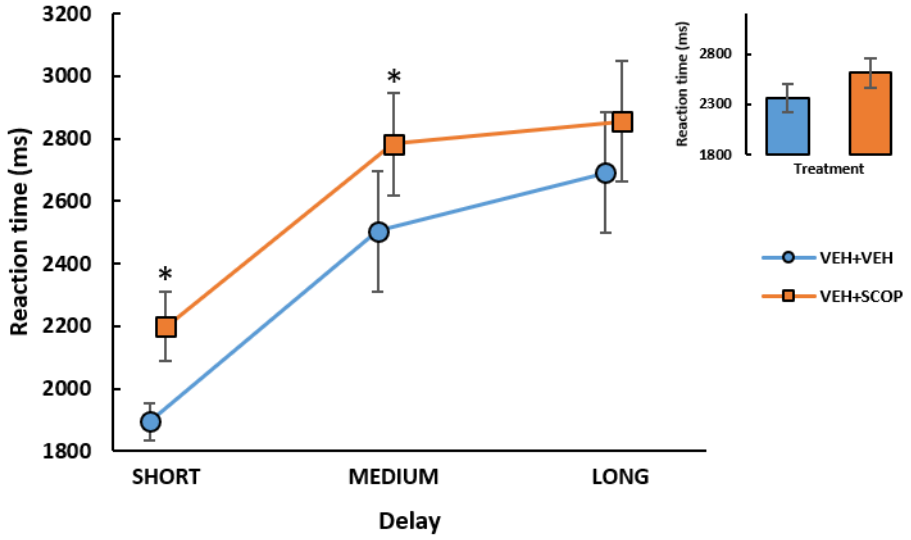


Figure 5. Effect of scopolamine (VEH+SCOP) treatment compared to vehicle (VEH+VEH) on average reaction time (RT) in the three delay categories (SHORT, MEDIUM, LONG). The inset illustrates that scopolamine treatment had no significant main effect on the pooled average RT compared to vehicle. There was no significant interaction between Treatment and Delay. The graph shows that the RT in medium delay was significantly slower compared to short delay, but no significant difference was found between medium and long delay for both vehicle and scopolamine treatment. Averages across N=6 subjects, error bars show s.e.m. For details on treatments see **Figure 1B**. Asterisks mark significant ($p<0.05$, post hoc) simple Treatment effects within each level of Delay.

The ANOVA of donepezil monotreatment compared to vehicle did not show a significant main effect of Treatment ($F_{1,5}=1.06$, $p=0.349$, $\eta_p^2=0.18$, see **Figure 6**). We found a significant main effect of Delay on RT ($F_{2,10}=21.953$, $p<0.001$, $\eta_p^2=0.81$), and significant

interaction between Treatment and Delay ($F_{2,10}$=7.152, p=0.012, η_p^2=0.59), which was probably due to a slightly stronger delay effect in the donepezil monotreatment condition (**Figure 6**). Post hoc tests showed that both delay effects for both the vehicle and donepezil treatments were significant (VEH+VEH: SHORT vs. MEDIUM post hoc p<0.001; MEDIUM vs. LONG post hoc p=0.007; DON200+VEH: SHORT vs. MEDIUM post hoc p<0.001; MEDIUM vs. LONG post hoc p<0.001). Donepezil monotreatment compared to vehicle resulted slower RT in short (VEH+VEH vs DON200+VEH post hoc p=0.009) and long (VEH+VEH vs DON200+VEH post hoc p=0.002) delay periods, however, it had no effect in the medium delay period (VEH+VEH vs DON200+VEH post hoc p=0.382).

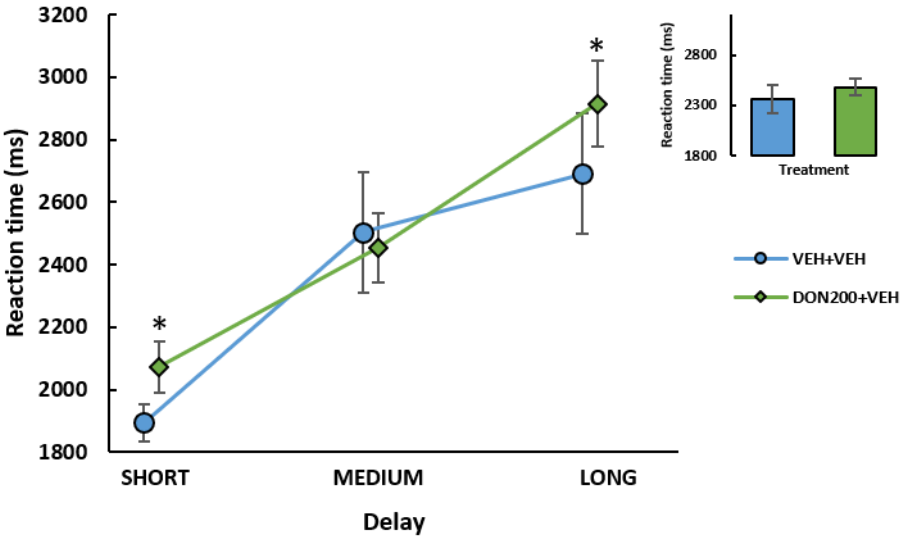


Figure 6. Effect of high-dose donepezil (DON200+VEH) monotreatment compared to double vehicle treatment (VEH+VEH) on average reaction time (RT) in the three delay categories (SHORT, MEDIUM, LONG). The inset illustrates that donepezil monotreatment had no significant main effect on average RT compared to vehicle. There was a significant interaction between treatment a delay. Donepezil monotreatment compared to vehicle resulted slower RT in short and long delay, however, it had no effect in the medium delay period. Averages across N=6 subjects, error bars show s.e.m. For details on treatments see **Figure 1B**.

Asterisks mark significant ($p<0.05$, post hoc) simple Treatment effects within each level of Delay.

We analyzed the effect of simultaneous administration of donepezil and scopolamine (DON100+SCOP; DON200+SCOP) compared to scopolamine monotreatment (VEH+SCOP, see Figure 8). There was no significant main effect of Treatment on RT ($F_{2,10}=1.643$, $p=0.242$, $\eta_p^2=0.25$). In contrast, ANOVA showed significant main effect of Delay ($F_{2,10}=25.392$, $p<0.001$, $\eta_p^2=0.84$). In the case of scopolamine treatment, RT in the medium delay was significantly slower compared to the short delay (SHORT vs. MEDIUM post hoc $p<0.001$), but no significant difference was found between medium and long (MEDIUM vs. LONG post hoc $p=0.515$) delay periods. In contrast, in the case of low and high dose of donepezil treatments, increasing delay resulted in slower RT across all delay periods (post hoc for DON100+SCOP: SHORT vs. MEDIUM $p<0.001$, MEDIUM vs. LONG $p=0.062$; for DON200+SCOP: SHORT vs. MEDIUM $p=0.004$, MEDIUM vs. LONG $p=0.003$). While this pattern is similar to that observed for PAs, in the case of RTs this was not supported by an interaction between Treatment and Delay ($F_{4,20}=1.079$, $p=0.393$, $\eta_p^2=0.18$).

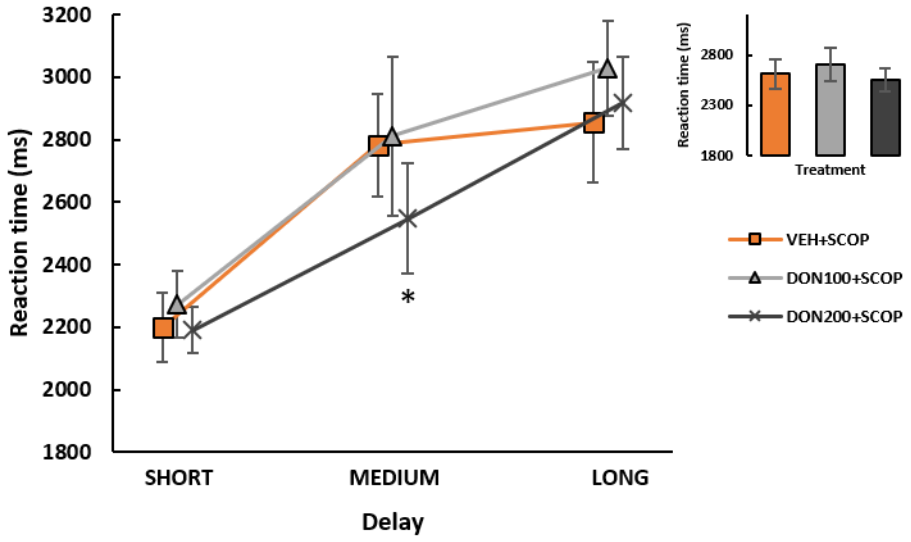


Figure 7. Effect of simultaneous administration of donepezil and scopolamine (DON100+VEH; DON200+VEH) treatments compared to scopolamine (VEH+SCOP) on average reaction time (RT) in three delay categories (SHORT, MEDIUM, LONG). The inset illustrates that donepezil treatments had no significant effect on average RT compared to scopolamine. In the case of scopolamine treatment, RT reached the plateau value in the medium delay, while in the donepezil treatments the RT increased continuously. This pattern was not supported by an interaction between treatment and delay. Averages across N=6 subjects, error bars show s.e.m. For details on treatments see **Figure 1B**. The asterisk marks the significant ($p<0.05$, post hoc) difference between RT in the VEH+SCOP and the DON200+SCOP treatment conditions in the medium delay condition.

4. Discussion

In the present study, we applied a DMTS paradigm to investigate the effects of cholinergic receptor modulation on macaque short-term memory in a delay-dependent manner. As also observed in the vehicle treatment condition in this study, continuous decrease in PA and increase in RT for vehicle treatment over the delay period indicated that VWM representations deteriorated with the passage of the delay period. We investigated how this simple behavioral index of decay in the speed and accuracy of memory performance is influenced by cholinergic receptor agents administered before the testing session: We tested the amnestic effects of the muscarinic acetylcholine receptor antagonist scopolamine and for the reversal of impairments the acetylcholinesterase inhibitor donepezil in combination with scopolamine.

We showed that scopolamine treatment decreased PA in the DMTS task, which – interestingly enough – reached an asymptotic low performance level earlier, in the medium delay condition, while in the vehicle (control) treatment PA decreased continuously through the entire course of the delay period. This is in accordance with the pioneering results of Bartus et al [29], who observed dose-dependent scopolamine-induced deficits that were more pronounced with longer delay in a modified delayed manual response task. In their task, the response accuracy was very high (80-90%) even for the highest delay (10 s) in the control condition, while with delay periods up to 76 s and the more challenging task used in our study, we could measure the course of delay-dependent short-term memory deterioration even in the control condition, and also how it changes as a result of pharmacological treatments. The wider delay range used in our study also revealed that under scopolamine treatment, performance reached the vehicle long-delay performance level already at the medium delay condition, however, it did not decrease further. This is suggestive of a rightward instead of a downward shift of the performance accuracy curve, implying that it is primarily the pace, not the depth of

delay-dependent short-term memory deterioration that changed as a result of scopolamine treatment. Importantly – and also in line with previous results [29,31] – short-delay responses were only weakly or not affected by scopolamine. Short-delay performance is generally thought to reflect iconic memory [35] [36] or more recently theorized intermediate memory states in this time range between iconic and *bona fide* working memory [37–41]. We suggest that these early VWM processes may be less reliant on muscarinic mechanisms, in contrast to processes during or transitions into later states.

Combined donepezil treatment was found to partially reverse the impairment caused by the administration of scopolamine with the same delay-dependent pattern, i.e. affecting performance accuracy only in the medium delay, partially reinstating the continuously decreasing performance pattern observed in the vehicle condition. There is abundant evidence that pro-cholinergic substances, including nicotinic agonists [42,43] and cholinesterase inhibitors [15,24,44] can mitigate scopolamine-induced deficits in short-term memory performance. Several studies by Buccafusco et al. [22,23,25,26,28] even used variable delay periods, however, in these studies the delay was mainly used to titrate task difficulty for each animal to optimize sensitivity of the design to detect pharmacological effects, and not to explicitly characterize the delay-dependence of such effects. The resulting variability in delay length between animals in these studies exceeds the plausible range of between-subject variation in the course of cognitive events during working memory maintenance, and is rather likely to reflect generic, delay-independent factors behind each animal's performance. Unexpectedly, donepezil monotreatment in our study affected only short and long delay performance, mainly in terms of response slowing. Some previous studies have shown cognitive enhancing effects of donepezil monotreatment in memory tasks in young and aged macaques [22,23]. Our study does not confirm such enhancing effect: Here we provide evidence for

donepezil to reverse scopolamine-induced impairment of performance accuracy, and not for an additive enhancing effect.

Reaction times also depended on the length of the delay period but showed less sensitivity to pharmacological manipulations both as main effects and when the delay was taken into account. Reaction times are thought to mainly reflect the time required for visual search in the probe array, which is known to be faster for shorter delay since shorter-lived memory traces provide better search templates and attentional guidance than memories stored for longer periods of time [45]. Since we used a touchscreen instrument without any restraint applied to the animals, our experimental set-up was not optimized for precise RT measurements, possibly not providing enough statistical power for detecting the delay-dependent effects of cholinergic agents on RT.

The most prominent model relates working memory maintenance to sustained neural activity, which is confirmed by single unit [46,47] and non-invasive [48] measurements as well. Modeling [33] and experimental data [14,49,50] suggest that muscarinic receptor activity is essential for persistent delay activity during working memory, and it is known that scopolamine applied locally in the dorsolateral prefrontal cortex diminishes delay-period firing rate and memory-related activity patterns [51] and also delay-period activity as measured by fMRI [14] in humans. Based on this, we hypothesize that the cholinergic modulation of the temporal persistence of working memory by scopolamine and donepezil observed in this study is probably due to disruption and partial recovery, respectively, of the muscarinic mechanisms supporting the maintenance of persistent stimulus-coding delay activity in the frontoparietal memory networks.

Recently, behavioral and neurophysiological data has convergently shown that the substrate of working memory dynamically changes with the passing of time, shifting from primary sensory areas to networks of frontal and parietal associative cortical and subcortical

areas [52–54]. This process is paralleled by traversing from detailed sensory images to more abstract, e.g. categorical representations [45]. Miller and Desimone [55] have shown that despite marked behavioral effects of scopolamine, neurons in the inferior temporal cortex, at the highest level of the visual representational hierarchy, remain unaffected by the drug. This is in accordance with our findings on the lack of cholinergic modulations early in the delay, when sensory cortical representations are thought to play a more prominent role in VWM maintenance. Relatedly, the dependence of mid-delay memory maintenance on muscarinergic mechanisms is supported by the findings of Aggelopoulos and colleagues [56], who have shown that scopolamine specifically hinders the formation of categorical, more abstract stimulus representations that are more prominent later in the delay period and also pave the way towards long-term memory encoding [14].

In conclusion, in macaques performing a DMTS VWM task, we have extended the classical results on delay-dependent cholinergic effects by using delay lengths up to 76 seconds. We showed that the delay-dependent scopolamine-induced impairments on VWM maintenance specifically affect the 10-30 seconds time range, suggesting that scopolamine speeds up the normal deterioration of short-term memory with the passage of time. In addition, we also tested how donepezil, a clinically widely used cholinesterase inhibitor with high translational relevance, could partially rescue medium-delay scopolamine impairments. The results are in line with our current knowledge on the role of muscarinic acetylcholine receptors in the maintenance of working memory. Taking the length of the delay period into account can be a valuable component of basic and preclinical pharmacological research on the behavioral manifestations of delay-dependent cholinergic mechanisms and could deepen our understanding of short-term memory and its age-related impairments.

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Conflict of Interest Statement

B.L. and G.L. are employees of Gedeon Richter Plc. This does not alter our adherence to journal policies on sharing data and materials. The remaining authors (V.O., B.K., A.T., I.H.) declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

V.O., A.T., B.L., G.L. and I.H. designed the research. V.O. conducted the experiments, V.O., A.T. and B.K. performed data analysis. V.O., B.K., B.L. and I.H. wrote and reviewed the manuscript.

Data Accessibility Statement

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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