

Central muscarinic cholinergic involvement in serial pattern learning: Atropine impairs acquisition and retention in a serial multiple choice (SMC) task in rats



Amber M. Chenoweth^{a,*}, Stephen B. Fountain^b

^a Department of Psychology, Hiram College, Hiram, OH 44234, United States

^b Department of Psychological Sciences, Kent State University, Kent, OH 44242, United States

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ABSTRACT

Atropine sulfate is a muscarinic cholinergic antagonist which impairs acquisition and retention performance on a variety of cognitive tasks. The present study examined the effects of atropine on acquisition and retention of a highly-structured serial pattern in a serial multiple choice (SMC) task. Rats were given daily intraperitoneal injections of either saline or atropine sulfate (50 mg/kg) and trained in an octagonal operant chamber equipped with a lever on each wall. They learned to press the levers in a particular order (the serial pattern) for brain-stimulation reward in a discrete-trial procedure with correction. The two groups learned a pattern composed of eight 3-element chunks ending with a violation element: 123–234–345–456–567–678–781–818 where the digits represent the clock-wise positions of levers in the chamber, dashes indicate 3-s pauses, and other intertrial intervals were 1 s. Central muscarinic cholinergic blockade by atropine caused profound impairments during acquisition, specifically in the encoding of chunk-boundary elements (the first element of chunks) and the violation element of the pattern, but had a significant but negligible effect on the encoding of within-chunk elements relative to saline-injected rats. These effects persisted when atropine was removed, and similar impairments were also observed in retention performance. The results indicate that intact central muscarinic cholinergic systems are necessary for learning and producing appropriate responses at places in sequences where pattern structure changes. The results also provide further evidence that multiple cognitive systems are recruited to learn and perform within-chunk, chunk-boundary, and violation elements of a serial pattern.

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1. Introduction

Evidence has accumulated indicating that, even in rats, acquiring and performing complex sequential behavior in the form of serial patterns depend on concurrently using multiple cognitive processes mediated by multiple dissociable brain systems (Fountain, Rowan, & Wollan, 2013; Fountain et al., 2012; Muller & Fountain, 2010). However, despite this claim, the neural substrates of these processes have not been fully characterized. The already-identified cognitive processes include discrimination learning involving simple stimulus–response (S–R) learning, timing/counting/serial position processes, multi-item sequential learning/memory, and lower-order and hierarchical rule learning (for reviews, see Fountain, 2008; Fountain et al., 2012). Earlier work provided evidence that rule learning could be dissociated

from discrimination learning in serial pattern learning tasks by exposing rats to trimethyltin, a neurotoxic organotin, or MK-801, an N-methyl-D-aspartate (NMDA) receptor antagonist (Fountain & Rowan, 2000; Fountain, Schenk, & Annau, 1985). More recent work has shown that chronic adolescent exposure to nicotine in rats produces sex-specific differential impairments of adult S–R learning and multi-item learning/memory with no effects on rule learning in either sex (Pickens, Rowan, Bevins, & Fountain, 2013; Renaud, Pickens, & Fountain, 2015), though the critical systems involved in these impairments have not been identified.

Recent work also shows that intact central cholinergic systems are likely critical for some, but not all, of the identified cognitive processes. For example, Fountain et al. (2013) examined cholinergic involvement in the performance of well-learned serial patterns. They trained rats on serial patterns to a high criterion, then treated rats daily before testing with atropine sulfate, a muscarinic cholinergic antagonist. Atropine treatment caused impaired performance for well-learned pattern elements typically encoded by S–R

* Corresponding author.

E-mail address: chenowetham@hiram.edu (A.M. Chenoweth).

learning and multi-item learning/memory in patterns. Performance for pattern elements typically encoded by rule learning was not affected by atropine in unambiguous patterns. The current article reports work that used daily treatment with atropine before training to examine the role of muscarinic cholinergic function in the acquisition of complex serial patterns in the serial multiple choice (SMC) task.

In the SMC task, rats were trained on variations of a sequential pattern consisting of eight 3-element chunks:

123-234-345-456-567-678-781-818-

where digits represent the clockwise position of eight levers in a circular array, dashes indicate 3-s temporal pauses, or “phrasing cues” (Kundey & Fountain, 2010; Muller & Fountain, 2010; Stempowski, Carman, & Fountain, 1999), and all other intertrial intervals were 1 s. The first element of each chunk (e.g., 123-234...) is termed a “chunk-boundary element,” with the following elements in each chunk designated as “within-chunk elements” (e.g., 123-234...). The last element of the pattern (i.e., -818) is called a “violation element” because the left turn required violates the within-chunk rule followed in the previous seven chunks, namely, “turn right within chunks.” Previous research utilizing the SMC paradigm has found evidence that multiple cognitive systems are employed concurrently in acquiring and maintaining sequential responses (Fountain, 2008; Fountain & Benson, 2006; Fountain & Rowan, 1995a, 1995b; Fountain, Wallace, & Rowan, 2002; Fountain et al., 2012), a hypothesis that has been further supported by dissociations observed in rats treated with MK-801, an NMDA receptor antagonist (Fountain & Rowan, 2000), and adolescent rats treated with nicotine, a nicotinic cholinergic agonist (Pickens et al., 2013; Renaud et al., 2015). Specifically, daily acute MK-801 exposure during acquisition of the foregoing serial pattern blocked violation-element learning but caused a less severe impairment for chunk-boundary-element learning in adult male rats (Fountain & Rowan, 2000). A later study showed that nicotine exposure during adolescence caused impairment of chunk-boundary-element learning without affecting violation-element learning in adult male rats (Pickens et al., 2013).

Fountain et al. (2013) explored the effects of atropine on retention performance on variations of the pattern described above. Rats were trained to a criteria performance (10 percent or fewer errors on any element type) without any pharmacological manipulation, and were then given intraperitoneal (i.p.) injections of 50 mg/kg atropine sulfate prior to test. Atropine-treated rats produced more errors at chunk-boundary and violation elements compared to criterion performance, and compared to within-chunk performance. These results corresponded to previous evidence that correct performance at chunk boundaries appears to depend primarily on learning stimulus–response (S–R) associations between phrasing cues as discriminative cues signaling when to make the chunk-boundary response, but evidence also indicates that rats concurrently encode the serial positions of phrasing cues (Muller & Fountain, 2010; Stempowski et al., 1999). Correct performance of violation elements also appears to rely on learning discriminative cues, such as location within the chamber, serial position, and serial position in relation to phrasing cues, with rats encoding multiple cues from preceding trials as well as apparatus cues, such as lever position, to signal where a violation occurs in the pattern (Kundey & Fountain, 2010; Muller & Fountain, 2010). Within-chunk element performance, unlike chunk-boundary and violation elements, appears to be governed by rule-learning and is unaffected by removal of phrasing cues or changes to the apparatus cues (Muller & Fountain, 2010), or to the presence of atropine (Fountain et al., 2013). Further, a comparison between injections of

50, 25, and 100 mg/kg of atropine sulfate to equivalent doses of atropine methyl nitrate (AMN), which does not cross the blood brain barrier but has peripheral effects equivalent to atropine sulfate, revealed that rats given AMN showed no impairments in performance. These results suggested that central muscarinic cholinergic systems were necessary for the retention and utilization of discriminative cues required to make correct responses at points in the patterns where structure changed – chunk-boundary and violation elements – but not for successful retention of rule-based within-chunk elements.

Fountain et al. (2013) demonstrated the role of central muscarinic cholinergic systems in rats’ ability to perform a well-learned serial pattern with a violation. However, the literature is mixed on the retention effects of muscarinic cholinergic antagonists. Some studies indicate that the observed impairment of retention performance reported by Fountain et al. (2013) was consistent with retention deficits in standard memory tasks (Buccafusco et al., 2008; Doguc et al., 2012; Higgins, Woodward, & Henningfield, 1989). However, others have reported that muscarinic cholinergic blockade impairs acquisition, not retention (Atri et al., 2004; Ghoneim & Mewaldt, 1975; Hasselmo, 2006; Hasselmo & McGaughy, 2004; Whishaw, 1989). This leaves an open question how a central muscarinic cholinergic antagonist would affect acquisition in the SMC task, with the literature indicating that an impairment in acquisition for rats treated with atropine should be expected. Further, due to the mixed literature on muscarinic cholinergic blockade effects on retention, replication of impaired retention performance in this task is prudent.

To examine these questions, three experiments were conducted. Experiment 1 compared acquisition of a serial pattern under 50 mg/kg atropine versus saline prior to each daily training session to assess whether rats treated with atropine would show impaired acquisition of the 24-element serial pattern containing chunk-boundary and within-chunk elements and a violation element described above from Fountain et al. (2013). Experiment 2 assessed persistent effects of atropine treatment in acquisition with an atropine-free test to determine whether rats treated with atropine in acquisition would show continued impairment, indicating that atropine did impair learning, not just rats’ ability to perform correct responses. Experiment 3 provided a partial replication of Fountain et al. (2013) by treating the saline control rats from Experiments 1 and 2 with atropine and assessing their performance on the well-learned serial pattern to determine whether or not atropine would impair rats’ performance for a well-learned serial pattern. The results of these experiments were expected to extend the work of Fountain et al. (2013) by determining which, if any, cognitive/behavioral learning and performance systems are vulnerable to a central muscarinic cholinergic challenge in acquisition of a serial pattern. Further, this extension would determine if the same systems implicated in retention in Fountain et al. (2013) were also required for acquisition of the serial pattern by examining if similar dissociations are observed between learning within-chunk elements, chunk-boundary elements, and violation elements.

2. Method

2.1. Subjects

Eleven naïve Long Evans hooded rats (*Rattus norvegicus*) at least 90 days old at the time of surgery served as the subjects for all three experiments. All subjects were implanted with bipolar electrodes (MS301, Plastic Products, Roanoke, VA) for unilateral hypothalamic brain stimulation reward (BSR; coordinates, skull level: 4.5 mm posterior, 1.5 mm lateral, 8.5 mm below the surface

of the skull). Prior to surgery, rats were given i.p. injections of 3.65 mg/kg xylazine and 0.05 mg/kg atropine, and then deeply anesthetized with isoflurane. Following surgery, rats were given antibiotics (60,000 units penicillin i.m.) to reduce the likelihood of infection. Rats were housed individually with food and water freely available in the home cage throughout the experiments. They were kept on a 15:9-h light–dark cycle, with testing occurring during the light portion of the cycle.

2.2. Apparatus

Two chambers (30 × 30 × 30 cm; Fountain & Rowan, 1995a, 1995b) equipped with a response lever on one wall and a commutating device mounted on the ceiling were used for shaping lever-press responses for BSR. Both chambers were made of clear Plexiglas with stainless steel rods for the floor. Each chamber was enclosed in a sound-attenuating cabinet made of particleboard (20 × 60 × 65 cm), located in a room separate from the test chambers.

Four octagonal test chambers (40 cm between parallel walls × 30 cm tall; Fountain & Rowan, 1995a, 1995b; Fountain et al., 2013) were constructed of clear Plexiglas with a response lever mounted on each wall, for a total of eight response levers numbered 1–8 consecutively, and a commutating device located centrally on the ceiling of the chamber, and a floor of stainless steel hardware cloth. Rats were connected to the stimulator via a flexible cord (Plastic Products MS304) attached to the commutator. Chambers were located in pairs separated by a melamine board in two separate rooms and controlled from an adjoining room with a microcomputer and interface (Med Associates, Inc., Fairfield, VT). All test chambers were monitored using closed circuit cameras mounted above the chambers.

2.3. Drugs

Rats given atropine (Experiments 1 and 3) received i.p. injections of 50 mg/kg atropine sulfate (Sigma), the dose common to both experiments reported in Fountain et al. (2013), dissolved in bacteriostatic water in a volume of 1.0 ml/kg. Rats given saline (Experiments 1 and 2) received i.p. injections of an equivalent volume to that received of atropine. All injections were given 30 min prior to daily training sessions.

2.4. Procedures

Rats began the shaping procedure after at least 1 week of recovery following surgery. Rats were shaped to lever-press for BSR in one of two shaping chambers. Rats received pulses of BSR as reinforcement for each correct response. Each pulse consisted of 250 ms of a 60-Hz sinusoidal pulse train from a constant current source. Parameters were adjusted as needed during shaping to maintain lever-press responding. Amperage was adjusted within the range of 20–80 μ A and the number of pulses was increased to as many as 3 pulses for each correct response. Once established,

these parameters were maintained throughout the remainder of the experiment. Rats were required to reach a criterion of at least 1000 responses within one 30-min session in order to remain in the experiment. Rats were given up to 3 sessions to meet this criterion.

The procedures utilized for each experiment are summarized in Table 1 and described in the following sub-sections.

2.4.1. Experiment 1: Atropine effects on acquisition of a serial pattern

After rats completed the shaping procedure, they were randomly assigned to one of two groups: atropine- or saline-treated, and began daily training sessions on a 24-element pattern with a violation (123-234-345-456-567-678-781-818), as described in Fountain et al. (2013). The first element of each chunk (e.g., 123-234...) is termed a “chunk-boundary element,” with the following elements in each chunk designated as “within-chunk elements” (e.g., 123-234...). The last element of the pattern (i.e., -818) is called a “violation element” because the left turn required violates the within-chunk rule followed in the previous seven chunks, namely, “turn right within chunks.” Over the first 4 days of training, the number of patterns rats experienced was gradually increased: over 4 consecutive daily sessions, rats received training on 5 patterns the first day, 10 patterns the second day, 15 patterns for the third day, and 20 patterns for the fourth day. This gradual increase in training over the first 4 days comprised Block 1, 50 total patterns. For the remainder of training and transfer, all rats received a 50-pattern block of training each day. A discrete trials procedure with correction was used, meaning that at the beginning of each trial all eight levers were present in the chamber and the rat was free to choose from any of the eight levers present. If the rat made a correct choice, all of the levers would retract and the rat would receive BSR. If the rat made an incorrect choice, all levers *except* the correct lever would retract. The rat would then select the correct lever, as it was the only option available, and then receive BSR. The process would then repeat for the next serial position in the pattern. The location of the first lever response produced, of the eight possible levers each rat could choose, was recorded for each trial. All rats received a total of twelve 50-pattern blocks of training in Experiment 1.

2.4.2. Experiment 2: Effects of discontinuing atropine

The procedure was the same as in Experiment 1 and occurred on Block 13 only, the day immediately following the last day of acquisition (Block 12). All rats used in both groups of Experiment 1 received i.p. injections of saline at a volume of 1.0 ml/kg.

2.4.3. Experiment 3: Atropine effects after drug-free training

Experiment 3 examined the effects of atropine on performance of a well-learned serial pattern. Rats in the saline control group continued training as per the procedure described in Experiment 1. Once all rats in the saline control group reached a criterion of less than 10% errors on any element type within a daily session ($M = 2.8$ daily blocks), on the next day of training they were treated

Table 1
Experimental design.

	Experiment 1: Blocks 1–12 Atropine effects on acquisition ^a	Experiment 2: Block 13 Effects of discontinuing atropine ^b	Experiment 3 ^c Atropine effects after drug-free training ^d
Group 1	Saline	Saline	Atropine
Group 2	Atropine	Saline (drug-free)	

^a Experiment 1: Comparing pattern acquisition under saline (Group 1) or under atropine (Group 2) across Blocks 1–12 of training.

^b Experiment 2: Comparing performance while drug-free on Block 13 for groups previously trained under saline (Group 1) or under atropine (Group 2).

^c Experiment 3 started the day after all rats in the saline control group reached a criterion of less than 10% errors on any element type within a daily block.

^d Experiment 3: Comparing effects of first-time exposure to atropine to asymptotic performance on the last daily block of saline training.

with atropine and tested on the same training procedure used in Experiments 1 and 2. To assure the drug was no longer present, rats were tested on saline the following day under the same training procedure as Experiments 1 and 2.

2.4.4. Statistical analysis

Analysis of variance (ANOVA) was used to examine the effects of atropine on rats' acquisition for each element type (within-chunk, chunk-boundary, and violation elements) across 50-pattern blocks of the experiment. Main effects and interactions were considered significant if $p < .05$. To assess differences in acquisition of pattern elements, a 2 (drug condition) \times 12 (50-pattern blocks) repeated measures ANOVA was conducted on rats' daily total correct responses for each element type. When significant effects were observed, planned comparisons based on the appropriate error term of the ANOVA (Fisher's Least Significant Difference tests) were conducted to determine the direction of the effect as well as specific 50-pattern blocks within acquisition when groups differed.

Analysis of variance (ANOVA) was also used to examine the effects of atropine on rats' performance for each element of the pattern collapsed across all 50-pattern blocks. Main effects and interactions were considered significant if $p < .05$. To assess differences in acquisition of pattern elements, a 2 (drug condition) \times 24 (pattern element) ANOVA was conducted on rats' mean number of errors for each element of the pattern. When significant effects were observed, planned comparisons based on the appropriate error term of the ANOVA (Fisher's Least Significant Difference tests) were conducted to determine the direction of the effect as well as specific elements of the pattern where groups differed.

The types of errors committed by rats trained under atropine or drug-free were analyzed by calculating the probability of error types at chunk-boundary elements and the violation element. Three possible error types were identified at chunk boundaries: perseveration errors (i.e., choosing the previously reinforced lever, as in 123-3 rather than 123-2), "back 2 levers" errors (i.e., choosing the lever a position one to the left of the correct lever, as in 123-1), or overextensions (i.e., choosing the lever that extends the chunk, as in 123-4). Errors that did not meet those definitions were considered "unassigned errors" (e.g., choosing a lever in the chamber that does not adhere to the defined categories, as in 123-8). An error type \times chunk (viz. a 3×8 design) repeated measures ANOVA was conducted comparing the three main types of errors across the eight chunk boundaries of the pattern. Main effects and interactions were considered significant if $p < .05$. Planned comparisons were conducted based on the appropriate error term from the appropriate ANOVA and were considered significant if $p < 0.05$.

3. Results

3.1. Experiment 1: Atropine impairs acquisition of a serial pattern

The results of Experiment 1 showed that atropine treatment impaired acquisition of a serial pattern composed of within-chunk elements, chunk-boundary elements, and a violation element. This conclusion was based on the results of a group \times block (viz., a 2×12 design, where drug group was the between-subjects factor; 2 = drug conditions of atropine or saline; 12 = 50-pattern blocks of acquisition) mixed design analysis of variance (ANOVA) conducted on rats' daily mean total number of errors (pooled across pattern elements). Main effects and interactions were considered significant if $p < 0.05$. Significant main effects were found for drug group, $F(1,9) = 59.50$, $p < 0.001$, and block, $F(11,99) = 69.20$, $p < 0.001$, and the drug group \times block interaction

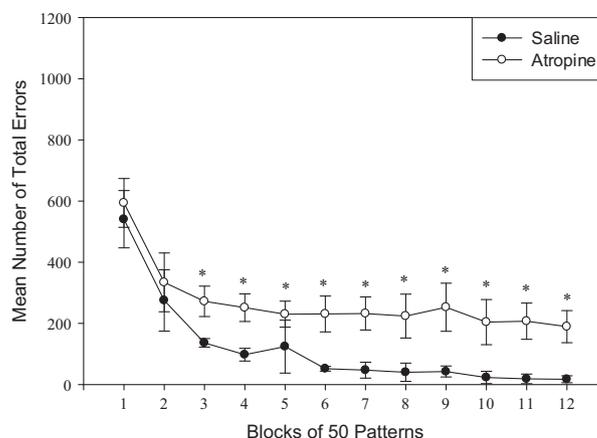


Fig. 1. Acquisition curves for the atropine-treated versus saline control groups over the twelve 50-pattern blocks of acquisition in Experiment 1. It should be noted that the data presented for Block 1 were collected over 4 consecutive daily sessions (5 patterns in session one, 10 patterns for session two, 15 patterns for session three, and 20 patterns for session four, equaling 50 total patterns), whereas Blocks 2–12 were collected as 50-pattern daily sessions. Block mean errors were averaged across all elements of the patterns. Error bars: \pm SEM; * indicates significant differences between groups, $p < 0.05$.

was found to be significant, $F(11,99) = 2.76$, $p = 0.004$. Planned comparisons (Fisher's Least Significant Difference tests) for comparing daily means between drug groups were conducted based on the appropriate error term from the ANOVA and were considered significant if $p < 0.05$. As shown in Fig. 1, planned comparisons showed that atropine-treated rats produced significantly more errors than saline controls on Blocks 3–12.

The results of Experiment 1 also showed that atropine injections severely impaired acquisition of chunk-boundary and violation elements, but only marginally disrupted acquisition of within-chunk elements of the pattern (see Fig. 2). This conclusion was based on the results of analyses conducted to determine differential effects of atropine by element type: within-chunk elements, chunk-boundary elements, and the violation element. A drug group \times block (viz., a 2×12 design, where drug group was the between-subjects factor) mixed design ANOVA was conducted for each element type. Main effects and interactions were considered significant if $p < 0.05$. As shown in the top panel of Fig. 2, the ANOVA for within-chunk elements indicated a main effect for drug group, $F(1,9) = 7.09$, $p = 0.026$, and block, $F(11,99) = 66.149$, $p < 0.001$, but the drug group \times block interaction was not significant ($p > 0.05$). When collapsing across groups for the entire acquisition phase, the saline group committed 4.16% ($M = 31.22$) errors while the atropine group committed 6.42% ($M = 48.18$) errors out of a total of 750 possible errors that could be made on within-chunk elements on a given block during acquisition. The ANOVA for chunk-boundary elements indicated main effects for group, $F(1,9) = 39.41$, $p < 0.001$, and block, $F(11,99) = 33.93$, $p < 0.001$, and a significant group \times block interaction, $F(11,99) = 2.28$, $p = 0.016$. Planned comparisons (Fisher's Least Significant Difference tests) for comparing daily means between drug groups for each element type were conducted based on the appropriate error term from the appropriate ANOVA and were considered significant if $p < 0.05$. Planned comparisons showed that atropine-treated rats produced significantly more errors compared to saline controls on Blocks 2–12 (see Fig. 2, middle panel). Further, the ANOVA for violation elements indicated main effects for group, $F(1,9) = 142.18$, $p < 0.001$, and block, $F(11,99) = 54.15$, $p < 0.001$, and a significant group \times block interaction, $F(11,99) = 28.68$, $p < 0.001$. Planned comparisons (Fisher's Least Significant Difference tests) for comparing daily means between drug groups

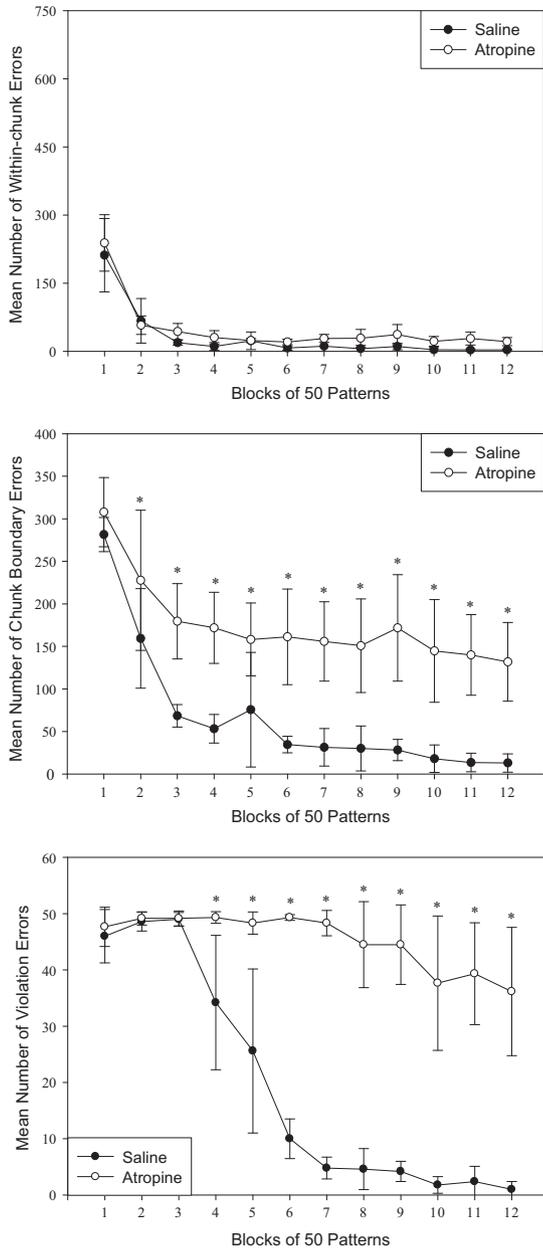


Fig. 2. Top panel: Acquisition of within-chunk elements over the 12 blocks of acquisition in Experiment 1 for the atropine-exposed and saline control groups. Block mean errors were averaged across all within-chunk elements of the patterns. Middle panel: Acquisition of chunk-boundary elements over the 12 blocks of acquisition in Experiment 1 for the atropine-exposed and saline control groups. Block mean errors were averaged across all chunk-boundary elements of the patterns. Bottom panel: Acquisition of the violation element over the 12 blocks of acquisition in Experiment 1 for the atropine-exposed and saline control groups. Block mean errors were averaged across all violation elements of the patterns. It should be noted that the data presented for Block 1 were collected over 4 consecutive daily sessions (5 patterns in session one, 10 patterns for session two, 15 patterns for session three, and 20 patterns for session four, equaling 50 total patterns), whereas Blocks 2–12 were collected as 50-pattern daily sessions. Error bars: \pm SEM; * indicates significant differences between groups, $p < 0.05$.

for each element type were conducted based on the appropriate error term from the appropriate ANOVA and were considered significant if $p < 0.05$. Planned comparisons showed that atropine-treated rats produced significantly more errors than saline controls on Blocks 4–12 (see Fig. 2, bottom panel).

This differential pattern of the overall impairment of acquisition by element type was even more pronounced when we examined

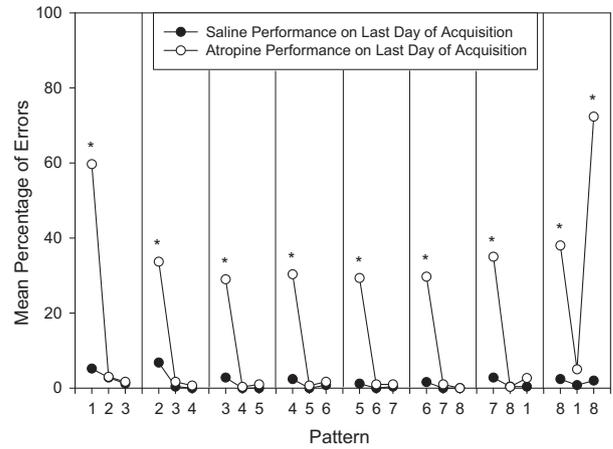


Fig. 3. Mean trial-by-trial errors for the atropine-treated versus saline control groups on the last day (Block 12) of acquisition in Experiment 1. Percentages of errors were averaged across the 50 patterns of the last day of acquisition. * indicates significant differences between groups, $p < 0.05$.

the last block of acquisition. A group \times chunk \times element (viz. a $2 \times 8 \times 3$ design, where drug group was the between-subjects factor) mixed design ANOVA was conducted on rats' mean percentage of errors (pooled across all 50 patterns for the last block of acquisition). Main effects and interactions were considered significant if $p < 0.05$. As shown in Fig. 3, the ANOVA indicated significant main effects for group, $F(1,9) = 51.04$, $p < 0.001$, chunk, $F(7,63) = 15.88$, $p < 0.001$, and element, $F(2,18) = 27.63$, $p < 0.001$, and significant interactions for group \times chunk, $F(7,63) = 13.54$, $p < 0.001$, group \times element, $F(2,18) = 19.89$, $p < 0.001$, chunk \times element, $F(14,126) = 9.18$, $p < 0.001$, and group \times chunk \times element, $F(14,126) = 8.16$, $p < 0.001$. Planned comparisons (Fisher's Least Significant Difference tests) for comparing means between drug groups for each chunk and element were conducted based on the appropriate error term from the appropriate ANOVA and were considered significant if $p < 0.05$. Planned comparisons showed that atropine-treated rats produced a significantly higher percentage of errors than saline controls on all chunk-boundary elements and the violation element.

The types of errors committed on the last day of acquisition (Block 12) by rats trained on atropine were also analyzed. Fig. 4 shows the mean proportion of error types at chunk-boundary elements and the violation element for atropine-treated rats on the last block of acquisition. Three possible error types were identified at chunk boundaries: perseveration errors (i.e., choosing the previously reinforced lever, as in 123-3 rather than 123-2), "back 2 levers" errors (i.e., choosing the lever a position one to the left of the correct lever, as in 123-1), or overextensions (i.e., choosing the lever that extends the chunk, as in 123-4). Errors that did not meet those definitions were considered "unassigned errors" (e.g., choosing a lever in the chamber that does not adhere to the defined categories, as in 123-8). An error type \times chunk (viz. a 3×8 design) repeated measures ANOVA was conducted comparing the three main types of errors across the eight chunk boundaries of the pattern and revealed a main effect for error type, $F(2,10) = 6.81$, $p = 0.014$. Planned comparisons were conducted based on the appropriate error term from the appropriate ANOVA and were considered significant if $p < 0.05$. They revealed that rats committed more perseveration than overextension errors, $t(5) = 4.25$, $p = 0.008$, and more back 2 levers than overextension errors, $t(5) = 3.28$, $p = 0.022$. There was no difference between the numbers of perseveration versus back 2 levers errors, indicating that rats made approximately the same number of each type of error across chunk-boundary elements.

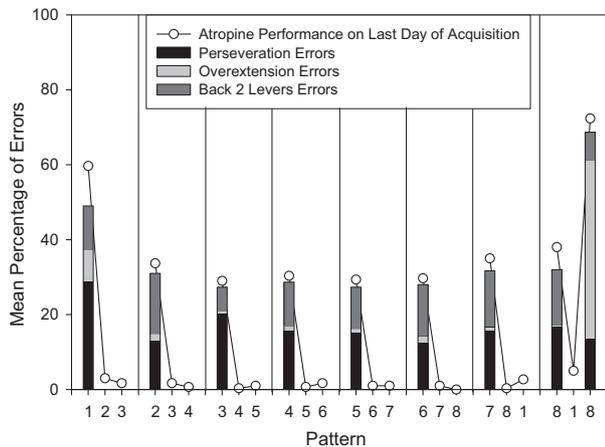


Fig. 4. Mean percentage of types of errors committed by atropine-treated rats on the last day of acquisition (Block 12) in Experiment 1. Percentages of error types were averaged across the 50 patterns on the last day of acquisition.

A similar analysis examined the types of errors made at the violation element and revealed a different pattern of most common error type committed. Rats made significantly more overextension (i.e., choosing the lever that would follow the “turn right until you reach a phrasing cue” rule set forth in the rest of the pattern, as in 812 rather than 818) than perseveration (i.e., 811) errors, $t(5) = 3.05$, $p = 0.028$, more overextension than back 2 levers (i.e., 817) errors, $t(5) = 4.02$, $p = 0.010$, and no difference between perseveration versus back 2 levers errors.

3.2. Experiment 2: Impairment from atropine persists when atropine is discontinued

The results of Experiment 2 showed that the impairment observed in rats exposed to atropine persisted when atropine was removed, providing further evidence that atropine disrupted acquisition of a serial pattern with a violation element. Fig. 5 shows the mean percentage of errors for both groups (atropine-treated and saline controls) on the last day of acquisition (Block 12) and the drug-free day (Block 13) pooled across all 50 patterns for each day. A group \times block \times chunk \times element (viz. a $2 \times 2 \times 8 \times 3$ design, where drug [training] group was the between-subjects

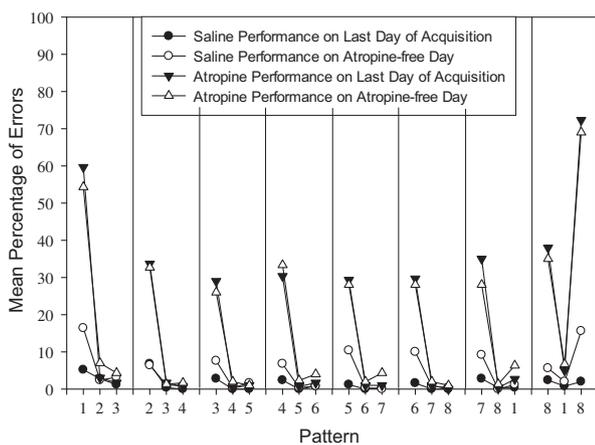


Fig. 5. Mean trial-by-trial errors for the atropine-treated versus saline control groups on the atropine-free day (Block 13) in Experiment 2 compared to the last day of acquisition (Block 12). Percentages of errors were averaged across the 50 patterns on atropine-free day. The comparison of interest is the atropine-treated group on the last day of acquisition versus the drug-free day, which yielded no significant difference ($p > 0.05$).

factor) mixed design ANOVA was conducted on rats' mean percentage of errors (pooled across all 50 patterns of the last block of acquisition and the block of the drug removal) and showed no significant improvement in the atropine group tested on saline on the drug-free day. Main effects and interactions were considered significant if $p < 0.05$. Significant main effects were found for group, $F(1,9) = 16.38$, $p = 0.003$, chunk, $F(7,63) = 19.23$, $p < 0.001$, and element, $F(2,18) = 26.14$, $p < 0.001$, and significant interactions were found for group \times chunk, $F(7,63) = 12.16$, $p < 0.001$, group \times element, $F(2,18) = 13.13$, $p < 0.001$, chunk \times element, $F(14,126) = 12.54$, $p < 0.001$, and group \times chunk \times element, $F(14,126) = 7.28$, $p < 0.001$. All other main effects and interactions were not significant. Planned comparisons (Fisher's Least Significant Difference tests) for comparing means between drug groups for each block as well as chunk and element were conducted based on the appropriate error term from the ANOVA and were considered significant if $p < 0.05$. Planned comparisons showed that atropine-treated rats produced significantly more errors than saline controls on both Blocks 12 and 13 on the chunk-boundary elements as well as the violation element; however, atropine-treated rats did not perform differently without atropine than they had on the last block of acquisition with atropine.

An error type \times chunk (viz. a 3×8 design) repeated measures ANOVA was conducted comparing the three main types of errors across the eight chunk boundaries of the pattern and revealed a main effect for error type, $F(2,10) = 5.86$, $p = 0.021$. Planned comparisons revealed that rats committed more perseveration than overextension errors, $t(5) = 4.35$, $p = 0.007$, and more back 2 levers than overextension errors, $t(5) = 2.70$, $p = 0.043$. There was no difference between the numbers of perseveration versus back 2 levers errors, indicating that rats made approximately the same number of each type of error across chunk-boundary elements on the drug-free day. A similar analysis examined the types of errors made at the violation element and revealed rats made significantly more overextension than back 2 levers errors, $t(5) = 3.39$, $p = 0.019$. Furthermore, Fig. 6 shows a comparison of the error types from the last day of acquisition and the drug-free day. Paired samples t -tests indicated that there were no significant differences ($p > 0.05$) between the types of errors made for each element type across the two days.

3.3. Experiment 3: Atropine impairs performance of a well-learned serial pattern with violation

The results of Experiment 3 showed that atropine impaired retention of a well-learned serial pattern with violation, as observed by treating the saline control rats with atropine. Fig. 7 shows the mean percentage of errors for the 50-pattern block of the atropine retention test compared to the daily 50-pattern blocks immediately prior to and after, pooled across all 50 patterns for each session. Results show atropine interfered with retention, especially at chunk boundaries and the violation element. A blocks \times chunk \times element (viz. a $3 \times 8 \times 3$ design, where all variables were within-subjects variables) repeated measures ANOVA was conducted on rats' mean percentage of errors (pooled across all 50 patterns for the block prior to test, the test block, and the block immediately following test). Main effects and interactions were considered significant if $p < 0.05$. Main effects were found for block, $F(2,8) = 12.27$, $p = 0.004$, chunk, $F(7,28) = 4.88$, $p = 0.001$, and element, $F(2,8) = 20.25$, $p = 0.001$, and significant interactions were found for block \times chunk, $F(14,56) = 2.62$, $p = 0.005$, block \times element, $F(4,16) = 18.98$, $p < 0.001$, chunk \times element, $F(14,56) = 5.52$, $p < 0.001$, and block \times chunk \times element, $F(28,112) = 4.35$, $p < 0.001$. Planned comparisons for comparing means between blocks as well as chunk and element were conducted based on the appropriate error term from the ANOVA and

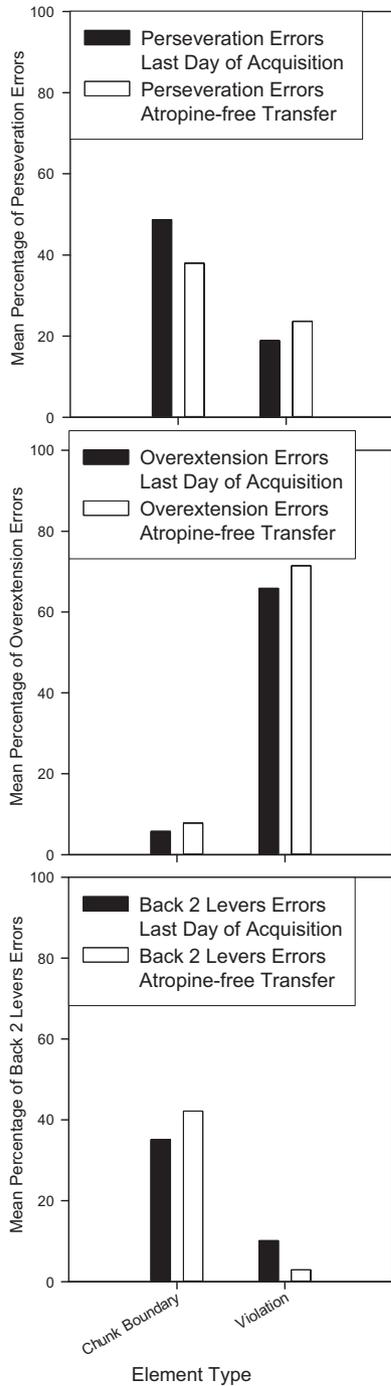


Fig. 6. Top panel: Mean percentage of perseveration errors at the chunk-boundary and violation elements for the atropine-treated rats on the atropine-free day (Block 13) in Experiment 2 compared to the last day of acquisition (Block 12). Percentages of perseveration errors were averaged across all chunk-boundary and violation elements of the 50 patterns on the atropine-free day and the last day of acquisition. Middle panel: Mean percentage of overextension errors at the chunk-boundary and violation elements for the atropine-treated rats on the atropine-free day (Block 13) in Experiment 2 compared to the last day of acquisition (Block 12). Percentages of overextension errors were averaged across all chunk-boundary and violation elements of the 50 patterns on the atropine-free day and the last day of acquisition. Bottom panel: Mean percentage of “back 2 levers” errors at the chunk-boundary and violation elements for the atropine-treated rats on the atropine-free day (Block 13) in Experiment 2 compared to the last day of acquisition (Block 12). Percentages of “back 2 levers” errors were averaged across all chunk-boundary and violation elements of the 50 patterns on the atropine-free day and the last day of acquisition.

were considered significant if $p < 0.05$. Planned comparisons indicated that performance was significantly impaired when rats were

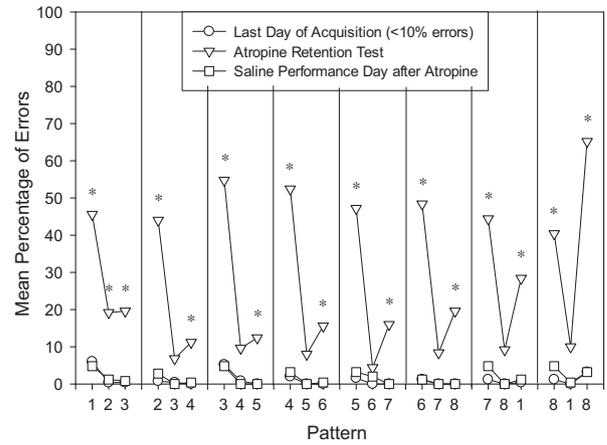


Fig. 7. Mean trial-by-trial errors for the saline control group on the atropine retention test in Experiment 3. Percentages of errors were averaged across the 50 patterns of the day immediate prior to the retention test (Block 12, the last day of acquisition), the retention test day (Block 13), and the day immediately following the retention test (Block 14). * indicates significant differences between groups, $p < 0.05$.

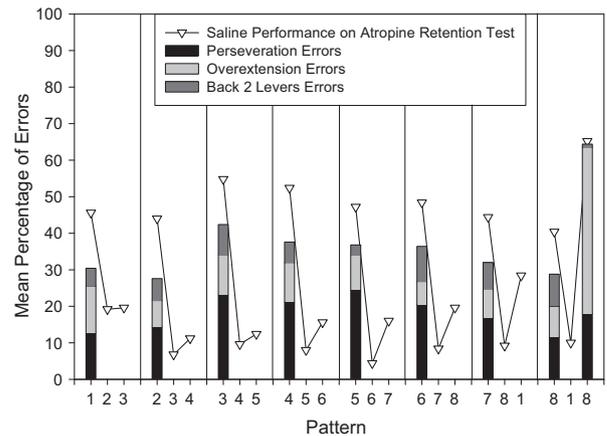


Fig. 8. Mean percentage of types of errors committed by saline control group on the atropine retention test in Experiment 3. Percentages of error types were averaged across the 50 patterns on the retention test day.

given atropine, especially at all chunk-boundary elements and the violation element. Atropine impaired performance on some of the within-chunk elements, primarily on element #3 of each chunk.

Fig. 8 shows the types of errors made at chunk-boundary elements and the violation element. Unlike in acquisition, rats in retention were not consistent in the types of errors made across chunk boundaries, with an element type \times chunk (viz. 3×8 design) repeated measures ANOVA confirming there were no significant differences. Analysis of the types of errors made at the violation element revealed that rats made significantly more overextension than back 2 levers errors, $t(4) = 3.57, p = 0.023$.

4. Discussion

The results of Experiments 1 and 2 showed clear evidence of a severe learning impairment in rats treated with atropine sulfate, with the effects persisting when the drug was removed. Moreover, Experiment 3 showed similar impairments in rats administered the drug after they had mastered the pattern. These data suggest that central muscarinic cholinergic systems played a critical role in the acquisition and retention of this sequential

pattern. Specifically, central cholinergic systems appeared to be necessary for encoding and accessing correct responses where structure changed in the pattern (i.e., chunk-boundary and violation elements).

In Experiment 1 we saw that rats in the atropine-treated group were impaired in learning the serial pattern, most notably at chunk-boundary and violation elements. The subtle effect of atropine on within-chunk elements, relative to the more severe impairment on acquisition of chunk-boundary and violation elements, supports that multiple processes were utilized when rats acquired this sequential task. The processes involved in encoding within-chunk responses were not sensitive to cholinergic impairment in the same way as chunk-boundary and violation elements. Thus, making discriminatory responses appeared to require intact central cholinergic systems, while the rule-learning required to successfully produce within-chunk element responses were not cholinergic-dependent, further supporting the multiple-item learning/memory claim (Fountain et al., 2013; Muller & Fountain, 2010). Evidence also indicated that rats made different types of errors at chunk-boundary and violation elements and therefore cannot be considered the same type of cognitive response, despite the same “turn left” motor response required to perform both responses correctly. In both responses, the rats demonstrated that a response that differed from the “turn right” within-chunk element response was required; however, the resulting error types indicated a different means for solving the problem depending on whether rats were anticipating a chunk-boundary or violation element response. At chunk boundaries rats primarily demonstrated a perseverative response (49% of errors; e.g., 123-3) and occasionally committed overextension errors (6%; e.g., 123-4). Violation element errors revealed that rats treated this chunk similar to the previous seven chunks of the pattern by committing a majority of overextension errors (66%; i.e., 812), and to a lesser extent perseveration errors (19%; i.e., 811). The violation element must have been encoded in such a way that rats could anticipate it, as there were no obvious cues (e.g., a phrasing cue) present immediately prior to the violation element to assist in making the correct response; rats must have encoded multiple items in order to make the correct response at the correct time, as demonstrated by Muller and Fountain (2010). The ability to correctly encode and produce the violation response was markedly sensitive to cholinergic antagonism, based on the results presented here showing significantly impaired acquisition of the violation element in atropine-treated rats compared to controls. Chunk-boundary elements, however, did have an additional cue (i.e., a phrasing cue) present immediately prior to their positions within the pattern, which allowed for a more direct S-R association to be made (Fountain et al., 2013; Muller & Fountain, 2010; Stempowski et al., 1999). These elements were also sensitive to cholinergic antagonism, but in a way that was different from the violation element, as evidenced by differences in acquisition rates (see Fig. 2, middle and bottom panels). Rats, while clearly impaired in their ability to make correct responses at chunk-boundary elements relative to saline-treated controls, were able to anticipate this element type. This did not appear to happen in the acquisition of the violation element, suggesting that atropine blocked the rats’ abilities to access the information needed to learn correct violation element responses.

Performance did not improve between the last day of atropine treatment in the acquisition phase of Experiment 1 and the atropine-free day in Experiment 2. This indicated that atropine did interfere with acquisition of the pattern and that these effects persisted even when atropine was not present in the system. Further evidence revealed a similar pattern of errors committed in both acquisition and in the drug-free day, confirming that atropine impaired learning in the acquisition phase. This pattern

of errors brings to mind the issue of an order schema addressed by Lashley (1951), in that “conflicting impulses may distort the order, although the primary determining tendency, the idea, remains the same” (p. 118). It is clear in both acquisition and the drug-free day that atropine-treated rats encoded the structural changes in the pattern differently than within-chunk elements. The observation that the majority of errors at these points remained in the same area of the chamber (e.g., perseverations and “back 2 levers”) suggests that the intention to produce the correct response was there, however, some “conflicting impulse” might have interfered with the production of the correct response at these structural changes. A future examination in which rats having experienced a central muscarinic blockade in initial training continued training in a drug-free state could examine if the rats are able to acquire the task at a normal rate, or if the initial deficit either impairs or enhances later learning. A study by Abdulla, Calaminci, Stephenson, and Slinden (1993) suggested that learning rates may actually be faster in rats having received chronic administration of a muscarinic antagonist relative to a saline control due to an up regulation of muscarinic cholinergic receptors. However, we are unable to speculate at this time if a similar enhancement of later training might occur with the SMC paradigm, particularly with the systemic injections employed in the present study.

Experiment 3 showed that atropine impaired performance in retention, partially replicating Fountain et al. (2013). Further, results indicated that performance was impaired in a similar manner as observed in the acquisition phase, with elevated errors at chunk-boundary and violation elements. Higgins et al. (1989) also found that atropine impaired retention performance of response sequences in humans, which supports the results observed here and by Fountain et al. (2013). Higgins et al. did note, however, that the impairment in acquisition was more severe than that observed in retention in that subjects produced more errors in acquisition. Additionally, a higher dose of atropine was required to produce errors in retention compared to the lower doses that disrupted acquisition (Higgins et al., 1989). The results of Higgins et al. differ from what was described here in that in Experiment 3 the same dose produced errors in retention of a well-learned pattern at or above the same level as that observed on the last day of the acquisition phase in rats exposed to atropine throughout acquisition. A more recent study by Doguc et al. (2012) found dose-dependent deficits in spatial working memory of rats receiving chronic administration of scopolamine after reaching asymptotic performance. Tissue analysis revealed that scopolamine appeared to have reduced expression of NMDA receptors. While it is unlikely that such changes occurred in our one-day retention test, this finding by Doguc et al. (2012) further strengthens the argument of the interaction of multiple neural systems, notably cholinergic and glutamatergic, in sequential learning and memory tasks.

The results of Experiment 3 added to the mixed results in the literature regarding the effects of anticholinergics on retention performance. Hasselmo (2006) and others have argued that administration of muscarinic cholinergic antagonists impair encoding of new information but not retrieval of previously stored information (Atri et al., 2004; Ghoneim & Mewaldt, 1975; Hasselmo & McGaughy, 2004; Whishaw, 1989). However, Fountain et al. (2013) demonstrated a clear disruption in retention performance, a result replicated in Experiment 3 of the present study, with the comparatively greater impairment of performance at chunk-boundary elements and the violation element than on the within-chunk elements. Why was this difference from the established literature observed? Doguc et al. (2012) argued that these differences may be due to multiple factors, such as drug and dosage, as well as motivation to complete the task requirements (e.g., escaping a water maze or navigating a radial arm maze for a food reward). It is unclear in the present study utilizing the

SMC task with systemic injections of atropine the exact factors implicated. Our dosage of atropine was comparable to those established in the literature (Whishaw, 1989), which suggests that perhaps motivation may be worth examining. However, as we did not directly measure motivational factors in this task, we are unable to offer further speculation.

A more probable explanation for the impaired acquisition and retention performance under atropine may be a disruption of attentional processes, as cholinergic antagonists have been shown to disrupt behavior in attentional tasks (Buccafusco et al., 2008; Bunge & Zelazo, 2006; Deiana, Platt, & Riedel, 2011; Dunnett, Everitt, & Robbins, 1991; Everitt & Robbins, 1997; Hodges, Allen, Sinden, Lantos, & Gray, 1991; Klinkenberg, Sambeth, & Blokland, 2011; Newman, Gupta, Climer, Monaghan, & Hasselmo, 2012; Okaichi & Jarrard, 1982; Spangler, Rigby, & Ingram, 1986). For example, a review by Everitt and Robbins (1997) describes the strong parallels in attentional deficits on a serial-reaction time task found in both patients with Alzheimer's disease and experimental animals with cholinergic basal forebrain lesions. In both populations, treatments with tacrine, a cholinesterase inhibitor, improved performance, while scopolamine treatment in rats impaired attention and accuracy on the task. In the current study, it appears as though the rats were aware that something happened at chunk-boundary elements that follow a phrasing cue, but a disruption in attention impaired rats' abilities to produce the correct response. Thus, based on evidence from the pattern of error types, it appears rats tended to choose the lever for which they last received reinforcement (i.e., a perseveration response). Further, impaired attention may also account for elevated errors at the violation element, as rats appear to have "lost track" of where they were in the pattern and overextended the within-chunk rule that was appropriate in the previous seven chunks of the pattern, producing an 812 response sequence rather than the correct 818 violation chunk sequence. It is interesting to note that this pattern of errors observed in the current study was similar to those observed by Fountain et al. (2013), who reported that rats trained on the same phrased violation pattern used in the present study and treated with atropine in a retention test committed a similar pattern of errors. Rats produced primarily perseveration errors (55%, compared to 38% observed in the present study) and overextension errors (27%, compared to 20% observed in the present study) at chunk boundaries and primarily overextension errors at the violation element (88%, compared to 70% observed in the present study). Taken together, these results indicate that the general pattern of results observed by both Fountain et al. (2013) and in the current study are robust.

While the pattern of impairment in rats treated with atropine in the present study appeared similar in both acquisition and retention, with elevated errors at chunk-boundary and violation elements, there were differences between the deficits shown in acquisition and those in retention. As shown in Fig. 4, rats given atropine in acquisition showed a somewhat predictable pattern not only where errors occurred in the pattern, but also what type of errors occurred. For example, atropine-treated rats showed a deficit at all chunk-boundary elements, but committed more errors on the first chunk-boundary element of the pattern relative to the remaining seven chunk boundaries. One possible explanation for this effect is that atropine-treated rats had difficulty learning the appropriate "8" violation response on the violation element that was the last element of the pattern, then on the next trial after the violation element—the first element of the next pattern—they simply produced a perseveration of the previously corrected "8" response.

In contrast, rats given atropine in retention showed a more generalized impairment in behavior. These rats were significantly

impaired in their performance at chunk-boundary and violation elements; however, errors were also elevated at within-chunk elements, namely the third element of chunks (see Fig. 8), a pattern not observed in atropine-treated rats in acquisition. Moreover, rats given atropine during the retention test did not show the same pattern of errors as those given atropine in acquisition. Rats treated with atropine in acquisition had more time to develop less-efficient associations to produce correct responses to reach an asymptotic performance of better than chance on all elements aside from the first element of the pattern and the violation element, but not to the point of mastering the pattern. Rats given atropine in retention had the ability to build more efficient associations in acquisition to master the pattern; however, under the influence of atropine, access to the more efficient associations to produce correct responses was blocked, causing rats to rely on less-efficient random lever choices to result in chance performance on chunk-boundary and violation elements.

A similar pattern of the impairments observed in the present study have also been observed in rats administered the *N*-methyl-D-aspartate (NMDA) receptor antagonist MK-801, providing evidence that the glutamatergic system is involved in sequential pattern acquisition (Fountain & Rowan, 2000). What is of interest is that both NMDA receptors and muscarinic cholinergic receptors are found in abundance in the hippocampus (Monaghan & Cotman, 1985; Woolf, 1991), a brain structure that has produced mixed results within the sequential literature when lesioned. Rats with hippocampal lesions have shown deficits in the acquisition of a sequential radial arm maze task (Kesner & Novak, 1982), as well as in retention of other sequential tasks (Agster, Fortin, & Eichenbaum, 2002; Fortin, Agster, & Eichenbaum, 2002; Olton, Shapiro, & Hulse, 1984). However, given the systemic injections used in the present study, further studies are needed to identify the specific brain structures that are implicated by NMDA and cholinergic blockades in the SMC task.

These experiments contribute to our understanding of the role of muscarinic cholinergic systems in the organization of serial pattern learning. Experiment 1 showed that muscarinic cholinergic blockade by atropine impaired learning, particularly for chunk-boundary and violation elements, not just performance. Thus the results of the present study add to those of previous studies of NMDA receptor blockade (Fountain & Rowan, 2000), central muscarinic blockade (Fountain et al., 2013), and adolescent nicotine exposure (Pickens et al., 2013; Renaud et al., 2015) supporting the view that multiple dissociable brain systems are engaged concurrently to acquire and retain serial patterns in the SMC task (cf. Fountain et al., 2012; Muller & Fountain, 2010). Experiment 2 showed that the atropine-induced learning impairment observed during acquisition persisted when atropine was removed. Finally, Experiment 3 showed that atropine also blocked retention performance of an already well-learned serial pattern, thus replicating the results of Fountain et al. (2013). Taken together, the results show that atropine did not simply inhibit or interfere with performance of the pattern. If that were so, rats treated with atropine in acquisition should have performed their pattern better when atropine was removed. Instead, the evidence indicates that atropine disrupted both sequence learning and sequence performance. Future studies with the SMC task should focus on further localizing and characterizing the cholinergic systems that subservise the multiple learning and memory systems involved in sequential learning, memory, and performance.

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Amber M. Chenoweth reported this work in another form as part of a thesis in partial fulfillment of the Master of Arts degree

in the Department of Psychology, Kent State University. The experiments reported in this paper complied with the current laws of the United States of America.

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