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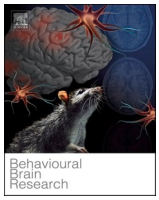

Research report

Muscarinic cholinergic receptor antagonism impairs spatial memory retrieval and minimizes retrieval-induced alterations in matrix metalloproteinase-9

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ABSTRACT

Cholinergic dysfunction in the hippocampus causes memory impairment, and degradation of the forebrain cholinergic system has been implicated in several neurological disorders. One such disorder, Alzheimer's Disease (AD) is associated with the abnormal expression of various proteins including matrix metalloproteinase-9 (MMP-9), an enzyme known to regulate hippocampus-dependent memory. Memory involves several stages including acquisition, consolidation, and retrieval, but the neurobiological correlates of retrieval have been studied much less than other stages of memory. We sought to investigate the potential relationship between cholinergic signaling and hippocampal MMP-9 expression and the involvement of each in spatial memory retrieval. We trained rats in the water maze until the task was well-learned, then, seven days later, we allowed some to retrieve the memory after an intracerebroventricular injection of scopolamine or vehicle. Western blot analysis of hippocampal tissue shows elevated levels of a truncated form of MMP-9 associated with spatial memory retrieval. Additionally, our results indicate that centrally administered scopolamine both impairs spatial memory retrieval and prevents retrieval-induced elevations in MMP-9. These findings provide evidence for a potential link between cholinergic dysregulation and abnormal MMP-9 levels seen in the brains of AD patients. An important, yet unresolved question is whether MMP-9 serves to support memory retrieval itself or if it is involved in maintaining the ongoing stability of a retrieved memory.

1. Introduction

Memory is a process by which organisms use past experiences to shape current and future behavior. As such, to be useful, memories must be initially acquired, subsequently consolidated, and ultimately retrieved with accuracy. In long-term declarative memory formation, each stage of acquisition, consolidation, and retrieval is underwritten by changes in hippocampo-cortical circuits in the brain [1,2].

Alzheimer's Disease (AD) is a neurodegenerative disease characterized by disruptions in the memory process. Among other pathophysiological changes, AD is associated with a loss of cholinergic function; as a result, the basal forebrain cholinergic system has been the main target of AD treatments (see [3] for review). In the rodent brain, the hippocampus receives cholinergic input from cells originating in the basal forebrain, specifically the medial septum and diagonal band of Broca. Numerous lines of research demonstrate that cholinergic inputs affect memory-related hippocampal function. Cholinergic antagonists reliably impair performance in rodent memory paradigms [4–6] while cholinergic agonists facilitate performance [7–9]. Furthermore, cholinergic antagonists alter electrophysiological regulation of the hippocampus (for review see [10]). Importantly, cholinergic modulation of hippocampus-dependent memory has been demonstrated at multiple stages of the memory process including acquisition [11] and consolidation [12]. However, the involvement of the cholinergic system in the retrieval phase of memory is less clear. Some studies show no effect of cholinergic manipulation on memory retrieval [13,14] while more recent evidence suggests otherwise [15,16].

In the brain, the extracellular matrix (ECM) exists as a mesh-like web of molecules in the intercellular space, providing structure and support to cells [17]. The ECM is malleable, and reconfiguration of the ECM is accomplished in part by the activity of matrix metalloproteinases (MMPs). MMPs are a family of over 20 individual endopeptidases that modify and regulate many ECM substrates [18]. MMP-9 stands out because of its importance to learning and memory as well as its

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involvement in various neurological disorders [19]. MMP-9 expression is subject to a significant amount of post-translational control (for review see [20]). Initial regulation comes from the fact that MMP-9 is transcribed as an inactive enzyme called pro-MMP-9 which requires cleavage of the pro-region to become activated. This activation can be accomplished by the tissue-plasminogen activator-plasmin system [21], cathepsin g [22], or by other MMPs, most notably MMP-3 [23]. In addition to its regulation by post-translational modification, extracellular MMP-9 can be sequestered to cell surface proteins, and/or its enzymatic activity can be inhibited by tissue-inhibitors of MMPs (TIMPs) [24].

MMP-9 has repeatedly been shown to be an essential regulator of hippocampus-dependent memory formation and hippocampal long-term potentiation (LTP) [25–30]. However, the specific involvement of MMP-9 in memory retrieval is unclear. To date, only one published study has investigated this question behaviorally. Moosavi and colleagues trained mice using the passive avoidance protocol and subsequently injected the cholinergic antagonist scopolamine 15 min before a retrieval test [31]. They showed that peripheral scopolamine injection affected associative memory retrieval and led to lower pro-MMP-9 levels compared to saline-injected controls [31]. Further investigation into the potential role of MMP-9 in memory retrieval is needed because important differences exist between associative fear conditioning and other forms of hippocampus-dependent memory such as those requiring spatial navigation. Because significant questions remain regarding the relationship between hippocampal MMP-9 expression and memory retrieval, the present study sought to characterize the relationship between spatial memory retrieval and MMP-9 expression. Furthermore, the present study investigated the effect of cholinergic antagonism on spatial memory retrieval and retrieval-induced alterations in hippocampal MMP-9 expression.

2. Materials and methods

2.1. Experimental animals

Twenty-eight male Sprague-Dawley rats were purchased from Envigo. The animals were quarantined for one week upon arrival and then were handled five minutes per day for three days before training to allow acclimatization to the researchers. At the beginning of the experiment, animals were 3–4 months old and weighed 290–380 g. Food and water were available ad libitum throughout the experiment. Animals were socially housed, two per cage, in AALAC-approved cages in a 22 °C room set at 30 % humidity with a 12-hr light/dark cycle beginning at 06:00. All animal testing was conducted during the light cycle between the hours of 10:00 and 15:00. All animal procedures were approved by the Institutional Animal Care and Use Committee of Concordia College and performed in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals.

2.2. Acquisition training

The water maze [32] is a circular pool 76 cm tall and 170 cm in diameter that is marked on the external surface with locations that represent the cardinal directions North (N), South (S), East (E), and West (W). The cardinal direction points thereby section the maze into four equal quadrants designated Northeast (NE), Northwest (NW), Southeast (SE), or Southwest (SW). Each day before use the maze was filled with 32 °C water to a depth of 34 cm. A clear, plastic escape pedestal (32.5 cm tall, 13.3 ×13.3 cm) could be submerged 1.5 cm below the water's surface. The room that housed the water maze was 3 square meters and its walls contained extra-maze cues consisting of large, geometric shapes high in visual contrast. All other visible extra-maze items (lamps, computer, researchers, etc.) were kept in a constant location throughout the experiment. A video camera was mounted on the ceiling above the maze and connected to a laboratory computer. The computer was equipped with AnyMaze software (Stoelting Co, Wood Dale) which allowed for automated data collection and analysis.

Acquisition training was carried out daily for six days with five trials per rat, per day. Each experimental animal was randomly assigned a target quadrant (NE, NW, SE, or SW), and the escape pedestal was placed 30 cm from the wall of the maze in the center of the target quadrant. The pedestal placement remained constant throughout acquisition training for each rat. An acquisition trial began by gently placing the rat into the pool facing the edge of the pool at the N, E, S, or W starting location. The starting location was pre-determined by semi-random assignment such that animals did not begin a trial from the same location twice in a row. A timer started immediately upon introduction to the water and rats were allowed 60 s to locate the pedestal. Once the pedestal was reached, the timer was stopped, and the rat was given a 30 s rest period. If the rat failed to locate the pedestal in 60 s, it was gently placed on the pedestal and then allowed a 30 s rest period. Following the rest period, a new trial was started, and this pattern continued until the rat completed five trials. Once five trials were completed, the animal was towel-dried and placed under a heat lamp for five minutes before being returned to its home cage.

2.3. Stereotaxic surgery

Following six days of acquisition training, all animals underwent intracerebroventricular (icv) cannulation surgery. Rats were anesthetized with equithesin without choral hydrate [33] (5 mg/kg; active ingredient sodium pentobarbital 35 mg/kg; Sigma #P-3761). Animals underwent stereotaxic (Stoelting, Wooddale, IL) surgery using previously described procedures [6]. Briefly, on the day after acquisition training was completed, animals were implanted with a guide cannula, fashioned from PE-60 tubing, above the right lateral ventricle using coordinates 1.0 mm posterior and 1.5 mm lateral from bregma. The cannula was secured to two stainless steel skull screws using dental cement. An injector was fashioned from stainless steel tubing and connected via 50 cm of PE-50 tubing to a 10 μl Hamilton syringe. When inserted into the surgically implanted guide cannula, the injector protruded 2.5 mm beyond the tip of the cannula such that it penetrated the lateral ventricle. Cannula placement was confirmed post-mortem via a 2 μl injection of fast-green dye (Sigma #F-7252) and histological examination.

2.4. Pharmacological manipulation

After a 5-day post-surgical recovery period, 7 days after the completion of acquisition training, rats were randomly assigned to one of three experimental conditions (see Fig. 1). A control group consisted of 8 rats that underwent acquisition training and cannulation surgery but did not receive retrieval testing. The 20 remaining rats were divided into an artificial cerebrospinal fluid (aCSF) group and a scopolamine group (N = 10 per group). Fifteen minutes prior to retrieval testing, animals in the scopolamine group received a 2 μl icv injection of scopolamine (5 μg/μl; Sigma #S-18755) dissolved in aCSF. The aCSF group received a 2 μl icv injection of aCSF. All injections were administered over 120 s

2.5. Retrieval testing and tissue harvest

Retrieval testing commenced 15 min after injection with either aCSF or scopolamine.

On the day of the retrieval test, the water maze was set up as usual except that the submersible escape pedestal was not placed in the maze. Animals in the aCSF and scopolamine groups were subjected to a single probe trial. The probe trial consisted of placing the animal into the maze at a randomized starting location and allowing it to swim for 120 s. Time in the target quadrant, average length of target quadrant visit, longest target quadrant visit, and swim speed were recorded. After the retrieval

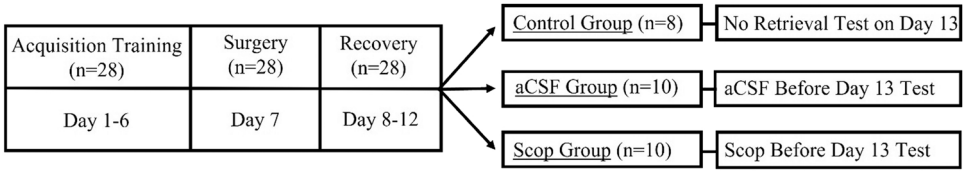

Fig. 1. Experimental design. Animals were assigned to control, aCSF, or Scopolamine (Scop) experimental conditions and progressed over 13 days through four phases of the project: Acquisition Training, Surgery, Recovery, and Retrieval Testing.

test, animals were towel dried, placed under a heat lamp for five minutes, and returned to their home cage. Two hours after the retrieval test, animals were sacrificed and the hippocampi were dissected, flash frozen in liquid nitrogen, and stored at −70 °C. Tissue from four animals per group were analyzed in the present study, and the remaining tissue was reserved for additional studies not reported in the present paper.

2.6. Western blotting

Hippocampal samples were removed from the −70 °C freezer, weighed, and homogenized at a 1:5 wet tissue/buffer ratio in homogenization buffer (1 M Tris HCl (ph 7.6), 1 M NaCl, 1 M $CaCl_2$, 1 % Brij-35). Homogenates were centrifuged at 12,000 rpm for 10 min at 4 °C. The supernatant fluid was removed and diluted with homogenization buffer at a 1:1 ratio. Protein levels from each sample were quantified using a commercial BCA protein assay kit (Thermo #23227). Protein concentrations were normalized to 10 μg/μl by adding additional homogenization buffer to each sample. Immediately before electrophoresis, samples were mixed with 4x Laemmli buffer (Bio-Rad #1610747) and heated at 100 °C for five minutes. SDS-PAGE was carried out using 10 % Tris HCL gels (Bio-Rad #4561033), and samples were then transferred to a nitrocellulose membrane (Bio-Rad #1620145). Following the transfer, the gel was washed in Tris-buffered saline (TBS) then blocked in a 5 % milk/TBS solution for one hour. After blocking, MMP-9 primary antibody (AbCam, #38898) was added to the 5 % TBS/milk solution at a 1:1000 concentration. The membrane was incubated overnight at 4 °C.

The following day the membranes were removed from the antibody-milk solution and washed alternately in TBS and 0.1 % Tween20-TBS (TTBS) for five washes of three minutes each. Membranes were then placed into a 5 % milk/TTBS solution, and an anti-rabbit horseradish peroxidase-conjugated secondary antibody was added (1:5000, Cell Signaling Technology #7074 S). Membranes were incubated in secondary antibody for one hour at room temperature and again exposed to a TBS-TTBS wash cycle. Finally, the membranes were placed in a chemiluminescent solution (Thermo #34500) for five minutes and exposed to X-OMAT film (Carestream Health Inc. #1611342). Following film exposure for MMP-9 levels, membranes were washed for five minutes in TBS and placed in stripping buffer (15 g Glycine, 1 g SDS, 10 ml Tween20, 1 L H_20, pH 2.2) for 10 min. Membranes were then subjected to five alternating TBS/TTBS washes of three minutes each and blocked with 5 % milk/TBS. Following this, membranes were stripped and then re-probed for β-Actin (1:5000 Sigma #A2228) using an anti-mouse secondary antibody (Cell Signaling Technology #7076 S) and the same methodology described above for MMP-9. Developed films for MMP-9 and β-Actin were digitally scanned and bands were quantified using CLIQS 1D software (TotaLab #1.2.040). To facilitate intergroup comparison, band densities were first normalized to β-Actin and then expressed as a percentage of the control group average.

2.7. Gelatin zymography

To assess MMP-9 enzymatic activity, gelatin zymography was performed on our samples. All zymography gels and reagents were purchased from Thermo and standard manufacturer protocols were used. Briefly, sample supernatants were prepared as described above for Western blotting, mixed 1:1 with 2X Tris-Glycine SDS sample buffer and electrophoresed through Novex Zymogram Plus Gels (Thermo #ZY00100). After electrophoresis, gels were incubated in a zymogram renaturing buffer (Thermo #LC2670) and then incubated overnight at 37 °C in a developing buffer (Thermo #LC2671). The next day, gels were rinsed three times with deionized water, stained two hours with a Coomassie blue solution and then washed with water. Whole-gel images were filtered and brightened with Apple Photos software to maximize band contrast. They were then quantified in the manner described in Section 2.6.

2.8. Statistical analyses

All results were analyzed for statistical significance using IBM SPSS Statistics software version 24.0. Acquisition data was analyzed using a 3 (group) X 6 (day) repeated-measures ANOVA and a one-way ANOVA on day 6 specifically. Retrieval test data was analyzed with independent-samples t-tests, and immunoblotting and enzyme assay data was analyzed with one-way ANOVAs. Tukey's post-hoc tests were conducted when warranted. Statistical normality tests were conducted, and any assumption violations were statistically corrected as appropriate.

3. Results

3.1. Acquisition training

A significant main effect for training day, $F(5, 125) = 69.36$, $p < 0.001$ indicated that animals' performance improved from days 1–6. The lack of a main effect for group, $F(2, 25) = 0.56$, $p = 0.59$ and the lack of an interaction effect $F(10, 125) = 0.84$, $p = 0.58$ demonstrated that animals from all groups learned the task at the same rate (see Fig. 2). In order to ensure there were no group differences at the end of the acquisition phase, a one-way ANOVA was performed for the final day of acquisition, and, importantly, there were no significant differences between groups, $F(2, 25) = 0.22$, $p = 0.81$.

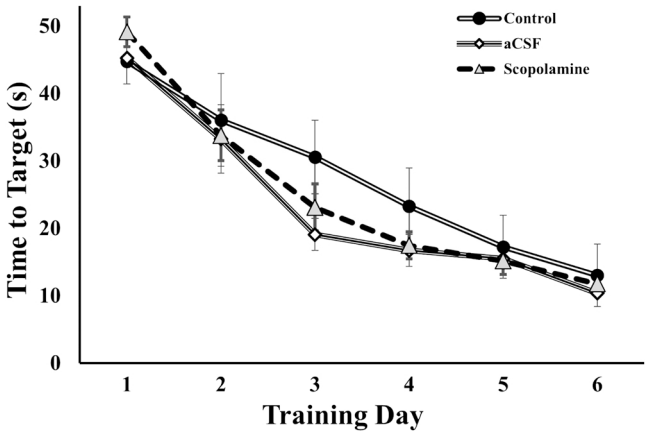

Fig. 2. Water maze acquisition training. Task acquisition of animals assigned to control, aCSF, and Scopolamine experimental conditions. Data are presented as mean +/- SEM.

3.2. Retrieval tests

Retrieval tests for animals in the aCSF and scopolamine groups were conducted on day 13 of the study (see Fig. 1). The data demonstrate that, compared to aCSF-injected animals, animals treated with scopolamine spent significantly less time in the target quadrant, $t(18) = 2.525$, $p = 0.021$, persisted less in the target quadrant on their longest visit $t(18) = 2.704$, $p = 0.015$, and on average spent less time per entry to the target quadrant $t(18) = 2.134$, $p = 0.047$ (see Fig. 3). However, there was no difference between the groups on swim speed $t(18) = -0.206$, $p = 0.839$, indicating the spatial memory retrieval deficits were not due to any potential motoric effects of scopolamine. The findings described above replicate nearly identical, unpublished findings from our laboratory using a separate cohort of aCSF and scopolamine animals.

3.3. Western blots

We detected six different MMP-9 immunoreactive bands in our samples at various molecular weights (see Fig. 4A). It is likely the top two bands of >150 and approximately 120 kD respectively represent MMP-9 multimers [20] or protein complexes that include MMP-9 and either TIMPs or cell adhesion molecules [34,35]. Our analysis shows another band at approximately 97 kD, a much fainter band at 67 kD (clearly visible after longer film exposure times), and two lower molecular weight bands at 47 and 41 kD. We believe the 97 kD band and the 67 kD band we observe are analogous to the 92 kD and 67 kD bands previously reported in the literature [23,30]. As Vandooren and colleagues discussed in their comprehensive review [20], it is important to remember that MMP-9 is a glycosylated protein, and, as such, MMP-9 exists as a heterogeneous mixture of glycoforms (see also [36]). In SDS-PAGE assays, this means that MMP-9 glycoforms migrate through the gel differently and present as a somewhat variable or hazy protein band [20]. Our contention that the 97 kD band we observe is pro-MMP-9 and analogous to the ~92 kD pro-MMP-9 band described elsewhere in the literature [30] is further supported by our zymography results (see Fig. 5). The two lower bands likely represent truncated forms of MMP-9 that have been characterized previously [23,37] and are believed to be degradation products of previously active MMP-9.

Comparing the levels of each of the immunoreactive MMP-9 bands with one-way ANOVAs, significant differences between groups were found only for the 47 kD truncated MMP-9 $F(2,9) = 9.06$, $p = 0.007$. Tukey's post-hoc tests revealed significantly higher levels in the aCSF group than both controls ($p = 0.008$) and scopolamine-injected rats ($p = 0.024$). There was no significant difference between control and scopolamine rats ($p = 0.744$; see Fig. 4C).

3.4. Gelatin zymography

Gelatin Zymography was conducted on our samples to determine whether there were group differences in MMP-9 enzymatic activity. Two gelatinase bands were observed: one at 97 kD and one at 67 kD. No significant differences were observed between the groups in either the 97 kD band ($F(2,9) = 0.628$, $p = 0.55667$) or the 67 kD band ($F(2,9) = 1.40$, $p = 0.295$; see Fig. 5). It is noteworthy that no gelatinase activity was detected in the 47 kD or 41 kD region of the gel.

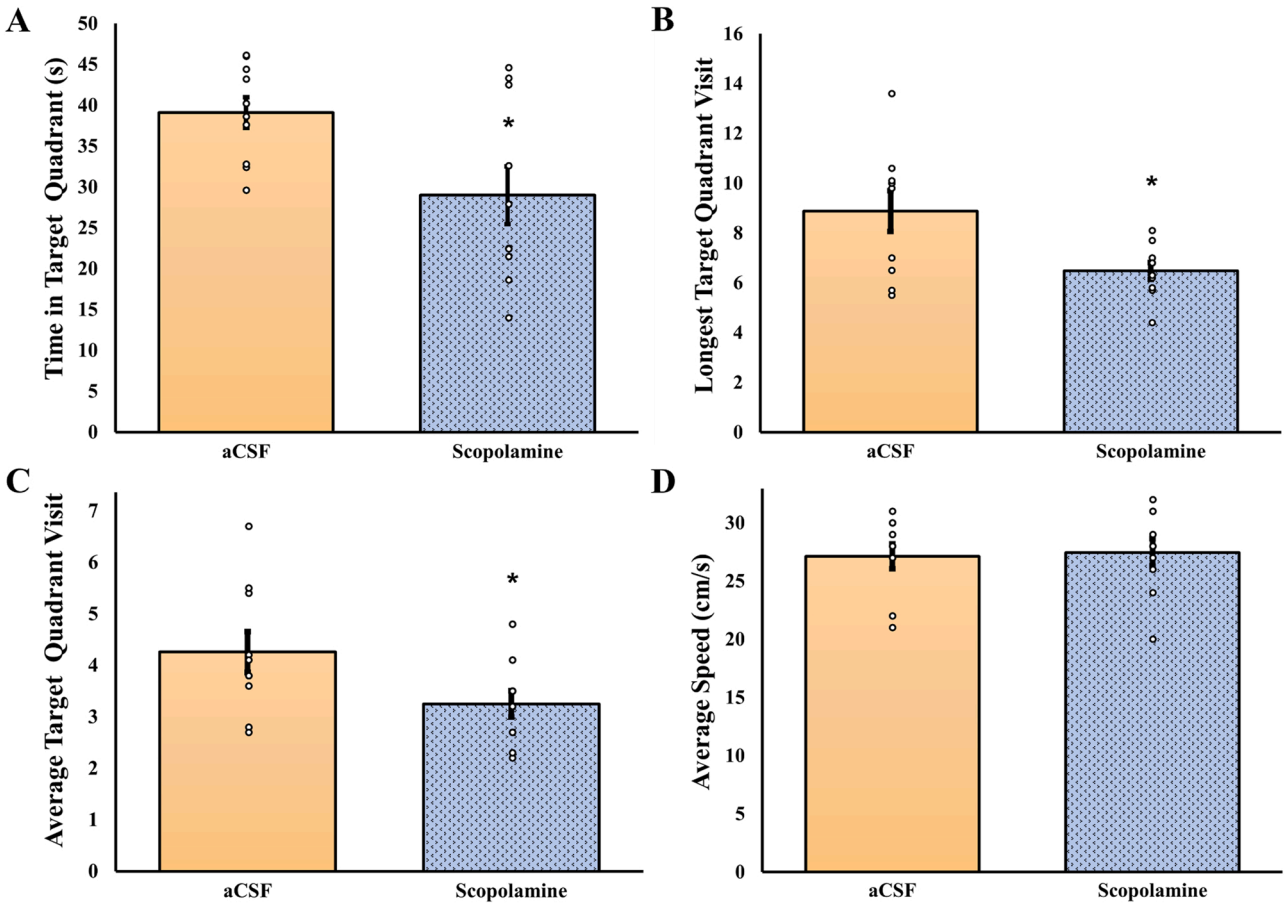

Fig. 3. Scopolamine impairs spatial memory retrieval. Retrieval test results for aCSF and Scopolamine conditions for time in target quadrant (A), longest target quadrant visit (B), average target quadrant visit (C), and average swim speed (D). *$p < 0.05$. Data are presented as mean + /- SEM with individual data points indicated.

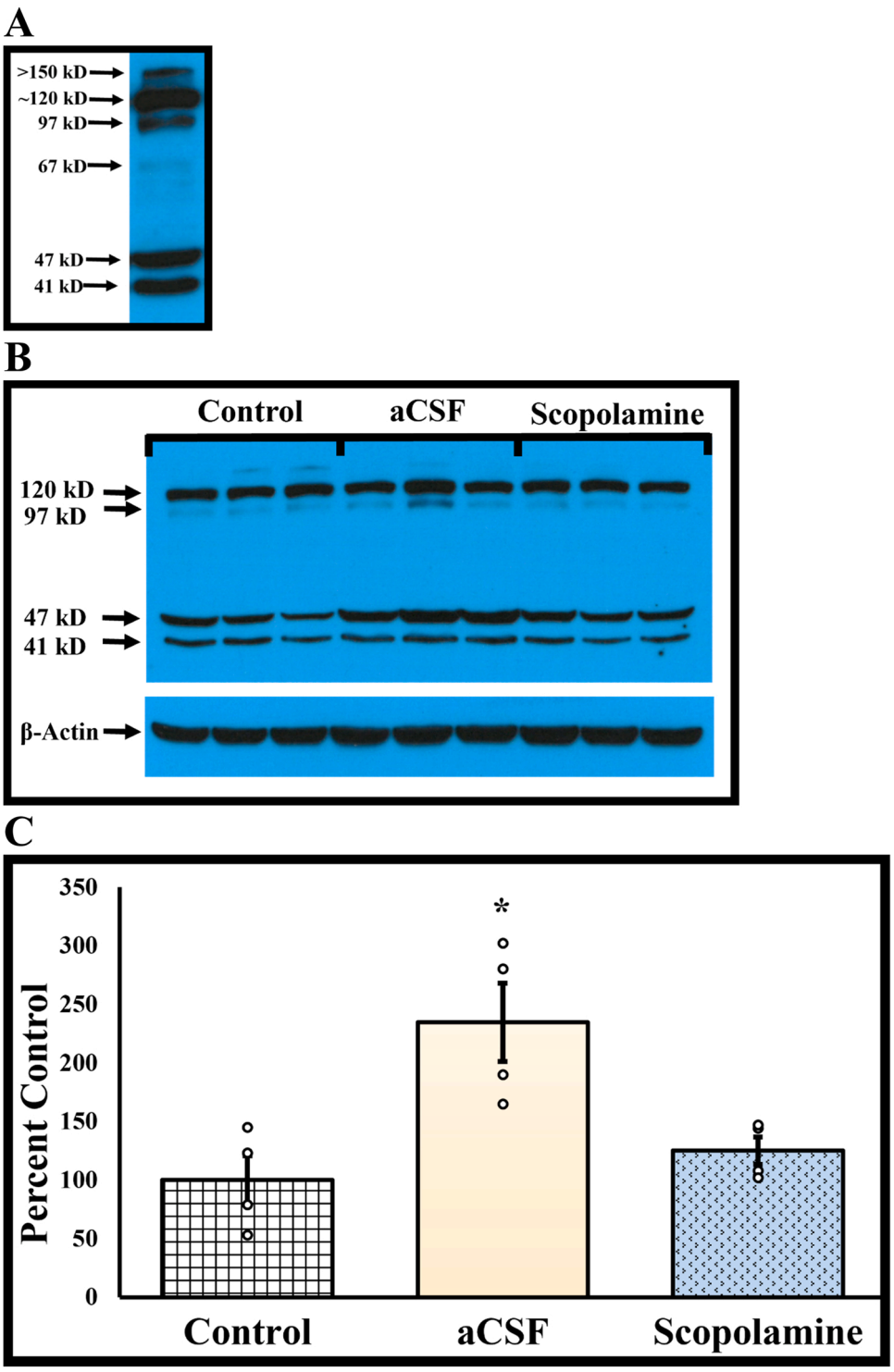

Fig. 4. MMP-9 expression 2 h post-retrieval. MMP-9 immunoreactivity is demonstrated for six isoforms (A). A truncated, 47 kD MMP-9 isoform is elevated in the aCSF condition versus both the Control and the Scopolamine conditions (B,C). *p < 0.05. Data are presented as mean +/- SEM with individual data points indicated.

4. Discussion

In the present study we tested the hypothesis that the cholinergic system is involved in the retrieval of a previously consolidated spatial memory. Our results show that central administration of the cholinergic antagonist scopolamine impairs the retrieval of spatial memories in the water maze. This finding supports previous research demonstrating the importance of cholinergic functionality in the retrieval of spatial and non-spatial memories [13,15,31,38], and it also lends support to findings demonstrating that the retrieval of a well-learned memory is impaired by cholinergic dysregulation [8,39].

We also tested the hypothesis that spatial memory retrieval is associated with changes in hippocampal MMP-9 expression. Specifically, our results demonstrate that a truncated form of MMP-9 is increased in association with spatial memory retrieval, and this MMP-9 increase is prevented by the mAChR antagonist scopolamine. To our knowledge, ours is the first demonstration of changes in hippocampal MMP-9 expression following spatial memory retrieval. It is also the first

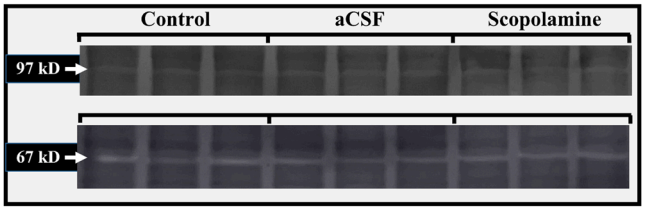

Fig. 5. MMP-9 enzymatic activity. Gelatinase activity is seen only for two MMP-9 isoforms and no differences are observed between experimental conditions.

demonstration of spatial memory retrieval-induced changes in MMP-9 expression being affected by the cholinergic antagonist scopolamine. Our findings are in agreement with the work of Moosavi and colleagues who demonstrated that MMP-9 levels were reduced in the hippocampus of rats intraperitoneally injected with scopolamine prior to retrieval of a non-spatial, associative memory [31]. Additionally, it was recently reported that rats injected with scopolamine expressed reduced hippocampal MMP-9 RNA [40]. Our findings therefore support a small but growing body of evidence connecting scopolamine-induced cholinergic inhibition to alterations in hippocampal MMP-9. One simple possible mechanism for scopolamine's effect on MMP-9 stems from the fact that mAChR activation in the hippocampus can play an important role in LTP and synaptic plasticity. A recent paper reported that post-operative dementia in mice was associated with reduced cholinergic signaling in the hippocampus and deficits in hippocampal LTP; furthermore, restoration of cholinergic signaling with galantamine improved memory and restored hippocampal LTP [41]. However, there is evidence from work in area CA2 of the hippocampus that cholinergic modulation of synaptic plasticity can be quite complex [42]. In hippocampal CA1, mAChR activation elevates glutamatergic signaling and produces NMDA-dependent LTP [43]. This mAChR-mediated enhancement of LTP at glutamatergic synapses in the hippocampus could either be a direct effect on CA1 cells or an astrocyte-mediated effect [44,45]. Regardless, given the involvement of MMP-9 in NMDA-dependent LTP [25,26,46], cholinergic modulation of NMDA-dependent LTP may be a mechanism by which scopolamine prevents retrieval-induced MMP-9 expression.

Due to previous research that identified gelatinase activity for truncated MMP-9 isoforms [23] we also analyzed our samples using gelatin zymography. We found gelatinase activity in the pro-MMP-9 region of the gel and the region of the 67 kD active MMP-9 band, but we observed no detectable gelatinase activity for truncated MMP-9. Further, we observed no differences in gelatinase activity between groups. It is important to note that denaturation of the pro-MMP-9 protein during SDS-PAGE exposes the catalytic domain of MMP-9 and thus allows for gelatinase activity stemming from the pro-enzyme; in *vivo*, the folded pro-enzyme would not be enzymatically active. Additionally, although we observed a 67 kD MMP-9 immunoreactive band on western blots, we cannot definitively attribute the observed gelatinase activity to the 67 kD active MMP-9 isoform. It is also possible that this gelatinase activity could be attributable to active MMP-2 or a combination of active MMP-9 and MMP-2. We can, however, definitively state that in our experiments we observe no gelatinase activity below 67 kD, thus demonstrating that the truncated MMP-9 isoform that correlated with spatial memory retrieval did not show gelatinolytic activity.

Previous studies have shown elevations in MMP-9 following the acquisition of spatial memory [30], cued fear conditioning [25], inhibitory avoidance learning [47], and contextual fear conditioning [48]. However, relatively little research has attempted to characterize the role of MMP-9 following memory retrieval. In the present study we observe a robust and reliable elevation in a truncated MMP-9 isoform two hours after spatial memory retrieval in the water maze. One possible explanation for the elevation in truncated MMP-9 we observed is that larger, activated forms of MMP-9 increased quickly after retrieval and were subsequently processed into deactivated degradation products. Truncated forms of MMP-9 have previously been characterized as degradation products of the activated protein [23,37] and may therefore be lagging indicators of active forms of MMP-9 occurring early post-retrieval. This possibility would suggest that elevations in active MMP-9 take place rapidly upon spatial memory retrieval. Indeed, locally induced MMP-9 activation at synapses stimulated by glutamate has been demonstrated to take place in *vitro* on a timeline of less than 15 min [46].

Furthermore, finely tuned regulation of MMP-9 activation is necessary for hippocampal LTP [26] and dendritic spine maturation and stability [49]. Additionally, the possibility that truncated MMP-9 has an as-yet unidentified role should not be automatically dismissed. Given the relative scarcity of research on memory retrieval and the incomplete understanding of MMP-9 substrates generally, it is possible that truncated forms of MMP-9 are themselves important regulators of cell functioning. Despite the lack of gelatinase activity we observed for truncated MMP-9, the possibility this MMP-9 isoform has other substrates or molecular targets remains. Indeed, others have stressed the need for more research into the roles truncated forms of MMP-9 play in cellular processes [20].

Future research should attempt to elucidate the function of MMP-9 elevation post-retrieval to determine whether it is a phenomenon associated with the retrieval itself or a regulator of post-retrieval memory stability (i.e., memory reconsolidation). Memory reconsolidation is thought to occur when memories are reactivated, as during retrieval, and the memory engram is brought into a state of increased malleability. Theoretically, this pliant state could facilitate the inclusion of new or additional information at the time of retrieval into the original memory. Clearly, this would be an adaptive feature that could strengthen memory accuracy by fine-tuning neural representations of the stored memory. To date, few studies have examined the role of MMPs in memory reconsolidation. Those that do show mixed results, and the only study to use a semi-specific MMP-9 inhibitor showed no effect on reconsolidation of a conditioned fear memory [50]. Further research investigating the role of MMP-9 inhibition is needed to help determine if preventing the retrieval-induced increase in MMP-9 we observe in our study impairs the ongoing stability of the memory (reconsolidation) or if it is a discretely retrieval-associated event. Potent and specific MMP-9 inhibitors are lacking and needed to delineate how MMP-9 is impacting synaptic changes that occur following retrieval and/or during memory reconsolidation. It is also worth emphasizing that the spatial memory retrieval test we conducted took place 7 days after training. Spatial memory retrieval at this timepoint likely involves a network of brain structures including the hippocampus and prefrontal cortex [51,52] and the retrosplenial cortex [53]. Due to the relative remoteness (7 days) of the retrieved memory in our study, and because hippocampal MMP-9 is a known plasticity marker [26], our findings lend support to the multiple trace model of episodic and spatial memory [54]. Future research should investigate the interplay between the hippocampus and cortical memory networks in order to further elucidate their importance for spatial memory retrieval. Finally, a potential limitation of our study stems from the fact that MMP-9 can be elevated following traumatic brain injury [55]. It is possible the cannulation surgery itself may have impacted MMP-9 levels, and this underscores the importance of the control, aCSF, and scopolamine groups all receiving cannulation surgery in our present study. Had the control group not received cannulation surgery, the observed elevation in truncated MMP-9 would be difficult to interpret due to this potential confound. Future research of the type described here should ensure that a surgical control is used; however, we acknowledge that future research may also benefit from the addition of a non-surgical, non-injected control and from the inclusion of female rats.

The memory deficit associated with AD has frequently been conceptualized as a failure of memory acquisition [56,57] or a failure of memory storage due to instability in the consolidated memory network [58,59]. Because cognitive tasks that assess memory acquisition and

storage also require memory retrieval, it has recently been suggested that task deficits attributed to memory acquisition or storage could be due to retrieval errors [60]. Roy and colleagues used a transgenic mouse model of early AD to demonstrate that although free retrieval of a previously learned contextual fear memory was impaired, optogenetic stimulation of cortical networks that constituted the engram led to robust expression of memory retrieval [60]. Their work demonstrates, at least in mouse models of AD, that memory deficits can be ascribed to a failure of retrieval rather than a failure of acquisition or storage. Our findings demonstrate that cholinergic function is important for spatial memory retrieval and suggest the cholinergic deficits known to be associated with AD [3] could result in memory retrieval failure.

Our findings indicate that mAChR-mediated cholinergic signaling in the forebrain is necessary for optimal spatial memory retrieval and for retrieval-induced changes in hippocampal MMP-9. Specifically, elevations in a truncated form of MMP-9 were associated with spatial memory retrieval. Additionally, centrally administered scopolamine impaired spatial memory retrieval and prevented retrieval-induced elevations in MMP-9. These findings provide evidence for a potential link between cholinergic dysregulation and abnormal MMP-9 levels seen in the brains of AD patients. An important, yet unresolved question concerns whether MMP-9 serves an important role in memory retrieval itself, or whether it is involved in maintaining the ongoing stability of the retrieved memory.

CRediT authorship contribution statement

Mikel Olson: Conceptualization, Methodology, Investigation, Analysis, Writing - Original Draft Preparation, Writing – Revision and Editing; **Bretton Badenoch:** Methodology, Analysis, Investigation, Writing – Original Draft Preparation; **Megan Blatti:** Analysis, Investigation, Writing – Original Draft Preparation; **Christine Buching:** Analysis, Investigation, Writing – Original Draft Preparation; **Nic Glewwe:** Analysis, Investigation, Writing – Original Draft Preparation.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Data availability

Data will be made available on request.

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