**Dorsal hippocampus- and ACC-projecting medial septum neurons differentially contribute to the recollection of episodic-like memory**

Tao Jin | Ruyan Chen | Mingshuo Shao | Xiao Yang | Lan Ma | Feifei Wang

Abstract

Episodic memory refers to the recollection of previous experiences containing specific temporal, spatial, and emotional information. The ability to recollect episodic memory requires coordination of multiple brain regions, including the hippocampus (HPC) and the cingulate cortex. While the afferents into HPC and cingulate cortex that orchestrate the episodic memory remain unclear. The medial septum (MS), one of the anatomical location of cholinergic centers, innervates not only the dorsal HPC (dHPC), but also the cingulate and entorhinal cortices. By using “What-Where-When” episodic-like memory (ELM) behavioral model and viral tracing, we found that MS neurons projected to dHPC and anterior cingulate cortex (ACC), which exerted distinct impacts on ELM recollection. Chemogenetic inhibition of the dHPC-projecting MS neurons disrupted “What-Where-When” ELM recollection as well as object location, object-in-place, and recency recognition memories recollection, while chemogenetic inhibition of the ACC-projecting MS neurons only disrupted “What-Where-When” ELM recollection. Moreover, neither dHPC- nor ACC-projecting MS neurons were involved in novel object recognition memory recollection or locomotor activity. Immunostaining showed that ACC- and dHPC-projecting MS neurons are partially overlapped populations. These findings reveal an unsuspected division of ELM processing and provide the potential mechanism that the recollection of episodic memory need the coordination of MS neurons projecting to dHPC and ACC.

**KEYWORDS**
anterior cingulate cortex, dorsal hippocampus, episodic-like memory, medial septum

Abbreviations: ACC, anterior cingulate cortex; AD, Alzheimer’s disease; AP, action potential; ChAT, choline acetyltransferase; CNO, clozapine N-oxide; Ctrl, control; dHPC, dorsal hippocampus; DNMS RAM, delayed nonmatch to sample eight arm radial maze; DREADD, designer receptors exclusively activated by designer drugs; ELM, episodic-like memory; GABA, γ-aminobutyric acid; HPC, hippocampus; MDD, major depression disorder; MS, medial septum; mPFC, medial prefrontal cortex; OFT, open field test; PTSD, post-traumatic stress disorder; TST, tail suspension test; vHPC, ventral hippocampus; VGLUT, vesicular glutamate transporter.
INTRODUCTION

Episodic memory was defined as the conscious recall of a personal unique experience not only about what happened, but also where and when happened which requires autonoetic awareness and a sense of subjective time in humans. Rodents, unlike humans, cannot verbalize a past experience, but they possess the ability to encode and recollect robust episode by forming associations between objects and the location (where) and/or occasion (when) it was last encountered, which was termed episodic-like memory (ELM). Due to the complexity of being an integrated and multidimensional memory, episodic memory is extremely vulnerable to diseases of neural system and easily disturbed. Episodic memory deficits were observed in neurodegenerative diseases such as Alzheimer’s Disease (AD) and also in other psychiatric diseases including Schizophrenia, Post-traumatic Stress Disorder (PTSD), and Major Depression Disorder (MDD). Therefore, episodic memory functions can be viewed as a highly sensitive marker of incipient brain pathology before the diseases become evident at the psychological and behavioral level. The research of episodic-like memory on rodents can help to pave the way for elucidating the neurobiological mechanisms that underlie episodic memory formation and recollection.

The dorsal hippocampus (dHPC) and its specific synaptic connections play an irreplaceable role in declarative memory, which consists of semantic and episodic memories. Blocking protein synthesis by stereotactic injection of anisomycin into this region impaired ELM consolidation, and cytotoxic lesion study showed that object location, object-in-place, and object temporal order memories were hippocampal dependent. Episodic memories were formed and retrieved through distinct hippocampal circuits. A recent research revealed a critical role of hippocampal CA1-projecting subiculum neurons in object-in-place learning, but the role of other afferents in regulating ELM was unclear. Successful episodic memory also requires the hippocampus to operate in concert with the neocortex, and of these, the anterior cingulate cortex (ACC) is of great interest. ACC was implicated in emotional processing, nociception, and motor control. Recent studies revealed the function of ACC and its synaptic connections in cognition. A causal link between ACC dysfunction and social deficits, as well as the neural circuit between ACC and ventral hippocampus (vHPC) controlling remote memory generalization have been found. Behavioral research on monkeys suggested the involvement of ACC in hierarchical reasoning.

GABAergic, cholinergic, and glutamatergic neurons in medial septum (MS) innervate the hippocampus and related cortical areas, contributing to the coordination of network activity and the regulation of learning and memory. Both dHPC and ACC receive strong inputs from MS. These studies lead us to speculate whether distinct afferents from MS can modulate episodic-like memory differentially. Here, we found that chemogenetic inactivation of dHPC-projecting MS neurons impaired “What-Where-When” ELM recollection as well as object location, object-in-place, and recency recognition memories, while inactivating MS neurons projecting to ACC only disrupted “What-Where-When” ELM recollection, unveiling the potential role that MS might contribute to precise cognitive function by coordinating ACC and dHPC.

MATERIALS AND METHODS

Animals

Adult C57BL/6J male mice were obtained from the SLAC laboratory animal company (Shanghai, China). All mice were housed (3-5 mice per cage) on a 12 hours light/dark cycle (light on from 8 AM to 8 PM) with access to food and water ad libitum. Male mice at 8-10 weeks of age were used for the study, which were randomly assigned to groups. All experiment procedures were strictly in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and were approved by Animal Care and Use Committee of the animal facility at Fudan University.

Viruses and stereotaxic surgery

All the viruses, AAV2-hSyn-retro-Cre, AAV2-hSyn-retro-Flp, AAV9-hSyn-DIO-mCherry, AAV9-hSyn-DIO-hM4Di-mCherry, AAV9-EF1α-DIO-EGFP, and AAV9-EF1α-fDIO-mCherry were purchased from Taitool Bioscience (Shanghai, China). Mice were anesthetized with 2% of isoflurane and placed in a stereotaxic instrument (Stoelting, Kiel, WI, USA). Microinjections were performed using 33-gauge needles connected to a 10 μL Hamilton syringe. The coordinates were: AP = 1.8 mm; ML ± 1.5 mm; DV = 1.4 mm for dHPC, AP + 1.1 mm; ML ± 0.3 mm; DV = 1.8 mm for ACC, and AP + 0.8 mm; ML ± 0.0 mm; DV = 4.0 mm for MS. The injection volume per site was 0.2 μL in ACC and dHPC and 0.5 μL (10 IU/mL AAV) in MS. All the mice were given at least 3 weeks to recover before behavioral experiments or electrophysiological recordings, and the efficiency of virus infection was verified by immunostaining. Only the mice with virus infection in correct place were chosen for further analysis.

Slice electrophysiology

Coronal sections (250 μm) containing MS was prepared as previously reported only with minor modifications. Briefly, the brains were cut on a vibratome (Thermo Fisher Scientific, Waltham, MA, USA).
2.4 | Immunohistochemistry

Mice were perfused transcardially with ice-cold saline followed by 4% of paraformaldehyde (dissolved in 0.1 M PBS). The brains were removed and fixed in 4% of paraformaldehyde overnight. Then, the brains were subjected to dehydrate in 30% of sucrose solutions at 4°C for 72 hours before being sliced into 30 μm coronal sections. Slices were incubated with primary antibodies in blocking solution containing 0.3% of Triton X-100 overnight at 4°C. Slices were washed with 0.1 M of PBS, and then, incubated in secondary antibody at room temperature for 2 hours. After washes in PBS, slices were mounted in anti-quenching mounting medium. Primary antibodies used were: anti-ChAT (AB143, Millipore, Darmstadt, Germany, 1:100), anti-GABA (A2052, Sigma, St Louis, WA, USA1:500), anti-NeuN (MAB377, Millipore, 1:500), anti-VGLUT1 (AB5905, Millipore, 1:200), anti-VGLUT2 (135403, Synaptic System, Gottingen, Germany, 1:800). Secondary antibodies used were: goat anti-rabbit 488 (111-545-144, Jackson ImmunoResearch West Grove, PA, USA, 1:50000), goat anti-rabbit 647 (111-605-003, Jackson ImmunoResearch, 1:50000), goat anti-guinea pig 647 (106-605-003, Jackson ImmunoResearch, 1:50000).

2.5 | Cell number quantification

For each mouse, 50 μm slices throughout the MS (coordinates from AP + 1.1 mm to AP + 0.4 mm) were collected. Images were acquired using a Nikon-A1 confocal microscope (Tokyo, Japan) with a 20x objective len. The cell numbers for EGFP, mCherry, or double labeling in each slice were counted with Image Pro Plus 6.0 (Media Cybernetics, Rockville, MD, US) and summed up as the total number. The quantitative analysis of overlap was calculated as follows: the total number of EGFP+ or mCherry+ neurons / the total number of EGFP+ neurons or the total number of mCherry+ neurons. The distribution of the labeling percentage was calculated as follows: the number of EGFP+ or mCherry+ neurons in each AP distance (eg., 1.1/1.05/1.0…) / the total number of EGFP+ or mCherry+ neurons in all AP distances (from +1.1 to +0.4), respectively.

2.6 | Behavioral tests

2.6.1 | “What-Where-When” episodic-like memory (ELM) task

The ELM paradigm was based on animals’ spontaneous preference for novel or less recently encountered stimuli or locations compared with familiar or more recently encountered ones.38-41 Exploration was occurred in an open chamber (60 × 60 cm²) at the illumination of 15 Lux to facilitate exploration and minimize anxiety levels. The stimuli presented were objects composed of wooden blocks varied in shape, color, height, and size with bottoms stuck to the chamber so they cannot be displaced. After being handled for 4 days, mice were habituated to the arena without stimuli for 5 minutes for consecutive 4 days. Each mouse received three phases, two sample phases and one test phase, with 1 h delay between each phase. In each sample phase, mice were allowed to explore two different objects placed in two unique locations of the arena for 10 minutes. In the test phase (5 minutes), mice were presented with all four objects but one object from each sample phase had been switched location. The proportion of time exploring was calculated as follows, time exploring each object / time exploring all objects, the discrimination ratios for when and where were calculated as follows, (time exploring the temporal distant or displaced objects-time exploring the temporal recent or stationary objects) / time exploring all objects. Experiments were camera recorded for subsequent analysis. The behavior videos were scored by two independent investigators who were blind to the group allocation.
2.6.2 | Novel object recognition (what) memory task

In this task, each mouse received one sample phase to explore two identical objects presented in two different places for 5 minutes, and after 1 hour delay, mice were put back to the chamber (3 minutes) with one object replaced by a novel one. The discrimination ratio was calculated as follows, (time exploring the novel object-time exploring the familiar object) / time exploring both objects.

2.6.3 | Object temporal order (When)/object recency memory task

This task consists of two sample phases and one test trial also with 1 hour delay. During each sample phase, mice were encountered two copies of objects for 5 minutes and in the test phase, mice were allowed to explore two objects each from sample phases for 3 minutes. The discrimination ratio was calculated as follows, (time exploring the temporal distant object-time exploring the temporal recent object) / time exploring both objects.

2.6.4 | Object location (Where) memory task

This memory task was comprised of one sample trial and one test trial separated by 1 hour delay. Each mouse received two identical objects in sample phase (5 minutes) with one of the objects moved to another place in the arena during test phase (3 minutes). The discrimination ratio was calculated as follows, (time exploring the displaced object-time exploring stationary object) / time exploring both objects.

2.6.5 | Object-in-place memory task

This task also involved one sample phase and one test phase separated by 1 hour delay. In sample phase, mice were presented with four objects for 10 minutes and in test phase, two objects were exchanged places and mice explored the objects for 5 minutes. The discrimination ratio was calculated as follows, (time exploring the displaced objects-time exploring stationary objects) / time exploring all objects.

Different cohorts of mice were used for each memory task and each mouse was given CNO injection for once. The time spent exploring each object in the memory tasks mentioned above were analyzed by experimenters who were blind to the project.

2.6.6 | Open field test (OFT)

Spontaneous locomotor activity was measured in an open field arena (40 x 40 cm²). Mice were placed in the center of the arena at the beginning of the test, and were allowed to freely explore the arena for 20 minutes. Distance traveled in the arena and time in center zone were quantified using a TopScan automated detection system (CleverSys, Reston, VA, USA).

2.6.7 | O-maze test

The O-maze was 70 cm in diameter and 70 cm high off the ground, and consisted of two open arms (7 cm wide) without walls, and two closed arms that were enclosed by vertical walls. Mice were gently placed in the open arms, and their behavior was recorded for 6 minutes via a TopScan automated detection system (CleverSys, Reston, VA, USA) located above the maze.

2.6.8 | Tail suspension test (TST)

Mice were suspended 20 cm above a solid surface by the use of adhesive tape applied to the tail. Their behavior was recorded for 6 minutes and automatically analyzed by Clever System software.

2.7 | Statistics

All data were presented as mean ± SEM. Statistical analyses were performed by SPSS 20.0 software (IBM, Armonk, NY, USA). The normality test of the data sets was performed by the Shapiro-Wilk test. Two-tailed unpaired t test was used for comparing two independent groups. Multiple group comparisons were assessed using two-way repeated measures (RM) ANOVA, followed by the Bonferroni’s post hoc test when significant main effects or interactions were detected. * $P < .05$, ** $P < .01$, and *** $P < .001$. All data are presented as mean ± SEM.

3 | RESULTS

3.1 | Medial septum (MS) neurons projecting to dorsal Hippocampus (dHPC) regulate episodic-like memory (ELM) recollection

To assess the role of dHPC-projecting MS neurons in ELM recollection, AAV2-retro-Cre was infected bilaterally
into the dHPC and a cre-dependent DREADD (designer receptors exclusively activated by designer drugs) virus AAV9-hSyn-DIO-hM4Di-mCherry (with AAV9-hSyn-DIO-mCherry as control) was infected into the medial septum (MS) (Figure 1A). Treatment with clozapine N-oxide (CNO), an activator of hM4Di, repressed the firing frequency of hM4Di-mCherry+ neurons in MS (Figure 1B-D). Immunostaining of the AAV-infected cells demonstrated that both mCherry+ and hM4Di-mCherry+ cells were co-labeling with choline acetyltransferase (ChAT, cholinergic marker), γ-aminobutyric acid (GABA), and vesicular glutamate transporter 2 (VGLUT2, glutamatergic marker) (Figure S2A-F). Then, open field test (OFT) was performed and CNO (1 mg/kg) was delivered by intraperitoneal (i.p.) injection 30 minutes before test, no significant difference was detected after inhibition of MS projections to dHPC, indicating mice’s locomotor activity was preserved after chemogenetic intervention (Figure S2H).

The “What-Where-When” episodic-like memory (ELM) paradigm evaluated mice’s ability to recollect the temporal occurrence (when) and spatial context (where) of a previously encountered object (what). In the two sample phases, mice were allowed to explore two different objects (object A and B or object C and D) placed in two unique locations of the arena separated by 1 hours. 30 minutes before the test session began, CNO was delivered by i.p. injection, and then, mice were presented with four objects while the positions of object B and object C had been switched and time spent exploring all four objects were measured (Figures 1E, and S1). Successful recollection of the what, where, when information of ELM would result in a pattern of preferential exploration so that most exploration was directed to the familiar location and temporally recent (object B). Exploring time of the familiar location but temporally distant (object A) or the novel location but temporally recent (object C) was less than that of object B, while the least exploration would be directed to the familiar location and temporally recent object (object D). As shown in Figure 1F, mice in the control (Ctrl) group displayed the expected pattern of exploration (B > (A, C) > D). Oppositely, ELM recollection was dramatically impaired in the chemogenetically inactivated group, showing no preference for the four objects. Discrimination ratio analysis confirmed that when disrupting the MS projections to dHPC, the ability of distinguishing the when and where components of ELM was significantly impaired (Figure 1G). Time spent exploring each object in sample phases and total objects exploration time in test phase between treatments did not differ (Figure 1H,I). These results suggested that mice’s ability to recall both the spatial and temporal information of ELM concerning one object’s prior occurrence was dependent on the functional interaction between MS and dHPC.

### 3.2 MS neurons projecting to dHPC regulate object location, object-in-place, and recency recognition memories recollection

In the ELM task, exploring the four objects cannot be considered independent, since more exploration of one object may be either a cause or consequence of less exploration of another one. Hence, we further examined this disassociation using a series of behavioral tasks to study MS-dHPC pathway in regulation of novel object preference, object location, object temporal order (recency), and object-in-place memories recollection. First, we confirmed that the basic novel object recognition (what) memory was preserved by using the what memory paradigm when inhibiting dHPC-projecting MS axons (Figure 2A,B). However, the recollection of individual memories for object temporal order (whent), object location (where), and object-in-place were significantly impaired comparing with control group by using respective behavioral protocols, and no obvious differences in exploring time were found between treatments (Figure 2C-H).

MS primarily consists of cholinergic, GABAergic, and glutamatergic neurons. Quantitative analysis in Figure S2g demonstrated that the percentage of GABAergic and glutamatergic neurons were similar, the percentage of cholinergic neurons was much higher, but there was no difference in composition and percentage of neuronal types between groups. These data indicated that cholinergic, GABAergic, and glutamatergic dHPC-projecting medial septal neurons together regulated ELM along with when, where, and object-in-place memories. O-maze test and tail suspension test (TST) were performed after CNO delivery, and no differences were detected between groups (Figure 2I,J), indicating the disturbance of these memories were not due to the impairment of depression or anxiety. Here, we conclude that MS neurons projecting to dHPC mediate the recollection of individual memories for when, where, and object-in-place without impairing the locomotion, anxiety, or depression level.

### 3.3 MS neurons projecting to anterior cingulate cortex (ACC) regulate ELM recollection, while have no effect on object location, object-in-place, and recency recognition memories recollection

To investigate the role of ACC-projecting MS neurons in ELM, AAV2-retro-Cre was infected bilaterally in ACC and AAV9-hSyn-DIO-mCherry or AAV9-hSyn-DIO-hM4Di-mCherry was infected in MS (Figure 3A). AAV expression and its inhibitory efficiency were verified the same as we did in Figure 1B-D (Figure 3B-D). OFT was performed and mice’s locomotor activity was not affected when inhibiting
FIGURE 1  Chemogenetic inhibition of MS neurons projecting to dHPC interferes with episodic-like memory (ELM) recollection. A, Diagram of AAV injection sites. B, Expression of hM4Di-mCherry in MS. Scale bar: 100 µm. C, Current-clamp recording of a representative mCherry+ neurons before and after application 10 µM CNO. D, Quantification of the firing frequency of mCherry+ neurons before and after application 10 µM CNO. mCherry+, n = 7, P = .850, hM4Di-mCherry+, n = 5, P < .001, two-tailed unpaired t test. E, Scheme of the episodic-like memory task. F, Distribution of exploring times per object in the test phase for Ctrl and hM4Di groups. F Treatment × object (3, 78) = 9.436, P < .001, two-way RM ANOVA by Bonferroni post hoc analysis. G, Discrimination ratio for where and when components of ELM. Where, P = .010, when, P < .001, two-tailed unpaired t test. H, Distribution of exploring times per object in the sample phases for each group. F Treatment × object (3, 78) = 0.842, P = .475, two-way RM ANOVA by Bonferroni post hoc analysis. I, Total exploring times in the test phase for each group. P = .509, two-tailed unpaired t test. Ctrl, n = 16, hM4Di, n = 12. Data are presented as mean ± S.E.M; *P < .05, ***P < .001
MS projections to ACC (Figure S3H). Immunostaining revealed mCherry+ and hM4Di-mCherry+ MS neurons colocalized with ChAT, GABA, and VGLUT2 and quantitative analysis exhibited the same percentage pattern as dHPC-projecting MS neurons (Figure 3A-G). After 3 week’s recovery, the episodic-like memory test was performed (Figure 3E). Disrupting the projections from MS to ACC impaired the ELM recollection. Separate calculations of discrimination ratios revealed the abolishment for the temporal and spatial components discrimination after CNO injection into the hM4Di-expressing mice (Figure 3F,G). In addition, hM4Di-expressing mice’s time spent in exploring objects in sample phases and the total exploration time in test phase were compatible with control group (Figure 3H,I). These data demonstrated that MS axons projecting to ACC was required for ELM recollection.
FIGURE 3  Chemogenetic inhibition of MS neurons projecting to ACC interferes with ELM recollection. A, Diagram of AAV injection sites. B, Expression of hM4Di-mCherry in MS. Scale bar: 100 µm. C, Current-clamp recording of a representative mCherry+ neuron and hM4Di-mCherry+ neuron before and after application 10 µM CNO. D, Quantification of the firing frequency of mCherry+ neurons and hM4Di-mCherry+ neurons before and after application 10 µM CNO. mCherry+, n = 7, P = .357, hM4Di-mCherry+, n = 4, P < .001, two-tailed unpaired t-test. E, Scheme of the episodic-like memory task. F, Distribution of exploring times per object in the test phase for Ctrl and hM4Di groups. F_{Treatment \times object (3, 63)} = 3.454, P = .022, two-way RM ANOVA by Bonferroni post hoc analysis. G, Discrimination ratio for where and when components of ELM. Where, P = .020, When, P = .038, two-tailed unpaired t-test. H, Distribution of exploring times per object in the sample phases for each group. F_{Treatment \times object (3, 63)} = .111, P = .953, two-way RM ANOVA by Bonferroni post hoc analysis. I, Total exploring times in the test phase for each group. P = .902, Mann-Whitney U test. Ctrl, n = 12, hM4Di, n = 11. Data are presented as mean ± S.E.M; *P < .05, **P < .01, ***P < .001.
Then, we used the separate behavioral paradigms to assess the role of ACC-projecting MS neurons in regulating individual memories recollection. What is interesting was that the what, where, when, and object-in-place memories were retained after chemogenetic inactivation of MS projections to ACC (Figure 4). General emotional states of the mice were tested (Figure S3I,J), and time in open arms in the O-maze test suggesting decreased anxiety level after intervention of ACC-projecting MS axons, consistent with the important role of ACC in emotional and nociceptive processing.26-28 Thus, we inferred that MS neurons projecting to ACC might play a vital role in higher-order cognition, like episodic-like memory.
but was not involved in processing basic what, where, when, and object-in-place individual memories.

3.4 | ACC- and dHPC-projecting MS neurons are partially overlapped populations

In order to investigate the spatial distribution of MS neurons that projected to ACC and dHPC, AAV<sub>2</sub>-retro-Cre and AAV<sub>2</sub>-retro-Flp was infected into ACC and dHPC, respectively, and AAV<sub>2</sub>-EF1a-DIO-EGFP, AAV<sub>2</sub>-EF1a-DIO-mCherry were infected into MS so that ACC-projecting populations could be labeled by EGFP and dHPC-projecting populations could be labeled by mCherry (Figure 5A). Brain slices containing the whole region of MS were imaged and analyzed. According to the statistical results (Figure 5C), there existed EGFP<sup>+</sup> mCherry<sup>+</sup> neurons (14% in EGFP<sup>+</sup> neurons and 28% in mCherry<sup>+</sup> neurons, respectively), indicating that partial MS neurons projected to both ACC and dHPC. Besides, quantification of EGFP<sup>+</sup> and mCherry<sup>+</sup> neurons across the AP axis (coronal distance from bregma +1.1 mm to +0.40 mm) illustrated that neurons in the whole MS take part in ACC- and dHPC-projections, while they were both primarily distributed in the middle and posterior MS region (Figure 5D). Further neuronal type analysis revealed that cholinergic, GABAergic, and glutamatergic neurons all contributed to the projections to both dHPC and ACC (Figure 5E-G). Here, we verified that majority of MS neurons projected to ACC and dHPC separately, but some of them projected to both regions simultaneously.

4 | DISCUSSION

Episodic memory was characterized as the replay of specific events in sequential order, including information about the time, location, as well as details of an event. However, episodic memory might not be unique to humans, a wide range of animal species also showed the ability to associate what, where, and when information with the event they experienced, which was termed episodic-like memory. The hippocampus has long been recognized as the hub region in encoding and recollecting episodic or episodic-like memory. However, there existed somehow paradoxical results, also most of the related researches were obtained through hippocampal lesion studies, more sophisticated mechanism and its regulation by inputs from other brain regions remained to be elucidated.

By using the delayed nonmatch to sample eight arm radial maze (DNMS RAM) behavioral task, Dougherty et al confirmed that the septocingulate cholinergic pathway was critically involved in working memory in a delay dependent manner while the septohippocampal cholinergic pathway might not. However, in our present study, we confirmed that dHPC received afferents from MS, a part of the basal forebrain that regulated ELM recollection as well as the object location, object temporal order, and object-in-place memories by using the “What-Where-When” model and individual memory tasks, suggesting that the dHPC-projecting MS neurons were not only responsible for integrating different information, but also recollecting the individual spatial and temporal information. Barker et al found that medial prefrontal cortex (mPFC) was important for the formation of associations between the object and place information. ACC could be considered a posterior portion of mPFC, but it was distinguished from typical mPFC at the level of the prelimbic cortex. Here, we thought the ACC-projecting MS neurons played an important role in integrating the spatial and temporal components of ELM, which meant that these neurons were responsible for processing higher-order cognitive function, like ELM or hierarchical reasoning, as for the basic recognition memories, ACC-projecting MS neurons did not need to be involved. However, neither dHPC- nor ACC-projecting MS neurons participated in the novel object recognition memory recollection, which was consistent with previous reports. Hence, the detrimental effect on recollecting ELM or the individual memories induced by chemogenetically inhibiting these two MS projections could not be explained by animals’ impairment of perceptual abilities or novelty preference, which could be further supported by the evidence of similar exploration time during sample and test phases between treatments. Suppressing MS input to either dHPC or ACC by systemic injection of CNO could achieve some level of circuit specificity. However, both in vivo and in vitro studies have implicated that clozapine has a strong effect on cholinergic signaling. Additionally, systemic CNO administration will suppress dHPC- or ACC-projecting MS neurons project to other brain areas, leading to the off-target effect. Thus, direct CNO administration to dHPC or ACC through a cannula, or optogenetic manipulation of these neurons will be more specific and contributes to our understanding of these two projections.

The retrograde virus tracing revealed the spatial distribution of dHPC- and ACC-projecting MS neurons, indicating they were partially overlapped. Immunostaining results demonstrated dHPC- and ACC-projecting MS neurons, including the overlapped proportion, consist of cholinergic, GABAergic, and glutamatergic neurons. One possibility was that MS cholinergic neurons possessed the ability to co-release GABA or glutamate like previously reported. Or alternatively, these three kinds of MS neurons together projected to dHPC and ACC and regulated the recollection of ELM and the individual memories, in that case, how cholinergic, GABAergic, and glutamatergic neurons interacted to orchestrate physiological behaviors remained to be investigated. It
has been proposed that glutamatergic neurons within the MS can excite cholinergic and GABAergic neurons, and exert anorexic effects. Probably these glutamatergic neurons projecting to ACC or dHPC are part of a connected excitatory network, which upon appropriate activation, may contribute to the observed behavioral effects.

ELM is sensitive to cerebral aging and neurodegenerative diseases due to its complexity. In the present study,
we elucidated the distinct contributions of dHPC- and ACC-projecting MS neurons to the recollection of ELM, providing the potential evidence that these two projections contributed to the maintenance of early cognitive function.

CONFLICT OF INTERESTS
The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS
F. Wang, T. Jin, and L. Ma designed research; T. Jin, R. Chen, M. Shao, and X. Yang performed research; T. Jin analyzed data and drafted the paper; L. Ma and F. Wang revised the paper.

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found online in the Supporting Information section.

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