Research

Muscarinic acetylcholine receptors act in synergy to facilitate learning and memory

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Understanding how episodic memories are formed and retrieved is necessary if we are to treat disorders in which they malfunction. Muscarinic acetylcholine receptors (mAChR) in the hippocampus and cortex underlie memory formation, but there is conflicting evidence regarding their role in memory retrieval. Additionally, there is no consensus on which mAChR subtypes are critical for memory processing. Using pharmacological and genetic approaches, we found that (1) encoding and retrieval of contextual memory requires mAChR in the dorsal hippocampus (DH) and retrosplenial cortex (RSC), (2) memory formation requires hippocampal M_3 and cooperative activity of RSC M_1 and M_3, and (3) memory retrieval is more impaired by inactivation of multiple M_1–M_4 mAChR in DH or RSC than inactivation of individual receptor subtypes. Contrary to the view that acetylcholine supports learning but is detrimental to memory retrieval, we found that coactivation of multiple mAChR is required for retrieval of both recently and remotely acquired context memories. Manipulations with higher receptor specificity were generally less potent than manipulations targeting multiple receptor subtypes, suggesting that mAChR act in synergy to regulate memory processes. These findings provide unique insight into the development of therapies for amnestic symptoms, suggesting that broadly acting, rather than receptor-specific, mAchR agonists and positive allosteric modulators may be the most effective therapeutic approach.

Central cholinergic signaling via mAChR has been implicated in learning and memory since the mid- to late-1960s (Meyers et al. 1964; Whitehouse 1964; Whitehouse et al. 1964; Meyers 1965; Vogel et al. 1967; Izquierdo et al. 1992). Yet, after nearly half a century of research, the exact role of acetylcholine in these processes remains elusive and subject to debate. Whether this neurotransmitter is a key player across phases of memory formation and retrieval, and even whether major components of cholinergic signaling contribute to such cognitive processes at all (Miyakawa et al. 2001) are still contentious topics. This may be due to the many complexities of the cholinergic system, including the sources and metabolism of acetylcholine, its diverse receptor subtypes, and the neuroanatomical and cell-type specificity of responses to this neurotransmitter.

Work by Hasselmo and colleagues has done much to unravel such complexities (Hasselmo and Schnell 1994; Kremin et al. 2006; Newman et al. 2013). They assert that activation of mAChR mediates attention to novel stimuli and enhanced sensitivity to relevant inputs to support learning, but may actually attenuate memory recall via those same mechanisms (Hasselmo and Bower 1993; Hasselmo and Giocomo 2006). Indeed, several researchers have reported null effects of intrahippocampally or systemically administered anti-muscarinics on memory retrieval (Rogers and Kesner 2003, 2004; Atri et al. 2004; Huang et al. 2011). However, recent evidence has accumulated, suggesting that these receptors support both the encoding and retrieval phases of memory (Soares et al. 2006; Azami et al. 2010; Souza et al. 2013; Soma et al. 2014). This conflicting evidence, in addition to the multifaceted nature of the cholinergic system, highlights the need for a systematic, selective, and regional approach to tease apart the role of specific components of muscarinic signaling in various stages of memory.

In addition to the controversial role of mAChR in memory retrieval, there is little consensus on the behavioral consequences of disrupting the function of each of the five mAChR subtypes. Although pharmacological and electrophysiological approaches often point to M_1 or M_2 as potent mediators of learning (Sen and Bhattacharya 1991; Fornari et al. 2000; Power et al. 2003; Soares et al. 2006; Figueredo et al. 2008; Ma et al. 2009), constitutive knockout of these receptors has no effect on learning in a variety of tasks (Anagnostaras et al. 2003; Bainbridge et al. 2008). Moreover, when such manipulations do impact learning, as is the case with constitutive M_3 deletion (Poulin et al. 2010), the neuroanatomical basis for the effect and the specific memory process(es) affected remain unknown.

In this series of studies, we aimed to identify the primary contributions of hippocampal and cortical mAChR subtypes to contextual learning and memory. We selected the retrosplenial cortex (RSC) as our cortical region of interest because of its involvement in both memory formation and retrieval (Keene and Bucci 2008a; Corcoran et al. 2011; Cowansage et al. 2014; Kwapis et al. 2015). We hypothesized that the RSC and dorsal hippocampus (DH) would similarly rely on the excitatory, postsynaptic M_1/M_3 class of receptors in both memory formation and retrieval, and that conditional knockdown of these individual receptors would delineate the specific contribution of each. Using intracranial infusions of general mAChR antagonists,

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M_1/M_3, or M_2/M_4 antagonists, and region-specific knockdown of M_1 or M_3, we demonstrated that memory formation required hippocampal M_3 and cooperative activity of RSC M_1 and M_3. Interestingly, we found that retrieval of recently acquired context memory required DH M_1/M_3 but RSC M_2/M_4 mAChR, whereas retrieval of remote memory involved all RSC mAChR subtypes. These experiments are the first to utilize conditional knockdown approaches to delineate specific, regional roles of mAChR subtypes in memory processes and demonstrate a neurochemical mechanism by which RSC supports the formation of a context memory.

Results

Contextual memory formation requires M_1/M_3 but not M_2/M_4 in DH and RSC

To examine whether mAChR in DH and/or RSC support contextual memory formation, we infused either the mAChR antagonist scopolamine or vehicle into DH or RSC prior to training, and then assessed freezing to the context the following day. Preconditioning drug infusions had no effect on levels of locomotor activity during context exploration prior to shock (scopolamine: DH $t_{14} = 0.835$, $P = 0.418$; RSC $t_{14} = 0.440$, $P = 0.667$; Fig. 1A, top; Telenzepine/AF-DX 116: DH $F_{(2,19)} = 1.821$, $P = 0.189$, Fig. 1B top; RSC $F_{(2,20)} = 1.435$, $P = 0.262$; Fig. 1C top) or on activity bursts in response to the shock (scopolamine: DH $t_{14} = 0.701$, $P = 0.495$; RSC $t_{14} = 1.390$, $P = 0.186$; Fig. 1A, middle; Telenzepine/AF-DX 116: DH $F_{(2,19)} = 0.266$, $P = 0.769$; Fig. 1B, middle; RSC $F_{(2,20)} = 1.605$, $P = 0.226$; Fig. 1C, middle), suggesting that these drugs did not affect baseline activity or shock sensitivity.

For both DH and RSC, independent samples *t*-tests indicated that scopolamine-treated mice showed reduced freezing compared with vehicle-treated mice (DH: $t_{14} = 2.199$, $P < 0.05$; RSC: $t_{14} = 3.084$, $P < 0.01$; Fig. 1A, bottom). We next attempted to delineate the class of mAChR subtype critical for memory formation in both the DH and RSC (Fig. 1B,C). We utilized the M_1/M_3 antagonist telenzepine and the M_2/M_4 antagonist AF-DX 116 to test the roles of these subtypes.

In DH, preconditioning infusion of telenzepine, but not AF-DX 116, resulted in reduced freezing compared with vehicle infusion (Fig. 1B, bottom), as indicated by a significant one-way ANOVA ($F_{(2,19)} = 15.925$, $P < 0.0001$) and subsequent post hoc tests (vehicle vs. AF-DX 116, $P = 0.902$; vehicle vs. telenzepine, $P < 0.0001$). Similarly, preconditioning RSC infusion of telenzepine, but not AF-DX 116, impaired freezing ($F_{(2,20)} = 5.641$, $P = 0.011$; vehicle vs. AF-DX 116, $P = 0.39$; vehicle vs. telenzepine, $P = 0.009$; Fig. 1C, bottom). These data indicate that M_1 and M_3 are likely the critical subtypes in both DH and RSC mediating contextual memory formation.

Conditional knockdown reveals significant roles of DH M_3 in memory formation

To further differentiate the roles of M_1 and M_3 during memory encoding, we obtained floxed mouse lines for each receptor and infused a Cre-expressing adeno-associated virus (Cre) or control virus (GFP) into RSC or DH prior to training (Fig. 2). Neither DH nor RSC M_1 knockdown caused any changes in contextual fear conditioning (Fig. 2A; DH: $t_9 = -0.325$, $P = 0.752$; RSC: $t_9 = 0.661$, $P = 0.525$) or baseline locomotor activity (Fig. 2B; DH: $t_9 = -0.36$, $P = 0.727$; RSC: $t_9 = -0.25$, $P = 0.808$), despite a roughly 30% reduction of M_1 RNA in DH ($t_9 = 2.84$, $P = 0.019$) and a better than 50% reduction of M_1 RNA in RSC ($t_9 = 6.23$, $P = 0.000$) (Fig. 2C). The levels of receptor knockdown were significant (Fig. 2C,F), even though they likely underrepresent the effectiveness of viral transfection, as our samples included some untransfected tissue.

Similar to M_1 knockdown, a significant knockdown of M_3 RNA in the RSC ($t_6 = 3.566$, $P = 0.012$) had no effect on contextual fear conditioning ($t_{20} = 0.0$, $P = 1.0$) or baseline locomotion ($t_{20} = -1.592$, $P = 0.127$) compared with control mice (Fig. 2D,E). In contrast, M_3 RNA knockdown in DH ($t_7 = 2.915$, $P = 0.023$) significantly impaired contextual fear conditioning ($t_{30} = 2.148$, $P = 0.040$), while leaving locomotor activity intact ($t_{30} = 0.600$, $P = 0.553$). To determine whether the effect of DH M_3 knockdown could be attributed to a retrieval effect, we first fear-conditioned mice and tested them for memory retrieval, then injected the Cre or GFP virus into DH, allowed the virus time to incubate, and then tested the mice again. In this case, there was no difference between groups ($t_{20} = 0.369$, $P = 0.716$; data not shown), suggesting that M_3 knockdown in DH prior to conditioning, but not prior to retrieval, impaired freezing.

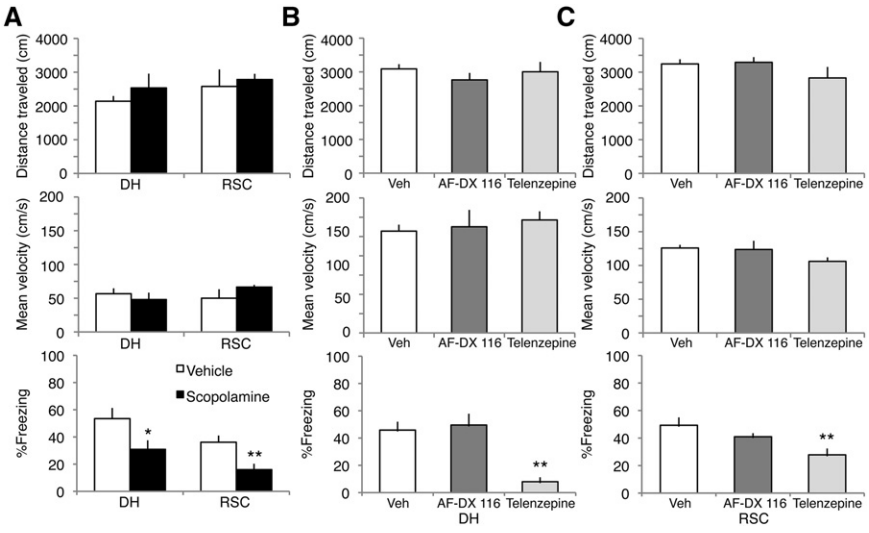

Figure 1. The effect of pharmacological inhibition of mAChR in DH and RSC on contextual memory formation. (*A*) Preconditioning infusion scopolamine into DH or RSC had no effect on preshock locomotor activity (*top*) or shock reactivity (*middle*), but impaired contextual fear conditioning, as indicated by decreased freezing during retrieval testing (*bottom*). Preconditioning infusions of AF-DX 116 or telenzepine into (*B*) DH or (*C*) RSC also did not affect locomotor activity (*top*) or shock reactivity (*middle*). Telenzepine impaired contextual fear conditioning when infused into either region, but AF-DX 116 did not (*bottom*). (*) $P < 0.05$; (**) $P < 0.01$ compared with vehicle.

Retrieval of recently acquired context memory requires DH M_1/M_3 and RSC M_2/M_4 activity

To determine whether mAChR support memory retrieval in addition to memory formation, we infused either scopolamine or vehicle into the DH or RSC prior to a retrieval test (Fig. 3A). After DH ($t_{10.664} = 5.065$, $P < 0.01$) or RSC ($t_{12} = 2.846$, $P < 0.05$) infusion, scopolamine-treated animals froze significantly less than their vehicle-treated controls. We pursued this effect by again utilizing

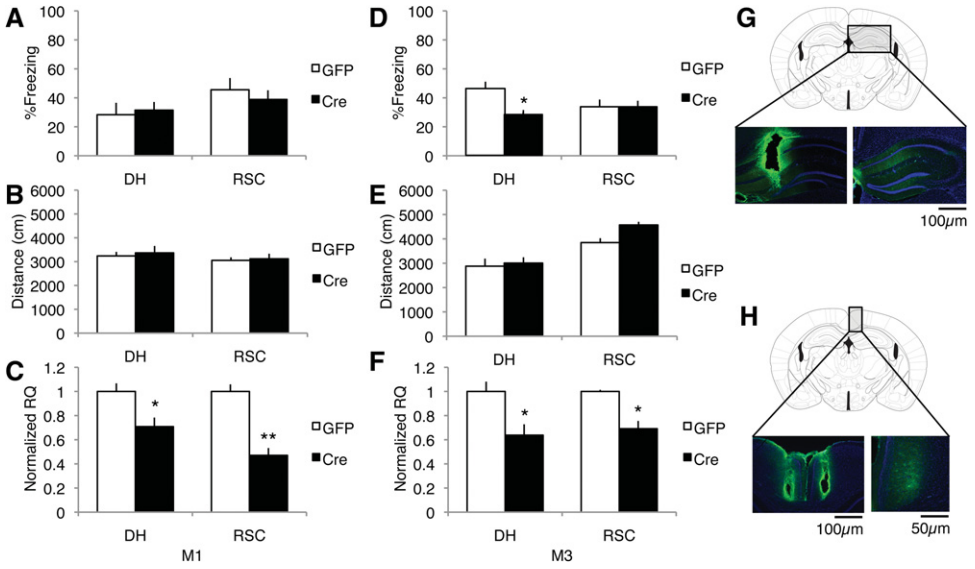

Figure 2. The effects of regional M1 (*A–C*) or M3 (*D–F*) knockdown on contextual fear conditioning. (*A*) Pretraining knockdown of M1 in either DH or RSC had no effect on contextual memory formation. (*B*) Locomotor activity prior to shock and (*C*) M1 mRNA expression in each group. (*D*) Regional M3 knockdown revealed a critical role for DH M3 in memory formation. (*E*) Locomotor activity prior to shock and (*F*) M3 mRNA expression in each group. Cre virus expression is shown in RSC (*G*) and DH (*H*). Representative sections were taken at (*left*) or anterior to (*right*) the site of infusion. GFP and DAPI are pseudocolored green and blue, respectively. (*) $P < 0.05$; (**) $P < 0.01$ compared with GFP.

AF-DX 116 (Fig. 3B) and telenzepine (Fig. 3C) to test the effects of combined M_1/M_3 inactivation or M_2/M_4 inactivation on retrieval in both the DH and RSC. In DH, preretrieval infusion of telenzepine significantly reduced freezing ($t_{14} = 2.169$, $P < 0.05$), whereas infusions AF-DX 116 ($t_{12} = 0.643$, $P = 0.532$) had no effect, compared with respective vehicle controls.

In RSC, however, telenzepine had no effect on retrieval ($t_{14} = 0.171$, $P = 0.866$). Rather, RSC infusion of AF-DX 116 significantly reduced freezing at retrieval ($t_7 = 3.307$, $P < 0.05$). These data indicate that DH uses similar mechanisms for contextual memory formation and retrieval (M_1/M_3), whereas RSC likely uses the M_1/M_3 receptor subtypes for formation, and the M_2/M_4 subtypes for retrieval.

Retrieval of remotely acquired context memories requires RSC M_1–M_4 activity

Given that RSC glutamatergic mechanisms of memory retrieval are retained at remote time points (Corcoran et al. 2011), we were curious as to whether the same was true for RSC muscarinic signaling. To test this possibility, we fear conditioned two groups of mice and tested them for memory retrieval 35 d later, first drug free to ensure the memory had been retained, and then on vehicle or scopolamine the following day (Fig. 4A). We have previously shown that a remote drug-free test does not diminish the role of RSC in memory retrieval during a second test the following day (i.e., the "remote" memory is not made "recent" by the first drug-free remote memory test; Corcoran et al. 2011). This single test is also not sufficient to cause extinction of the freezing response (Huh et al. 2009; Corcoran et al. 2013). Repeated-measures ANOVA indicated significant effects of day ($F_{(1,15)} = 14.417$, $P = 0.002$) and drug ($F_{(1,15)} = 12.445$, $P = 0.003$), and a significant day by drug interaction ($F_{(1,15)} = 10.540$, $P = 0.005$). Pairwise comparisons showed that scopolamine-treated, but not vehicle-treated animals froze significantly less on drug than off drug ($P < 0.01$), suggesting that scopolamine impaired retrieval of the remotely acquired contextual memory. We then carried out a second experiment with the same experimental design, this time utilizing AF-DX 116, and telenzepine (Fig. 4B). Repeated-measures ANOVA indicated significant effects of day ($F_{(1,23)} = 32.545$, $P < 0.01$) and a significant day by drug interaction ($F_{(2,23)} = 3.514$, $P < 0.05$). The overall effect of the drug condition was not significant ($F_{(2,23)} = 1.157$, $P = 0.332$). Subsequent pairwise comparisons indicated that both the AF-DX 116- ($P < 0.01$) and telenzepine-treated groups ($P < 0.01$) showed reduced freezing on drug compared with off drug, whereas the vehicle-treated group did not.

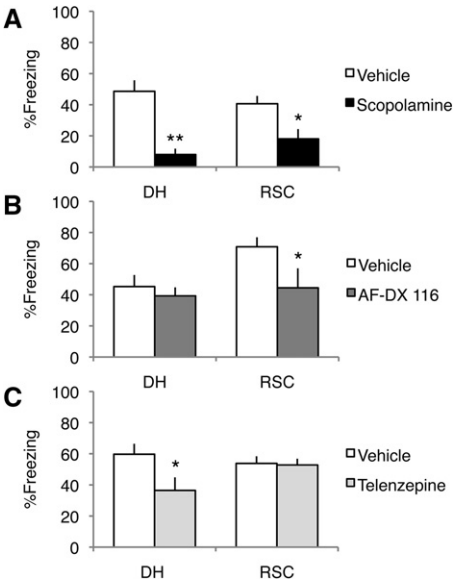

Figure 3. Effects of preretrieval mAChR inhibition in DH or RSC. (*A*) Scopolamine impaired retrieval if delivered into either region. (*B*) AF-DX 116 impaired retrieval if infused into RSC, but not DH, whereas (*C*) telenzepine impaired retrieval if applied to DH, but not RSC. (*) $P < 0.05$; (**) $P < 0.01$ compared with vehicle.

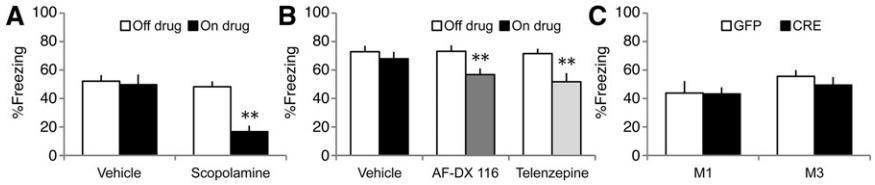

Figure 4. Effects of mAChR manipulations in RSC on remotely acquired memory. Intra-RSC infusion of scopolamine (*A*), AF-DX 116, or telenzepine (*B*) significantly impaired remote memory retrieval. (*C*) Knockdown of neither M1 nor M3 affected remote memory. (**) $P < 0.01$ compared with off-drug test.

Although memory retrieval 1 d after contextual fear conditioning was not impaired in the RSC M_1 and M_3 knockdown mice (Fig. 2), it is possible that a retrieval phenotype could emerge at remote time points. For example, M_1 knockout mice show increased forgetting compared with wild-type controls in a contextual memory test 30 d post-fear conditioning (Anagnostaras et al. 2003). To determine the role of RSC M_1 and M_3 receptors in remote memory, mice were fear conditioned, and then virus was injected into RSC of M_1 and M_3 floxed mice. The mice were then tested along with their respective controls 35 d post-conditioning (Fig. 4C). We found that neither M_1 ($t_{12.41} = 0.064$; $P = 0.950$) nor M_3 ($t_{16} = 0.820$; $P = 0.424$) knockdown in RSC impaired retrieval of remotely acquired memories.

Discussion

With these experiments, we have shown that cholinergic neurotransmission in DH and RSC is required for contextual fear conditioning (via M_1/M_3) and retrieval (via M_1–M_4). Importantly, whereas other researchers have shown that gross lesions or protein synthesis inhibition in the RSC disrupt task performance (Keene and Bucci 2008b; Kwapis et al. 2015), this is the first time that a specific mechanism (acetylcholine signaling) in RSC has been demonstrated to disrupt memory formation. Additionally, the use of conditional, regional knockdown of M_1 and M_3 mAChR subtypes provided novel evidence for their differential involvement in DH versus RSC mechanisms underlying memory. Data from pharmacological and knockdown experiments suggest that in DH, M_3 plays a more prominent role than M_1 in memory formation. The function of these two receptors in DH during memory recall may overlap or compensate for one another, as no deficits in retrieval were observed with DH-M_1 knockdown or post-conditioning DH-M_3 knockdown, even though combined M_1/M_3 antagonism impaired freezing at test. Similarly, in RSC, both M_1 and M_3 were required for contextual memory formation, suggesting that they operate synergistically. Our data also suggest that RSC utilizes a different class (M_2/M_4) of muscarinic receptors than DH (M_1/M_3) in recent retrieval, but that all subunits in RSC are involved in remote retrieval.

Our inferences for the effects of cholinergic drugs on memory were made from analyses of freezing behavior in response to a context paired with footshock (Gale et al. 2001). We previously established, using non-associative (context only) and pseudoconditioned (immediate shock followed by context) control groups, that freezing behavior induced by paired context-shock presentation reflects associative learning (Stanciu et al. 2001; Sananbenesi et al. 2002), with trained mice typically freezing 40%–60% of the time during the context test and non-associative controls freezing 5% or less. Both M1 and M3 floxed strains exhibited somewhat lower freezing than wild-type mice; nonetheless, this was a specific response because they still froze significantly more to the training context than to a novel context (data not shown). Thus, freezing deficits caused by cholinergic manipulations at training were most likely due to interference with associative learning. Pretest manipulations are more difficult to interpret because freezing impairments could be due to direct drug effects unrelated to memory retrieval. Given that a limitation of our study is the unknown within-experiment baseline behavior of the animals (i.e., context only control), it is difficult to provide a definitive argument for memory retrieval relative to alternative interpretations, such as effects on motor activity and expression of freezing. The strongest support for the former comes from our activity data that were automatically collected at training, showing that neither scopolamine, AF-DX 116, nor telenzepine had any effects on locomotor activity (Fig. 1). Similarly, activity was unaffected by the conditional knockdown of M1 and M3 (Fig. 2). We also believe it to be unlikely that general effects would have resulted in region- and memory phase-specific reductions of freezing. Finally, some of the freezing impairments might have been due to state-dependent effects, but that can be ruled out, because the dose of scopolamine used in this study does not produce such effects in the contextual fear conditioning paradigm (Jovasevic et al. 2015). Notably, cholinergic drugs can also interfere with behavior by increasing anxiety (Smythe et al. 1998). Such confound is not likely, however, given that the treated mice exhibited decreased rather than increased freezing behavior.

As for the doses of cholinergic antagonists used in this study, their choice proved to be somewhat challenging because most work on the dorsohippocampal muscarinic mechanisms has so far been performed with rats, and mainly with scopolamine with doses ranging from 1 to 80 μg/hippocampus. The drug has been used over a wide range of doses, and the effects were variable and dependent on the learning paradigm and time of infusion (relative to training or memory testing), so that doses as low as 2 μg/hippocampus had learning impairing effects in some studies (Izquierdo et al. 1992), and doses of 80 μg/hippocampus being ineffective in others (Farr et al. 2000). Conversely, clear impairments with increasing (25 and 50 μg/hippocampus), but not low (5 μg/hippocampus) doses have been found with contextual fear conditioning (Gale et al. 2001; Wallenstein and Vago 2001). We previously showed that 1 μg/hippocampus was sufficient to impair learning (Radulovic et al. 2000); however, this was found in Balb/c mice, which show atypical responses to cholinergic drugs (Messier et al. 1999). We therefore performed pilot studies for each antagonist, and selected the doses based on their ability to affect both fear conditioning and memory retrieval. Given that the selected doses had no side effects (as discussed above) and fall within the range of doses that were earlier characterized in rats, we do not anticipate that the drugs exerted non-specific actions.

The finding that conditional DH M_3 but not M_1 knockdown impaired memory formation was consistent with findings in constitutive M_1 and M_3 knockouts (Miyakawa et al. 2001; Anagnostaras et al. 2003). In the RSC, however, only the M_1/M_3 antagonist telenzepine, but neither M_1 nor M_3 knockdown, impaired learning, indicating that these receptor subtypes have redundant function in memory formation. This redundancy of cortical M_1 and M_3 could be particularly evident when manipulations are relatively long lasting, such as genetic ablation, compared with acute pharmacological interventions. For example, even though systemic administration of the M_1 antagonist dicyclomine impairs contextual fear conditioning (Fornari et al. 2000), and similarly, systemic administration of an M_1 potentiator rescues the effect of scopolamine on contextual fear conditioning (Ma et al.

2009), M1 knockout or null mutant mice show no impairment in contextual fear conditioning (Miyakawa et al. 2001; Anagnostaras et al. 2003). A similar argument may be made for the DH's utilization of these receptors during retrieval. An alternative explanation is that these effects may be mediated by the amygdala or other brain areas (Young and Thomas 2014).

Although much emphasis has been placed on the role of M_1 in learning deficits such as those observed in models of Alzheimer's disease (Medeiros et al. 2011; Puri et al. 2015), our finding that M_3 knockdown in DH had a greater impact on learning than M_1 knockdown suggests a more important role for M_3 in context memory. This is supported by work showing that both M_3 knockout mice and mice with M_3 phosphorylation deficiency have deficits in contextual fear conditioning (Poulin et al. 2010). Interestingly, whereas M_1 rather than M_3 is the predominant mediator of muscarinic potentiation of hippocampal LTP (Anagnostaras et al. 2003; Shinoe et al. 2005; Anisuzzaman et al. 2013; Dennis et al. 2016), a putative physical substrate for learning, M_3 modulates the inhibition of excitatory synaptic transmission in CA1 (de Vin et al. 2015). One route by which M_3 in DH could support learning is by increasing the excitability and intrinsic oscillatory activity of CCK+ interneurons. These neurons may support or drive the hippocampal theta rhythm (Ylinen et al. 1995; Cea-del Rio et al. 2011), which is thought to underlie CS–US associations (Anagnostaras et al. 1999) and encoding of episodic information (Maren et al. 1994; Hasselmo 2005).

The experiments herein do not differentiate between the potential contributions of RSC M_2 and M_4 to memory retrieval, however, based on findings with knockout mice, a more prominent role of M_2 is expected. Mice lacking the M_4 receptor exhibit normal working and long-term memory (Degroot and Nomikos 2006; Koshimizu et al. 2012), although they do have impairments in some social and addiction-like behaviors (de la Cour et al. 2015; Koshimizu et al. 2012; Schmidt et al. 2011). In contrast, M_2 knockout mice have a variety of learning-related phenotypes, such as altered LTP (Seeger et al. 2004; Zheng et al. 2012), deficits in behavioral flexibility during learning tasks (Seeger et al. 2004), impaired working memory, and poor acquisition of a passive avoidance task (Bainbridge et al. 2008). However, they do not display any impairment in cued or contextual fear conditioning (Bainbridge et al. 2008), suggesting that M_2/M_4 coactivation might be critical for the observed effect.

Previous work from our laboratory (Corcoran et al. 2011) showed that RSC NMDAR and, in particular, NR2A-containing receptors are required for retrieval of both recently and remotely acquired memory. The current findings that intra-RSC scopolamine also impaired retrieval of contextual memories regardless of the memory age demonstrates the important contribution of cholinergic signaling. Although we did not perform direct comparisons between the DH and RSC (because the experiments were performed separately, at different times, and in different behavioral rooms, resulting in different freezing levels in vehicle controls), the observed effects point toward a model of muscarinic contribution to retrieval that is multifaceted and non-uniform across brain regions. Unlike other neurotransmitter receptors such as NMDAR (Gao et al. 2010), AMPA receptors (Schiapparelli et al. 2006; Bannerman 2009), adrenergic receptors (Gibbs and Summers 2002; Galeotti et al. 2004), and dopamine receptors (Sarinana et al. 2014; Sarinana and Tonegawa 2016) for which the roles of specific receptor subunits or subtypes are clearly discriminable in various memory processes, it seems that activation of several mAChR subtypes may be necessary to maximally effect one process. This model is consistent with findings using electrophysiological approaches, which demonstrate that cortical M_1, M_2, and M_4 together exert a "triad of effects" (M_1 increases neuronal firing rates, M_2 mediates a decrease in cellular inhibition, and M_4 depresses excitatory transmission), which may underlie attention and learning (Gigout et al. 2012). Together, these effects might allow for the selective activation of neural ensembles required for recall.

The shift from M_1/M_3- to $M_{2/4}$-dependent memory retrieval by RSC might also reflect changes in the neural circuits or contribute to changes in intracellular signaling that occur as memories age. It is thought that memory retrieval initially relies upon hippocampal mechanisms, but over time comes to require a distributed network of cortical sites (Squire et al. 2004). Additionally, retrieval of remote memories for contextual fear conditioning requires the activation of the cAMP-PKA-CREB signaling pathway in RSC, whereas recent retrieval does not (Corcoran et al. 2013). RSC receives inputs from both hippocampus and cortical areas necessary for remote memory retrieval, such as anterior cingulate cortex (Frankland et al. 2004); thus, M_2 and M_4 may become engaged and cAMP-dependent signaling comes online as memories age and cortical inputs to RSC begin to take precedence over hippocampal inputs. Functionally, the diffusion of activity across multiple mAChR in RSC may contribute to the increase in "fuzziness" of memory retrieval that occurs as memories age (Winocur et al. 2010).

A cooperative contribution of mAChR to mnemonic processes is also in line with our pattern of results in RSC which show that as manipulations of mAChR activity became more specific (i.e., from nonselective inhibition by scopolamine to M_1/M_3 inhibition by telenzepine, to conditional knockdown of either M_1 or M_3), the effect on memory grew weaker. The hypothesis that activity of a single mAChR subtype is not as potent as all working in synergy to promote learning and memory processes, supports, counterintuitively, the potential of less selective mAChR-targeting compounds as therapies for memory-related neurological disorders. Although the recent development of highly specific pharmacological tools will help to elucidate the neurobiological function of mAChR subtypes, higher-order functions such as lasting episodic memory may benefit more from drugs with broader mAChR selectivity.

Materials and Methods

Subjects

For pharmacological experiments, wild-type male C57BL/6N mice aged 8–9 wk were obtained from Harlan. Mice having the floxed *CHRM1* or *CHRM3* gene were generated as described previously (Gautam et al. 2006; Kamsler et al. 2010). Both strains were backcrossed to the C57BL/6N background for at least 10 generations. Homozygous floxed (ff) mice of either strain were utilized in experiments beginning at 8–9 wk of age.

All mice were individually housed on a 12-h light–dark cycle and allowed ad libitum access to food and water. All procedures were approved by Northwestern University's Animal Care and Use Committee in compliance with National Institutes of Health standards.

Surgery

Mice were anesthetized with 1.2% Avertin and implanted with double guide cannulas (26 gauge; Plastics One) targeted to either DH (1.7 mm posterior, ±1.0 mm lateral, 2.0 mm ventral to bregma) or RSC (1.8 mm posterior, ±0.4 mm lateral, 0.75 mm ventral to bregma). In previous studies, we have shown that single infusions of pharmacological agents at these coordinates can have profound effects on memory processes (Corcoran et al. 2011, 2013; Leaderbrand et al. 2014; Jovasevic et al. 2015). Cannulas were fixed in place with dental cement, and mice were allowed to recover for at least 72 h prior to behavior experiments or viral infusions. Correct placement was verified after experiments via methylene blue infusion and subsequent examination of thin coronal sections throughout the targeted brain region, unless

tissue was collected for RNA expression or immunohistochemical studies.

Drug and virus infusions

Infusions via cannula were made using 28 gauge injectors that extended 0.5–1.0 mm beyond the guide cannulas. All drug and corresponding vehicle infusions were delivered in a volume of 0.2 (RSC) or 0.25 (DH) μL per side at a rate of 0.6 μL/min. Drug concentrations and diluents were as follows: scopolamine hydrobromide (Tocris), non-specific muscarinic receptor antagonist that does not have known off-target interactions (B Roth and W Kroeze, University of North Carolina, pers. comm.), 50 mg/mL in aCSF (25 μg/DH; 20 μg/RSC); telenzepine dihydrochloride (Tocris), M1/M3 antagonist, 75 mg/mL in aCSF (37.5 μg/DH, 30 μg/RSC); AF-DX 116 (Tocris), M2/M4 antagonist, 4 mg/mL in 50% DMSO (2 μg/DH, 1.6 μg/RSC). To maximize drug efficacy, we used the highest doses for each drug that did not affect locomotion, shock responses, or any other detectable changes of behavior, but was able to block memory processes (pilot data for lower doses not shown). All drugs/vehicles were delivered 30 min prior to fear conditioning or retrieval test.

Viruses were delivered in a volume of 0.4 (RSC) or 0.5 (DH) μL per side at a rate of 0.5 μL/min, and injectors were left in place for 5 min after the end of the viral infusion. The adeno-associated virus expressing the Cre recombinase (Cre) enzyme (AAV2.hSyn.iCre.IRES.GFP.bGH) was obtained from the Penn Vector Core in the School of Medicine Gene Therapy Program at the University of Pennsylvania. One of two control viruses of the same serotype expressing GFP were utilized: rAAV2/TRUFR-eGFP(ssCMV-GFP) from the Gene Therapy Center Vector Core at the University of North Carolina at Chapel Hill, or the AAV2-GFP Control Virus (AAV-302) from Cell Biolabs, Inc. After viral infusions, mice were allowed an interval of 4 wk before any behavioral testing to allow for knockdown of the floxed M_1 or M_3 gene.

We did not use any drug or viral manipulations of M_5 mAChR, because this receptor is not significantly expressed in rodent hippocampus or cortex (Weiner et al. 1990).

Contextual fear conditioning

Fear conditioning was performed in a 35 × 20 × 20 cm Plexiglas chamber with a stainless steel rod floor, housed in a sound-attenuating cabinet, as described previously (Radulovic et al. 1998). Mice were individually placed in the chamber and allowed to explore for 3 min before a 2-sec, 0.8 mA constant current footshock was delivered. Mice were then immediately removed from the chamber and returned 24 h later for a 3-min retrieval test. The chamber was cleaned with 70% ethanol between each mouse.

Memory retrieval was assessed via fear to the chamber, as expressed by freezing behavior. Freezing was defined as the absence of all movement save for respiration, and was scored every 5 sec by a blind observer. Data were expressed as the percentage of the total number of observations that mice spent freezing.

Immunohistochemistry

At the end of behavioral experiments, a subset of virally infused M_1(ff) and M_3(ff) mice were intracardially perfused with ice-cold 4% paraformaldehyde, and their brains were removed and post-fixed for an additional 24 h. Brains were then cryopreserved with 30% sucrose and cryosectioned at 50 μm thickness. Selected coronal sections near the virus infusion site were then used for free-floating immunohistochemistry with a primary antibody against GFP (1:1500, Millipore). Signal amplification was attained with biotinylated secondary antibodies (1:200) and ABC complex (Vector Laboratories), and immunostaining was visualized with fluoresceinisothiocyanate. Sections were then counter-stained with DAPI and mounted in Vectashield (Vector Laboratories). The tissue was then imaged at 5× or 10× magnification with a cooled color charge-couple camera and SPOT software (Diagnostic Instruments) to confirm accurate placement and viral spread (see Fig. 2G–H).

At the end of behavioral experiments, a subset of M_1(ff) and M_3(ff) mice were killed via cervical dislocation and RSC or DH was rapidly dissected on ice. Tissue was immediately frozen over liquid nitrogen and transferred to −80°C until total RNA extraction with the PureLink RNA Mini Kit (Life Technologies). RNA was then subjected to reverse transcription with Taqman reagents (Applied Biosytems) and the resulting cDNA was subjected to real-time PCR using SYBR Green master mix (Applied Biosystems) and primers for either the M_1 or M_3 receptor. The housekeeping gene GAPDH was used as an internal control. RQ values generated by the Applied Biosystems 7300 Sequence Detection Software were compared across Cre- and GFP-treated groups to detect M_1 or M_3 knockdown. The M_1 primers used were 5′-AGT CCC AAC ATC ACC GTC TTG-3′ (forward) and 5′-TCC CGA TGA ATG CCA CTT G-3′ (reverse). The M_3 primers used were 5′-CCT CTT GAA GTG CTG CGT TCT GAC C-3′ (forward) and 5′-TGC CAG GAA GCC AGT CAA GAA TGC-3′ (reverse). The GAPDH primers used were 5′-AAC TTT GGC ATT GTG GAA GG-3′ (forward) and 5′-ACA CAT TGG GGG TAG GAA CA-3′ (reverse).

Data analysis

All statistical analyses were performed using SPSS. Statistical differences were detected by two-tailed independent *t*-tests, paired-samples *t*-test, one-way ANOVA, or repeated-measures ANOVA, as appropriate and indicated in the text. Equality of variances was assessed by Levene's test, and the assumption of sphericity was assessed by Mauchly's sphericity test. Where equal variance and sphericity were violated, fractional degrees of freedom were used to determine significance of the *t*- or *F*-tests. Post hoc comparisons following significant main or interaction effects in the ANOVAs were performed using Tukey's test. Data are presented as mean ± SEM.

Competing interest statement

The authors declare that there are no conflicts of interest regarding this article.

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