

Selective cognitive dysfunction in acetylcholine M₁ muscarinic receptor mutant mice

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Blockade of cholinergic neurotransmission by muscarinic receptor antagonists produces profound deficits in attention and memory. However, the antagonists used in previous studies bind to more than one of the five muscarinic receptor subtypes. Here we examined memory in mice with a null mutation of the gene coding the M₁ receptor, the most densely distributed muscarinic receptor in the hippocampus and forebrain. In contrast with previous studies using nonselective pharmacological antagonists, the M₁ receptor deletion produced a selective phenotype that included both enhancements and deficits in memory. Long-term potentiation (LTP) in response to theta burst stimulation in the hippocampus was also reduced in mutant mice. M₁ null mutant mice showed normal or enhanced memory for tasks that involved matching-to-sample problems, but they were severely impaired in non-matching-to-sample working memory as well as consolidation. Our results suggest that the M₁ receptor is specifically involved in memory processes for which the cortex and hippocampus interact.

Muscarinic, cholinergic receptor blockade produces an array of profound deficits in attentional processing, memory acquisition and memory consolidation. Five muscarinic receptor subtypes and their corresponding genes, termed M₁–M₅, have been identified and cloned, but their high degree of sequence similarity has hindered the development of highly selective ligands¹. Therefore, the broad array of deficits produced by antagonists such as scopolamine or atropine and more selective ligands (for example, pirenzepine or dicyclomine for M₁) could result from action at multiple receptor subtypes^{1–3}.

The M₁ subtype is the most abundant of the muscarinic receptors in the forebrain and hippocampus^{4,5}, and some evidence suggests an important role for M₁ receptors in memory and cognition. M₁ receptors are colocalized with NMDA receptors in hippocampal pyramidal neurons, and coactivation with NMDA receptors results in amplified NMDA currents⁶. Moreover, M₁ receptors are required for muscarinic activation of mitogen-activated protein kinase (MAPK) in the cortex⁷.

Therefore, we examined memory and synaptic plasticity in mice with a targeted null mutation of *Chrm1*, the gene that encodes the M₁ receptor⁸. These mice (M₁^{-/-}) were examined for fear conditioning acquisition and consolidation, spatial working memory on the Olton radial arm maze, spatial reference memory on the Morris watermaze and social discrimination memory, all of which are sensitive to anticholinergic drugs and lesions of the hippocampus^{9–16}. We also examined long-term potentiation (LTP) in the hippocampus¹⁷. Although M₁ mutant mice were

severely impaired on certain tasks, they showed remarkable sparing and even enhancement of performance on others. The present results are not consistent with a general role for M₁ receptors in memory formation or initial stability of memory in the hippocampus. Rather, our findings suggest a complex role in the processing of information that requires interaction between the hippocampus and cortex.

RESULTS

Enhanced contextual fear acquisition

To explore the role of M₁ receptors in memory tasks previously shown to be sensitive to anticholinergic treatment, we began by examining hippocampus-dependent contextual fear conditioning^{10,18,19}. Contextual fear conditioning is severely disrupted by systemic^{9,20} or intra-hippocampal scopolamine^{21,22}. We first examined contextual fear acquisition by a protocol in which mice received one unsignaled shock (with no warning cue) per day for four consecutive days²³. Surprisingly, M₁^{-/-} mice ($n = 12$) showed better context freezing acquisition than wild-type (WT) controls ($n = 12$) (Fig. 1a; main effect of genotype, $F_{1,22} = 22.0$, $P < 0.0001$; group \times day interaction, $F_{3,66} = 16.6$, $P < 0.0001$; day 1, $P > 0.25$; day 2, $P = 0.07$; days 3 and 4, P values < 0.0002). To evaluate whether the enhancement in contextual freezing was due to changes in activity, we examined activity suppression scores (Fig. 1b), which can be used to adjust for differences in baseline activity²⁴. The suppression score is computed as: (activity on test)/(activity on test + baseline activity from day 1 before

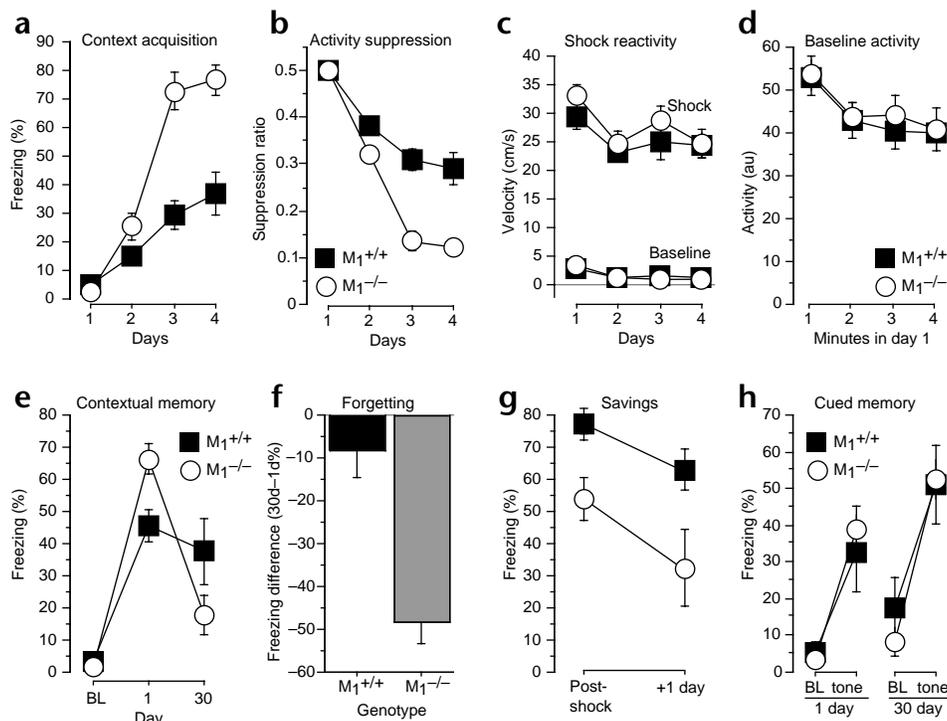


Fig. 1. Pavlovian contextual and cued fear conditioning. **(a)** Context fear acquisition was assessed by giving mice one shock per day 4 min after placement in a conditioning chamber for 4 consecutive days; freezing fear behavior is shown for the 4 min before each shock. $M_1^{-/-}$ mice showed markedly greater context freezing than did WT littermates, especially after day 2. **(b)** Activity suppression (test activity/(baseline activity + test activity)), which controls for any possible differences in baseline activity, is shown for the same days as in **(a)**. **(c)** Shock reactivity (the mouse's velocity during the 2 s when the current was on (shock) compared with the 2 s just before the shock (baseline)) was unaffected in $M_1^{-/-}$ mice across the 4 d of training. **(d)** Baseline activity (arbitrary units based on pixel change scored by computer) during the 4 min before the first shock on the first conditioning day is shown. M_1 mutants did not differ from littermate controls. **(e)** Contextual memory was assessed by giving naive mice two tone–shock pairings after a 2-min baseline period (BL). They were then given contextual memory tests 1 and 30 d after training. $M_1^{-/-}$ mice showed enhanced acquisition, but very unstable memory compared to WT 30 days later. This was not due to extinction, because mice were given additional training at the end of the day-1 testing (Methods). **(f)** Forgetting was assessed as the difference in performance from day 1 to day 30. $M_1^{-/-}$ mice showed drastic forgetting despite their enhanced acquisition. **(g)** Savings was assessed by giving mice two additional unsignaled shocks on day 32, immediately followed by a post-shock freezing test for 4 min, and an additional context test 1 d later. M_1 mutants apparently lost their enhanced memory, as they showed no evidence of savings even after reinstatement by the shock. **(h)** Cued memory was assessed in a novel context 1 d after the 1 and 30 d context memory tests. Mice were placed in a novel context, and after a 2-min baseline period (BL), the training tone was played for 3 min. In contrast to their contextual memory, $M_1^{-/-}$ mice showed normal acquisition and retention of cued memory. Data points depict mean \pm standard error (s.e.m.).

shock)^{23–25}. We found results that were similar to freezing (although, in this case, the day 2 difference was significant, $P = 0.01$). These differences in conditioning were also not due to a change in shock reactivity, measured as a change in velocity in response to shock²³. $M_1^{-/-}$ mice showed shock reactivity equivalent to WT on each of the days tested (Fig. 1c; main effect of genotype for all shock periods, $F_{1,22} = 0.8$, $P > 0.3$; baseline versus shock period, each day $F_{1,22}$ values > 175 , $P < 0.0001$; interaction with genotype, n.s.). There was no group difference in computer-scored activity during the 4-minute baseline period prior to shock on the first conditioning day (Fig. 1d; $F_{1,22} < 0.1$, $P > 0.7$); there was also intact habituation of activity to the context (main effect of minute, $F_{3,66} = 29.7$, $P < 0.0001$; interaction with genotype, n.s.), a process that is impaired after lesions to the hippocampus^{9,10,24,26}. Together, these data indicate a pronounced enhancement in contextual fear conditioning that is not attributable to a change in generalized activity or shock sensitivity.

Impaired contextual memory but normal cued memory

We examined the consolidation of contextual fear conditioning, during a time period when contextual memory becomes indepen-

dent of the hippocampus^{10,18,26}. To determine if the enhancement in contextual fear conditioning was specific to hippocampus-dependent memory, we also investigated hippocampus-independent cued (tone) fear conditioning^{10,18,19}. A naïve group of mice was given two-tone shock pairings, followed by contextual memory testing 1 and 30 days after training (Fig. 1e). There was a significant interaction of day and genotype ($F_{2,22} = 10.6$, $P < 0.001$); mutants ($n = 7$) did not differ from WT ($n = 6$) on baseline freezing ($F_{1,11} = 1.9$, $P > 0.2$), but were enhanced on day 1 ($F_{1,11} = 9.5$, $P = 0.01$) and dropped back to the level of controls (or below) by day 30 ($F_{1,11} = 3.0$, $P = 0.11$). Most striking was the forgetting in M_1 mutants from day 1 to 30. This decrease was not due to extinction because we inserted an additional training trial on day 3 before the 30-day interval (Methods). To generate a forgetting score, we subtracted day 1 performance from that on day 30 (Fig. 1f). Mutants showed a much larger forgetting score than wild-type controls ($F_{1,11} = 27.5$, $P < 0.001$). To ensure that this reflected a true loss of memory rather than a failure of recall, mice were given reinstatement training with two unsignaled shocks that were followed immediately by a post-shock freezing test and then an additional context test one day later (Fig. 1g). $M_1^{-/-}$ mice showed no evidence of their

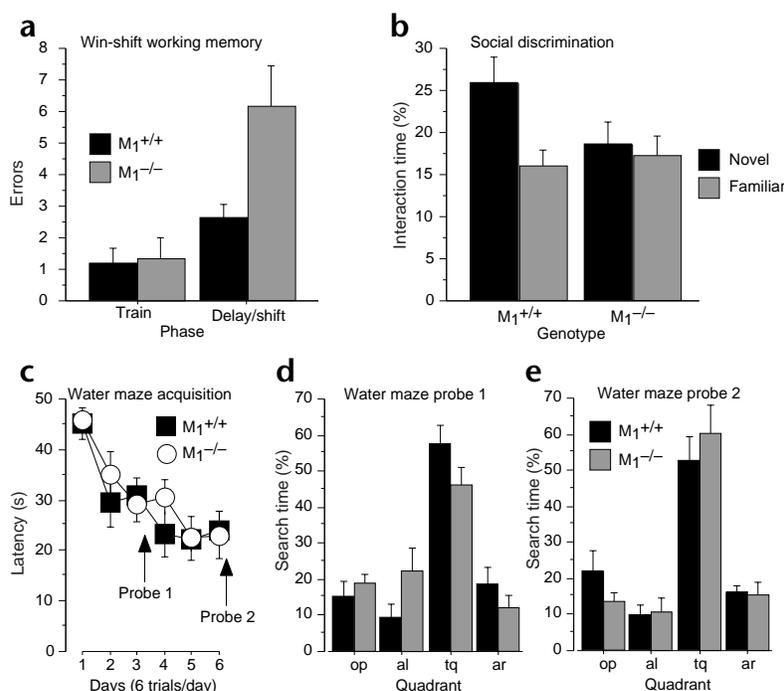


Fig. 2. Win-shift spatial working memory, social discrimination and Morris water maze learning. **(a)** Win-shift working memory, in which mice were trained to search for pellets on an eight-arm radial maze, in a two-phase working memory task. On the training phase of each day, four randomly chosen arms were open and baited. After a 2-min delay, the shifted (not previously baited) arms were baited, and all eight arms were open. This was repeated for 15 d, after which the delay was increased to 60 min for an additional 3 d. Shown here are the average number of errors for the training phase and 60-min delay phase. $M_1^{-/-}$ mice performed normally on the training phase, but were severely impaired on the working memory phase. The mice also tended to be impaired at the 2-min delay (data not plotted). **(b)** Social discrimination memory was assessed by exposing mutant or WT male mice to ovariectomized female mice and assessing interaction time. Subjects were exposed for 4 min to two novel mice, followed by exposure to one novel and one familiar mouse 1 day later. The interaction time (%) with the novel and familiar mouse during this test is shown here. Wild-type mice showed a preference for the novel female, indicating a memory for the familiar female, but M_1 mutants did not. **(c)** Water maze acquisition (six trials per day, 60-s maximum per trial); latency to reach a hidden platform in a fixed spatial location is shown. $M_1^{-/-}$ mice did not differ from controls. **(d)** Probe trial 1, in which the platform was removed at the end of day 3. $M_1^{-/-}$ and WT mice searched selectively during the 30-s probe trial in the target quadrant in the target quadrant (tq) and did not differ. op, opposite; al, adjacent left; ar, adjacent right. **(e)** Probe trial 2, at the end of day 6. Mice searched selectively, and the two groups did not differ. Data points depict mean \pm s.e.m.

previously enhanced acquisition, as they were significantly lower on both savings tests ($F_{1,11} = 9.7$, $P < 0.01$), indicating a failure to reinstate previously enhanced memory²⁵. We also examined cued fear conditioning, in a novel context, one day after each of the first two context tests (on days 2 and 31; Fig. 1h). In both the 1-day and the 30-day memory tests, there were no group differences in freezing ($F_{1,11}$ values < 0.2 , P values > 0.6), and both genotypes showed a large increase in freezing during the tone compared with the baseline period ($F_{1,11}$ values > 33 , P values < 0.0001 ; interaction, n.s.). Taken together, these results reproduce and extend the results of the first experiment: $M_1^{-/-}$ mice showed an enhancement of contextual fear acquisition. It was also important to examine context conditioning under signaled conditions, because it has been suggested that unsignaled context conditioning may be less sensitive to hippocampal lesions²⁷. It should be noted, however, that we and others have found that hippocampal lesions interfere with both instances of context conditioning, particularly when made after training^{23,26,28,29}. Nonetheless, in the present study, equivalent results were found; contextual fear acquisition was enhanced whether under signaled or unsignaled training conditions.

We also found marked forgetting, however, during the time period when contextual memory shifts from a hippocampus-dependent to an independent (and presumed cortical) state^{25,26,30}. Moreover, we found intact hippocampus-independent cued fear conditioning, regardless of the training-to-testing interval.

These results differ from those obtained with anti-muscarinic pharmacological treatment. Scopolamine produces significant hyperactivity in the fear conditioning chambers, severe deficits in contextual fear acquisition⁹ and, to a lesser extent, retrograde amnesia for contextual fear^{20,22}. Likewise, dicyclomine, arguably a selective M_1 antagonist, produces effects similar to scopolamine on fear conditioning². As with most selective muscarinic agents, however, dicyclomine has considerable affinity for other receptor subtypes: it binds M_3 receptors with essentially equivalent affinity as M_1 receptors, and has only one order of magnitude higher affinity for M_1/M_3 compared with M_4/M_5 receptors³. Therefore, the effects of scopolamine or dicyclomine on learning cannot be solely attributed to M_1 receptors. Nevertheless, our data are consistent with a role for M_1 receptors in the acquisition and consolidation of contextual fear conditioning.

Impaired working memory on the radial arm maze

To further explore the memory phenotype of M_1 mutant mice, we examined win-shift spatial working memory (Methods), which is dependent on the hippocampus and prefrontal cortex, and disrupted by anti-muscarinic treatment¹². Compared with WT ($n = 7$), M_1 mutants ($n = 6$) were selectively impaired on the delay (60 minutes) phase, but not on the training phase (Fig. 2a; genotype \times measure interaction, $F_{1,11} = 5.0$, $P < 0.05$; training phase, $F_{1,11} < 0.1$, $P > 0.8$; delay phase, $F_{1,11} = 6.6$, $P < 0.03$).

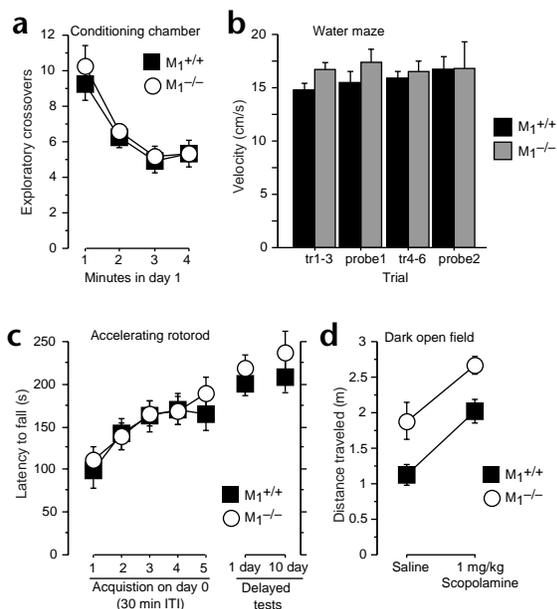


Fig. 3. Generalized activity and motor performance. **(a)** Exploratory activity (full cage crossovers) was assessed in the fear conditioning chambers before any shock (same time period as Fig. 1d). $M_1^{-/-}$ mice did not show hyperactivity in the small, well-lit conditioning chambers. **(b)** Watermaze swim velocity was assessed by computer during the training (tr, training trial days) and probe trials. M_1 mutants showed no evidence of an increase or change in activity. **(c)** Motor skill acquisition and retention were assessed on the accelerating rotarod. Mice were given five training trials (300 s maximum) 30 min apart, and tested again 1 and 10 d afterward. $M_1^{-/-}$ mice showed normal performance and retention of this motor skill. **(d)** Exploratory activity was also assessed on a large, very dark and quiet open field for 5 min, after injection of saline or 1 mg/kg scopolamine HBr. $M_1^{-/-}$ mutants showed hyperactivity on this open field, and remained sensitive to scopolamine-induced hyperactivity. Data points depict mean \pm s.e.m.

$M_1^{-/-}$ mice were also mildly impaired when they were tested at a short 2-minute delay, especially early in training (data not shown, days 1–3, $F_{1,11} = 6.3$, $P < 0.03$; days 4–15, n.s.). The results are consistent with the effects of scopolamine, and they suggest a critical role for M_1 receptors in working memory.

Impaired social discrimination memory

To further explore memory sensitive to nonselective anti-muscarinic agents and hippocampal lesions^{15,16}, we examined 1-day social discrimination memory in WT ($n = 12$) and mutant ($n = 11$) mice (Fig. 2b). Wild-type mice showed significant discrimination ability ($F_{1,11} = 7.4$, $P = 0.02$), whereas mutants did not ($F_{1,10} < 0.2$, $P > 0.7$). These data indicate an essential role for M_1 receptors in social discrimination memory. One notable feature that social discrimination memory has in common with win-shift memory is that both tasks consist of a delayed non-matching-to-sample problem; contextual fear conditioning, in contrast, consists of a match-to-sample problem, and the finding that it was not impaired in mutant mice suggests that this difference may be at the core of deficits in M_1 mutants. As the deficit in M_1 mutants was reflected in less exploration of the novel female rather than more exploration of the familiar female, another possible interpretation of the deficit could indicate diminished attention or exploration in M_1 mutants. Discriminating between these possibilities will require further investigation.

Intact spatial memory on the Morris watermaze

To determine whether non-matching-to-sample problems may be selectively impaired in M_1 mutants, we further examined anticholinergic-sensitive and hippocampus-dependent memory by testing reference spatial memory on the hidden-platform version of the watermaze^{13,14}. This complex task shares similar spatial learning requirements with the win-shift radial arm maze, but consists of a matching, rather than non-matching-to-sample problem. The time taken to find the escape platform (latency) throughout acquisition did not differ between the $M_1^{-/-}$ ($n = 8$) and WT ($n = 8$) (Fig. 2c; $F_{1,14} < 1$, $P > 0.4$). In addition, both groups showed significantly shorter latencies with training ($F_{5,14} = 13.9$, $P < 0.0001$; interaction, n.s.), which indicated that learning had occurred. Unexpectedly, search time in each quadrant also did not differ between mutants and controls during the first probe trial at the end of day 3 (Fig. 2d; $F_{1,14} < 0.9$, $P > 0.3$), and both groups showed a strong preference for the target quadrant (main effect of quadrant, $F_{3,14} = 18.6$, $P < 0.0001$; interaction, n.s.). Similar results were obtained for the second probe trial given at the end of day 6 (Fig. 2d; $F_{1,14} = 2.0$, $P = 0.2$; main effect of quadrant, $F_{3,14} = 19.6$, $P < 0.0001$; interaction, n.s.), demonstrating normal performance in $M_1^{-/-}$ mice. To ensure that over-training did not mask a group difference, we examined cumulative proximity to the hidden platform during training. We found no significant group differences early or late in training (data not shown). We also carried out an additional probe trial 10 days after probe 2, and found good retention in both groups at this training-to-test interval (data not shown, main effect of group, $F_{1,13} < 0.2$, $P > 0.7$; main effect of quadrant, $F_{3,13} = 7.9$, $P < 0.001$; interaction, n.s.). In contrast to win-shift spatial working memory on the radial arm maze, spatial reference memory remained intact in M_1 mutants for this task, which is highly sensitive to non-selective anticholinergic treatment and hippocampal lesions. To determine the contribution of M_1 receptors to amnesia induced by anti-muscarinic treatment, we examined the effects of scopolamine on water-maze performance¹³. This task was chosen because there was no baseline difference in performance between $M_1^{-/-}$ and WT, unlike other tasks. Mice were trained for six trials per day for 3 days to induce relatively weak performance (susceptible to disruption), and were given 1 mg/kg scopolamine (i.p.) or saline (on alternating counterbalanced days) during performance of probe trials on the hidden-platform task. Scopolamine substantially impaired water-maze performance, reducing it to near chance (data not shown; target quadrant time, scopolamine versus saline, $F_{1,15} = 11.3$, $P < 0.01$). $M_1^{-/-}$ mice again did not differ significantly from WT ($F_{1,15} = 2.9$, $P > 0.1$), and scopolamine did not have a differential effect across genotypes (interaction, $F_{1,15} = 0.09$, $P > 0.9$). Indeed, scopolamine produced a nearly identical deficit in both groups, reducing target platform time by about one-third in both mutant and wild-type mice. These data provide further evidence that scopolamine does not produce memory deficits purely by acting through M_1 receptors, as it had the same effect in wild-type mice as it did in mice that lack functional M_1 receptors.

Selectively hyperactive and scopolamine-sensitive

Anticholinergic treatment produces a robust motor hyperactivity that can confound the interpretation of learning deficits⁹. We examined motor activity and performance under a number of conditions to determine the contribution of M_1 receptors to scopolamine-induced hyperactivity. We first assessed exploratory activity in the fear conditioning chambers by measuring ambulatory crossovers before the first shock (Fig. 3a). This measure

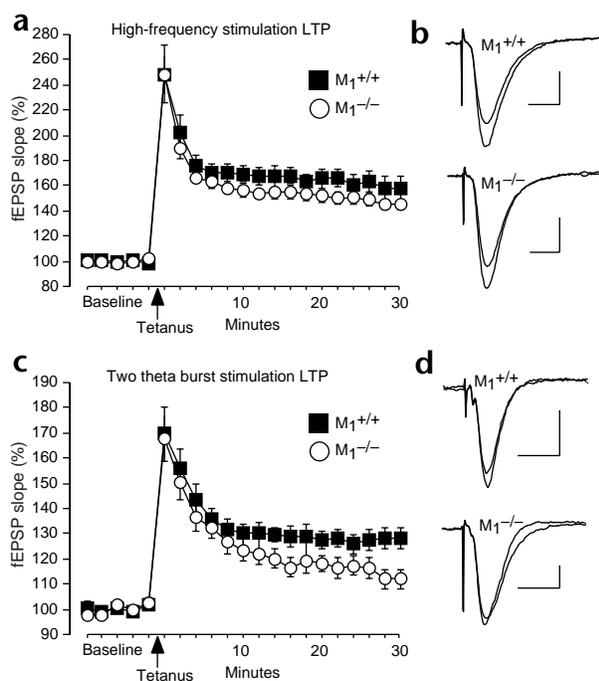


Fig. 4. Hippocampal CA1 long-term potentiation (LTP) *in vitro*. **(a)** After a 10-min baseline period, high-frequency stimulation (100 Hz \times 1 s) was delivered to the Schaffer collateral–CA1 pathway, and LTP was observed for 30 min. LTP was similar in mutants and WT controls. **(b)** Representative recording from area CA1 during the 10-min baseline and after LTP induction by HFS during the last 10 min of recording (average of five traces). **(c)** LTP after two theta-burst stimulation (TBS, two bursts separated by 200 ms, each burst four pulses at 100 Hz) was significantly reduced in $M_1^{-/-}$ mice. **(d)** Representative recording from area CA1 during baseline and after induction of LTP by TBS. Scale bars, 0.25 mV and 10 ms.

was more sensitive to changes in exploratory behavior than that reported in Fig. 1d. Hippocampal lesions and scopolamine administration produce large increases in activity during the same pre-shock period^{9,10,24,26}. During this period, however, $M_1^{-/-}$ mice were not significantly more hyperactive than WT ($F_{1,22} < 0.3$, $P > 0.6$; main effect of habituation $F_{3,66} = 28.0$, $P < 0.0001$; interaction, n.s.). Analogously, activity in the watermaze, measured by swim speed during every acquisition and probe trial (Fig. 3b), did not differ between mutants and WT ($F_{1,14} = 1.3$, $P > 0.2$). Casual observation suggested that unlike scopolamine-treated animals³¹, the $M_1^{-/-}$ mutants showed normal swimming. We also examined motor coordination, which is also affected by scopolamine. $M_1^{-/-}$ ($n = 18$) mice did not differ from WT ($n = 17$) during acquisition of the accelerating rotor-rod task (Fig. 3c; $F_{1,33} < 0.2$, $P > 0.7$; interaction, n.s.), a common test of motor coordination (Methods); the groups also did not differ when tested again 1 day later ($F_{1,33} = 0.5$, $P > 0.4$). Similar results were obtained when a representative subset of these mutants ($n = 11$) and WT ($n = 12$) were tested again 10 days later ($F_{1,21} = 1.1$, $P > 0.3$).

We investigated scopolamine-induced hyperactivity by measuring exploratory activity on a large, dark open field (Methods). On alternate days, we gave animals either saline or 1 mg/kg scopolamine intraperitoneally (i.p.). Fifteen minutes after the injections, they were placed on the open field for a 5-min test. Although M_1 mutants were hyperactive after both saline and scopolamine administration (Fig. 3d; $F_{1,11} = 11.7$, $P < 0.01$), both groups reacted to scopolamine with an equivalent increase in

activity ($F_{1,11} = 25.3$, $P < 0.001$; interaction, n.s.). Therefore, scopolamine-induced hyperactivity must not be mediated through M_1 receptors alone. Moreover, scopolamine produces hyperactivity in conditioning chambers, but these same M_1 mutant mice were not hyperactive when they were subsequently placed in the fear conditioning chambers for 4 min (without drug, $F_{1,11} < 0.3$, $P > 0.6$; data not shown but similar to Fig. 1d). These data indicate that M_1 mutants can be hyperactive under certain conditions, such as during exploration under low anxiety. Consistent with this, M_1 mutants also showed hyperactivity on the radial arm maze. They completed trials faster (both training and delay) than did wild-type mice (main effect of latency on the same days as depicted in Fig. 2a, $F_{1,11} = 6.1$, $P < 0.03$; interaction, n.s.). Hyperactivity was probably not the cause of the increased number of errors in M_1 mutants, as they showed similar hyperactivity on both the training and delay phases, but made more errors only on the delay phase (Fig. 2a).

Taken together, these data indicate that the M_1 mutation causes a hyperactivity that is limited to certain conditions and is milder than scopolamine-induced hyperactivity. It has recently been shown that hyperactivity observed in M_1 mutants originates in disturbed monoamine signaling in the striatum³². The hyperactivity observed in M_1 mutants should not confound the behavioral measures used to assess memory, as the mutants showed normal learning and memory in tasks known to be affected by hyperactivity (but see ref. 33).

Selective impairment in hippocampal LTP

We also examined Schaffer collateral LTP in the hippocampal slice—a cellular model of learning. We first examined LTP after high-frequency stimulation (HFS, 100 Hz \times 1 s). Both WT ($n = 7$ mice) and $M_1^{-/-}$ ($n = 11$) showed significant LTP (Fig. 4a and b; average of 10 min baseline versus average of last 10 min; WT, $F_{1,6} = 76.2$, $P = 0.0001$; mutant, $F_{1,10} = 101.2$, $P < 0.0001$), and its magnitude did not differ between mutants and WT (last 10 min, $F_{1,16} = 2.2$, $P > 0.15$). We followed LTP in a subset of mutant and wild-type mice for up to 70 min after induction. LTP remained robust and did not differ across genotype (data not shown). We further examined LTP after two theta burst stimulations (TBS, two bursts 200 ms apart, each burst 4 pulses at 100 Hz). This stimulation protocol is thought to mimic endogenous physiological activity induced by cholinergic activation during exploration (Fig. 4c and d)¹⁷. Under these more physiologically relevant conditions, the LTP induced by TBS in mutants is significantly less than in WT ($F_{1,20} = 4.5$, $P < 0.05$). Nevertheless, both WT ($n = 9$) and $M_1^{-/-}$ ($n = 13$) mice showed significant LTP (WT, $F_{1,8} = 59.1$, $P < 0.0001$; mutant, $F_{1,12} = 18.8$, $P = 0.001$). We followed a subset of animals for up to 60 min after TBS (data not shown). No further decline in LTP was observed after 30 min, suggesting the deficit observed in $M_1^{-/-}$ is maximal by 30 min. We also found no group difference in basal synaptic transmission (input-output function, data not shown). These data suggest a modest role for M_1 receptors in synaptic plasticity.

DISCUSSION

A notable finding of the present study is that contextual fear memory, win-shift spatial working memory, social discrimination and watermaze spatial reference memory, which are thought to depend on similar processes in the hippocampus^{26,34}, were found to be dissociable by the M_1 null mutation. These mice showed enhanced context conditioning, severely impaired win-shift and social discrimination learning, and normal watermaze learning. Most molecular studies of memory have focused on

general disruptions of the acquisition or maintenance of information within a specific structure^{35–37}, most often the hippocampus, implying commonality of the molecular substrates subserving different forms of hippocampus-dependent memory. The M_1 mutants, however, showed changes in memory function that cannot be characterized as broad alterations in either the acquisition or maintenance of information in a neural system. Our findings also indicate that the profound memory acquisition deficits produced by scopolamine or atropine treatment cannot be solely attributed to blockade of M_1 receptors².

Another recent study³³ suggests that M_1 receptor knockout mice are not globally impaired on hippocampus-dependent tasks (but see ref. 38). We did not find global deficits in hippocampus-dependent tasks, either. Instead, an intriguing pattern of spared function, deficits and enhancement emerged clustered around tasks that are hippocampus-dependent. Accordingly, electrophysiological analysis showed only mild deficits in hippocampal synaptic plasticity. Notably, the behavioral abnormalities were mnemonic in nature and were dissociable from a hyperactivity that was observed only under limited conditions.

Contextual fear conditioning was initially enhanced, and hidden-platform watermaze learning was normal in the M_1 mutants. Both of these tasks are considered reference memory tasks—they require only the use of invariant trial-independent information, and can be thought of as a matching-to-sample problem that can be computationally solved with a Hebbian-like generalization process across trials³⁹. In contrast, win-shift working memory, also dependent on the hippocampus, was severely impaired. This task requires working memory and the use of trial-independent information, and consists of a non-matching-to-sample computational problem that potentially requires the buildup of inhibition against generalization^{12,39–41}. This buildup of inhibition against matching to previously visited arms could, in theory, require a more complex, hierarchical computational mechanism in addition to Hebbian-like generalization processes⁴¹. Indeed, a recently described computational model for matching versus non-matching⁴² is consistent with many of the dissociations we found in M_1 mutants. This model, based on electrophysiological data, proposes a hippocampal-cortical circuit that could mediate both match inhibition and non-match enhancement. The dissociations found in the behavioral phenotype of the $M_1^{-/-}$ mice are consistent with this model, suggesting that the critical mnemonic role of M_1 receptor signaling may lie in this circuit.

In general, non-matching and working memory tasks, such as win-shift radial arm maze learning or object discrimination, require the prefrontal cortex, whereas the matching tasks do not, perhaps because of the differential need for working memory and inhibition^{41,43,44}. Therefore, the selective deficit of M_1 mutants in both of the non-matching-to-sample tasks that we examined (win-shift and social discrimination) may suggest prefrontal or other cortical deficiency. Indeed, during the delay phase only, $M_1^{-/-}$ mice tended to revisit previously baited (that is, match) arms at a very high rate, rather than making within-trial or random errors (data not shown), a pattern sometimes observed after prefrontal lesions^{41,43}. Moreover, contextual fear conditioning, which was initially enhanced, showed a deficit in consolidation over the time period when it becomes hippocampus-independent and presumably stored in the neocortex^{10,18,25,26,30}. These data suggest that M_1 mutant mice have cortical memory dysfunction, or impaired hippocampal-cortical interaction, that is specific to working memory and remote reference memory.

Our findings indicate that M_1 receptors are not general modulators of NMDA receptor-dependent coincidence detection⁶, as

previously suggested by the effects of anticholinergic treatment^{2,9,22}. A more complex view of cholinergic function in memory processing has been suggested in network models^{42,45,46} in which the cholinergic signal functions as a switch between inflow (encoding or write-in) and outflow (recall or write-out) modes of the hippocampus. Furthermore, these models suggest how the cholinergic signal may help coordinate the hippocampal role in acquiring recent memory and the cortical role in storing remote memory⁴⁵. High cholinergic activation, such as during exploration when theta rhythm is present, sets the appropriate hippocampal dynamics for inflow of information by suppressing feedback connections, both within the hippocampus and between the hippocampus and the association cortex. This prevents both the spread of information within the hippocampus and hippocampal retrieval from distorting existing representations in the cortex. At low cholinergic activation, such as during slow-wave sleep when hippocampal sharp waves are present, there is a release from cholinergic suppression. This permits outflow of activity along feedback connections, both within the hippocampus and to the cortex. During a theta stage, the model predicts strong encoding of new representations in the hippocampus, with little interference from and disruption to remote memories in the cortex. During a sharp-wave stage, strong repetition and spread of recently acquired traces become consolidated and integrated with permanent representations in the cortex.

This model offers a framework that may help to interpret the complex behavioral phenotype of the M_1 mutants: M_1 mutant mice may be biased to a hippocampus-inflow mode, perhaps because M_1 activation is required to bias processing away from the hippocampus and to the cortex. This could explain the deficit in cortex-dependent working memory and consolidation of remote memory because the loss of the M_1 receptors would bias processing away from the cortex. The model may also explain the enhancement in the acquisition of contextual memories in the M_1 mutants because in these mice, contextual memories would be solely dependent on hippocampal processing and free of possible interference from previously established cortical traces⁴⁵. Accordingly, the mutants are particularly adept at solving hippocampus-dependent matching-to-sample problems, but are deficient in non-matching and show impaired consolidation, both functions that require hippocampal-cortical interaction^{41,44}. A complementary model⁴² suggests that the entorhinal cortex could be a site for match suppression and non-match enhancement. Nonetheless, both types of problems involve hippocampal processing; however, because acquisition of recent memory versus recall of remote memory, or processing matching versus non-matching memory problems, seem to require incongruous processing by the hippocampus, there must be mechanisms by which the hippocampus can shift between these processing modes. The activation of M_1 receptors may be one of these mechanisms. Further investigation will be required to test this hypothesis, and many other interpretations are plausible, but it is now apparent that a fairly complex cognitive model will be necessary to account for the multifaceted phenotype we observed in M_1 mutant mice.

Our data suggest that M_1 receptors are important in cortical memory function and in the interaction between the cortex and the hippocampus, rather than being required for memory acquisition by the hippocampus *per se*. These data also indicate that the broader effects of nonselective muscarinic antagonists are almost certainly attributable to action at multiple receptor subtypes. Recent data suggest that M_3 and M_5 receptors may be important in activating septohippocampal neurons and in establishing theta rhythm⁴⁷. Therefore, a full characterization of mice that are deficient in other muscarinic receptor subtypes will be useful in understanding the precise roles of each receptor in mem-

ory formation. Further study is also required to investigate differences in muscarinic receptor localization between mice and rats, and also between rodents and humans. Nevertheless, M_1 receptors are the most abundant in the hippocampus and cortex of all three species^{8,48,49}. The present results indicate an important role in memory for M_1 receptors, and suggest that they remain a valid therapeutic site for memory disorders, especially Alzheimer's disease (AD). Indeed, M_1 receptor immunoreactivity is lower both in the hippocampus and surrounding cortex in the brains of patients with AD⁴⁸, and the memory deficits associated with AD probably involve both the hippocampus and cortex⁵⁰.

In recent years, molecular manipulations have offered important insights into the processes of memory acquisition and maintenance^{35–37}. The present findings show that molecular manipulations can also reveal complex cognitive phenotypes that offer compelling insights into the interaction of multiple systems involved in memory.

METHODS

Animals. The generation of mice lacking M_1 receptors by homologous recombination has been previously described⁸. Mice were originally generated from 129SvJ embryonic stem (ES) cells and crossed with C57Bl/6 mice at the University of Washington (Seattle, Washington). These were backcrossed five generations onto the C57Bl/6 background to generate nearly pure C57Bl/6 heterozygous mice. These were crossed to generate littermate wild-type and mutant mice, and shipped to UCLA (Los Angeles, California), where they were used in the present studies. Genotyping was by Southern blot and/or polymerase chain reaction (PCR). All experiments were done blind with respect to genotype and were conducted with the approval of the UCLA Animal Research Committee of the Chancellor's Office of Protection of Research Subjects, under continuous supervision of the campus veterinarian.

Fear conditioning: context acquisition. Mice were placed into a novel conditioning chamber, and after a 4-min baseline period, received a shock (2 s, 0.75 mA). After an additional minute, they were returned to their home cages. This was repeated for four days. Freezing and activity data were collected by computer during the 4-min period before the shock on each day to form an acquisition curve. Shock reactivity on each conditioning day, and ambulatory crossovers during the 4-min period before the shock on the first day, were also assessed. The apparatus and these procedures have been described elsewhere²³.

Fear conditioning: consolidation and cued conditioning. Mice were placed into a novel conditioning chamber and, after 2 min, were given two tone-shock pairings (tone: 30 s, 85 dB, 2.8 kHz; shock: 2 s, 0.75 mA) separated by 1 min each. After an additional minute, they were returned to their home cages. One day later, they were returned to the fear conditioning chambers for a 4-min context fear test (1-d test). The next day, they were brought to a novel environment and, after a 2-min baseline period, the training tone was presented for 3 min. We also wanted to re-test these mice after a 30-day interval, while avoiding extinction from these initial tests. Therefore, 1 d later, we returned them to the original training context, and the mice received an additional tone-shock pairing (as before). Thirty d later, the mice were again given context and tone tests on separate days as before.

Olton win-shift radial arm maze. Mice were food-deprived to 85% of their free-feeding body weight. They were pre-exposed to eight 20-mg rodent chow pellets in novel chambers in the same food cups that would be found on the radial maze, until they consumed all pellets. Mice were then given 15 d of win-shift training and testing on an eight-arm radial maze¹². Each day consisted of two phases. In the training phase, four randomly selected arms were baited and open. Mice had 5 min to retrieve all pellets. An error was defined as a re-entry into a previously baited arm. After retrieval of the pellets, the mouse was placed in a holding cage, and after a 2-min delay, was returned for the testing phase, in which all eight arms were now open,

and in which only the four previously unbaited arms were now baited. An error was defined as a re-entry into a previously baited arm, either between or within-phase. After 15 d with a 2-min interphase delay, mice were tested an additional 3 d with a 60-min interphase delay.

Morris watermaze. The basic protocol for the watermaze experiments has been described elsewhere^{25,37}. Our pool is made from white nylon, is 1.2 m in diameter and has an 11-cm diameter platform submerged 1 cm below the water surface. The water is made opaque with white non-toxic paint and maintained at 27 °C. Mouse position was tracked by computer (VPS118, HVS Image, UK). Mice were given six training trials per day (3 blocks of 2 trials, each block separated by approximately 1 h), from a random start location, with a hidden platform in a fixed location. For each training trial, mice had a maximum of 60 s swim time to mount the platform (mice were placed manually if 60 s elapsed), and they were then allowed to rest on the platform for an additional 20 s. At the end of days 3 and 6, mice were given a 60-s probe trial in which the platform had been removed. In a separate experiment, naive mice were tested for scopolamine-induced memory deficits. After 3 d of training as above, mice were given 1 mg/kg scopolamine HBr, i.p. or saline 20 min before a probe trial in which the platform had been removed. All mice received scopolamine and saline in counterbalanced order on two probe trial days, each trial 1-d apart.

Social discrimination. Male mutant or WT mice were placed in a clean acrylic cage, and after 4 min, two novel ovariectomized female Swiss-Webster mice were placed in the cage for 4 min. One day later, one novel and one familiar female were introduced into the cage for a 1-d memory test. Interaction time, defined as the male mouse's nose touching each female, was time-sampled by observers blind to genotype.

Open field testing. Fifteen minutes after receiving an i.p. saline injection, mice were placed for 4 min into a square open field (60 × 60 cm) made of white acrylic and illuminated only by indirect light provided by an overhead red 25-W bulb. One day later, mice received 1 mg/kg scopolamine HBr, i.p., and 15 min later were placed on the open field for an additional 4 min. Quiet (65 dB) background noise was provided by an air cleaner for both tests. Mouse position was tracked by computer.

Accelerating rotorod. For each trial, mice were placed on an accelerating rotorod, which accelerated from 4 to 40 rpm over 5 min. Latency to fall was recorded by the automated apparatus. Mice were given 5 trials of acquisition, with each trial separated by 30 min. They were also re-tested again 1 and 10 d later to assess memory for this motor skill.

Hippocampal LTP. Recordings were made using transverse hippocampal slices (400 μm thick) in a submerged recording chamber perfused (2 ml/min) with artificial cerebrospinal fluid containing 120 mM NaCl, 3.5 mM KCl, 2.5 mM CaCl₂, 1.3 mM MgSO₄, 1.25 mM NaH₂PO₄, 26 mM NaHCO₃ and 10 mM D-glucose at 31 °C. Extracellular excitatory postsynaptic field potentials (EPSPs) were recorded in CA1 stratum radiatum with a platinum-iridium recording electrode. EPSPs were evoked in separate pathways within the Schaffer collateral/commissural afferents with 100 ms test pulses via two stimulating electrodes placed one on either side of the recording electrode (~300 μm from the recording electrode). Test pulses were alternated each minute between the two electrodes throughout the duration of the experiment. After a 10-min baseline period, LTP was induced with a single tetanus delivered to one pathway (the test pathway), according to a high-frequency stimulation (HFS) protocol (100 Hz for 1 s) or two-theta burst stimulation (TBS) protocol (two bursts, each burst 4 pulses at 100 Hz, 200 ms inter-burst interval). The untargeted pathway served as a control pathway. Slices in which there was significant drift in the control pathway were excluded from further analysis. When multiple slices were used from a single animal, data were averaged and then entered into analysis as a single subject. Thus, all data reported reflect individual mice rather than individual slices.

Statistics. Data were entered into a general multivariate analysis of variance (MANOVA). After a significant omnibus comparison, pairwise com-

parisons were made using the Wald test. For brevity, nonsignificant (n.s.) interactions or other uninformative effects are omitted from text.

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Competing interests statement

The authors declare that they have no competing financial interests.

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