Behavioral/Systems/Cognitive

M_2 Muscarinic Acetylcholine Receptor Knock-Out Mice Show Deficits in Behavioral Flexibility, Working Memory, and Hippocampal Plasticity

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Muscarinic acetylcholine receptors are known to play key roles in facilitating cognitive processes. However, the specific roles of the individual muscarinic receptor subtypes (M_1–M_5) in learning and memory are not well understood at present. In the present study, we used wild-type ($M2^{+/+}$) and M_2 receptor-deficient ($M2^{-/-}$) mice to examine the potential role of M_2 receptors in learning and memory and hippocampal synaptic plasticity. $M2^{-/-}$ mice showed significant deficits in behavioral flexibility and working memory in the Barnes circular maze and the T-maze delayed alternation tests, respectively. The behavioral deficits of $M2^{-/-}$ mice were associated with profound changes in neuronal plasticity studied at the Schaffer–CA1 synapse of hippocampal slices. Strikingly, short-term potentiation (STP) was abolished, and long-term potentiation (LTP) was drastically reduced after high-frequency stimulation of $M2^{-/-}$ hippocampi. Treatment of $M2^{-/-}$ hippocampal slices with the $GABA_A$ receptor antagonist, bicuculline, restored STP and significantly increased LTP. Whole-cell recordings from CA1 pyramidal cells demonstrated a much stronger disinhibition of GABAergic than glutamatergic transmission in $M2^{-/-}$ hippocampi, which was particularly prominent during stimulus trains. Increased strength of GABAergic inhibition is thus a likely mechanism underlying the impaired synaptic plasticity observed with $M2^{-/-}$ hippocampi. Moreover, the persistent enhancement of excitatory synaptic transmission in CA1 pyramidal cells induced by the transient application of a low concentration of a muscarinic agonist (referred to as LTP_m) was totally abolished in $M2^{-/-}$ mice. Because impaired muscarinic cholinergic neurotransmission is associated with Alzheimer's disease and normal aging processes, these findings should be of considerable therapeutic relevance.

Key words: **acetylcholine; hippocampus; knock-out mice; learning and memory; long-term potentiation; muscarinic receptors; synaptic plasticity**

Introduction

Muscarinic acetylcholine (ACh) receptors are known to play central roles in facilitating cognitive functions (Bartus et al., 1982; Coyle et al., 1983; Fibiger et al., 1991; Iversen, 1997). Various lines of evidence, including lesion experiments and studies involving the central administration of muscarinic antagonists, indicate that muscarinic receptors located in the hippocampus are of particular importance for learning and memory processes (Bartus et al., 1982; Fibiger et al., 1991; Iversen, 1997).

The muscarinic receptor family (M_1–M_5) consists of five molecularly distinct subtypes, all of which are expressed in the hippocampus (Vilaro et al., 1993; Levey et al., 1995). The potential roles of the individual muscarinic receptors in learning and memory are not well understood at present, primarily because of the lack of ligands endowed with a high degree of receptor subtype selectivity (Caulfield and Birdsall, 1998).

Pharmacological evidence suggests that activation of M_1 muscarinic receptors may play a critical role in mediating the cognition-enhancing effects of acetylcholine (Coyle et al., 1983; Mash et al., 1985; Quirion et al., 1989). However, studies with M_1 receptor mutant mice revealed that the lack of M_1 receptors was not associated with major cognitive deficits in different hippocampus-dependent learning tasks (Miyakawa et al., 2001), indicative of a key role of non-M_1 muscarinic receptors in learning and memory.

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M_2 receptors, like M_1 receptors, are expressed in the hippocampus (both presynaptically and postsynaptically) and most other brain regions implicated in learning and memory processes (Levey, 1993; Levey et al., 1995; Rouse et al., 1997). Pharmacological studies using different M_2 receptor-preferring antagonists have led to contradictory results regarding the potential role of the M_2 receptor subtype in cognition. Whereas some studies suggested that blockade of central M_2 receptors enhances learning and memory in various experimental settings (Quirion et al., 1995; Carey et al., 2001), other investigators arrived at the opposite conclusion (Messer and Miller, 1988; Daniel and Dohanich, 2001).

In the present study, we used M_2 receptor-deficient ($M2^{-/-}$) mice (Gomeza et al., 1999) as novel experimental tools to investigate the potential role of the M_2 receptor subtype in cognition and hippocampal plasticity. Specifically, we subjected $M2^{+/+}$ and $M2^{-/-}$ mice to a series of behavioral tests, including two different hippocampus-dependent spatial learning and memory tasks, the Barnes circular maze test (Barnes, 1979, 1988; Bach et al., 1995) and the T-maze delayed alternation task (Crawley, 1999). Moreover, because gene disruption studies in mice have established a good correlation between impaired spatial learning and memory and reduced long-term potentiation (LTP) in the Schaffer collateral pathway to the hippocampal CA1 region (for review see Chen and Tonegawa, 1997; Silva et al., 1997; Mayford and Kandel, 1999), we also examined whether $M2^{-/-}$ mice showed activity-dependent changes in neuronal plasticity at the Schaffer collateral–CA1 synapse.

We found that $M2^{-/-}$ mice showed significant deficits in behavioral flexibility and working memory associated with pronounced impairments in neuronal plasticity at the Schaffer collateral–CA1 synapse. These new findings provide clear evidence that M_2 muscarinic receptors play an important role in mediating specific aspects of the cognition-enhancing actions of acetylcholine.

Materials and Methods

Animals. The M_2 muscarinic receptor gene was inactivated in mice by using standard gene-targeting techniques (Gomeza et al., 1999). $M2^{+/+}$ and $M2^{-/-}$ mice (genetic background, 129′J1′/CF1; 50/50%) were generated as described previously (Gomeza et al., 1999). Mouse genotyping was performed by PCR analysis of mouse tail DNA. All experiments were performed with adult male mice.

General behavioral studies. All behavioral studies, including the learning and memory tests (see below), were performed between 9:00 A.M. and 5:00 P.M. using adult male mice that were at least 8 weeks of age.

To assess spontaneous locomotor activity, mice were placed into the center of an open-field apparatus (40 × 40 cm; Columbus Instruments, Columbus, OH) under dim lighting. Motor activity parameters (distance traveled, number of vertical and stereotypic movements, ambulating and resting times) were then monitored and recorded over a 30 min period.

To study motor coordination and balance, mice were placed on an accelerating rotarod (drum diameter, 3 cm; Columbus Instruments), and the time each animal was able to maintain its balance on the rod was measured. The speed of the rotarod accelerated from 4 to 40 rpm over a 5 min test period.

The apparatus used for the light–dark transition test consisted of a cage (25 × 40 × 20 cm) equally divided into two by a black partition containing a small opening. One chamber was made of white plastic and was brightly illuminated, whereas the other chamber was black and dark. Mice were placed into the lit compartment and allowed to move freely between the two chambers for 5 min. The number of transitions between the two compartments, time spent in each chamber, and latency until the first transition were recorded.

The elevated plus-maze consisted of two open (45 × 5 cm) and two enclosed arms of the same size, with walls 15 cm high. The arms were constructed of black acrylic radiating from a central platform (5 × 5 cm) to form a plus sign. The entire apparatus was elevated to a height of 30 cm above floor level. Each mouse was placed in the central platform facing one of the open arms. The number of entries into the open and closed arms and the time spent on the open and closed arms were recorded during a 5 min test period.

Barnes circular maze test. The Barnes circular maze apparatus used was similar to the one described by Bach et al. (1995). The maze consisted of a white acrylic disc (122 cm in diameter) that was elevated 90 cm above the floor and contained 40 holes, each 5 cm in diameter, equally spaced around the perimeter of the circle. One of the holes led to a black Plexiglas escape tunnel (5 × 5 × 11 cm).

To familiarize mice with the maze and the existence of the escape tunnel, they were subjected to two habituation sessions on two consecutive days (one session per day). The position of the tunnel was varied randomly from mouse to mouse but remained constant throughout testing for a given mouse. Each mouse was placed in the middle of the maze under a start chamber (a square black box), and a buzzer (80 dB) and light were turned on. After 10 sec, the chamber was lifted and the mouse was guided to the escape tunnel. When the mouse entered the escape tunnel, the buzzer and light were turned off, and the mouse was allowed to remain in the tunnel for 1 min. On the following 18 d, actual test trials (one trial per day) were performed under identical conditions, except that the mice needed to locate the escape tunnel by themselves. Each trial ended when the mouse entered the goal tunnel or after 5 min had elapsed. The amount of time that the mice took to enter the tunnel (escape latency) and the number of errors (defined by the animal placing its nose in a hole that did not lead to the escape chamber) were recorded for each trial.

One month after the last training trial, the mice were retested to evaluate memory retention. The position of the target hole was the same as during the training period. One week after memory retention testing, the escape tunnel was moved to a new position opposite of the original (reversal learning). Mice were then subjected to five consecutive trials to locate the new position of the escape hole using the same procedure as described above.

T-maze delayed alternation task. The delayed alternation test was conducted in a black plastic T-maze (stem, 38 × 9 cm; arms, 30 × 9 cm; walls, 15 cm high). Sliding doors separated the first 14 cm of the stem as the starting compartment, and the arms from the stem 10 cm from the intersection. The end of each arm contained a small black plastic cup (1 cm in diameter) into which a food reward could be placed. A variety of fixed extramaze clues surrounded the apparatus.

Mice were kept on a maintenance diet throughout the course of all T-maze experiments. This diet resulted in a weight loss of ~10% after 2 d and ~15% after the first week of dieting. This reduced weight was maintained throughout all subsequent experiments. After 2 d of dieting, animals were subjected to three 10 min adaptation sessions (one session per day for three consecutive days), during which they were allowed to freely explore the T-maze with all doors open and both arms baited with food (pieces of Froot Loop cereal; Kellogg's, Battle Creek, MI). On the day after the last adaptation session, mice were subjected to a forced alternation protocol for five consecutive days (one session consisting of 11 trials per day; cutoff time, 10 min). The mice were forced (by blocking access to the previously visited arm) to visit one arm at the time, eat the food reward, and return to the starting compartment. Mice were confined to the starting compartment for 5 sec between trials. For the initial trial, both arms were baited with food. The door leading into the straight alley was opened, and the mouse was able to freely choose either the right or left arm of the T-maze and consume the food reward. The mouse was then allowed to return to the starting compartment. In the subsequent trial, the previously visited arm was blocked so that the mouse was forced to choose the previously nonvisited arm containing a food reward. This forced alternation procedure was then repeated nine additional times.

Actual training sessions commenced 1 d after the last forced alternation session. In this case, animals were subjected to daily sessions (one session per day) consisting of 11 continuous trials (one initial trial followed by 10 test trials). On the first trial, both arms were baited. After the

mouse had chosen one arm and eaten the food reward, the door to the unvisited arm was closed. The mouse was allowed to return to the starting compartment alone and was confined there for 5 sec. During the following 10 trials, the food reward was always located in the arm not visited in the previous trial. A correct choice was made if the mouse entered the previously unvisited arm. The baited arm remained the same until visited, even if the mouse chose the incorrect arm repeatedly. These daily training sessions were continued for 18 d (intertrial interval, 5 sec).

To set higher demands on working memory, the delay time (intertrial interval) was increased from 5 to 20 sec. Approximately 1 month after completion of the 5 sec task, the same set of animals was subjected for 10 consecutive days to the same training protocol as described above.

The T-maze was cleaned with mild detergent and water between trials. The number (percentage) of correct choices (alternation rate) was recorded.

Electrophysiology. Using standard procedures, transverse hippocampal slices (350–400 μm thick) were prepared from the brain of adult male mice (1–3 months of age). All procedures were performed according to the guidelines and with the approval of the Animal Care Committees at the Universities of Munich and Kiel. After dissection, slices were incubated in modified artificial CSF (ACSF) in which $MgCl_2$ was elevated to 3.5 mM and $CaCl_2$ was reduced to 0.5 mM for at least 2 hr before they were transferred to the recording chamber. Slices for field potential recordings were kept at room temperature (21–24°C), whereas slices for whole-cell recordings were initially incubated in warmed (35°C) ACSF for 20 min and then maintained at room temperature in the same solution. In the storage and recording chambers, slices were kept submerged, and ACSF was constantly gassed with 95% O_2-5% CO_2. Normal ACSF contained the following (in mM): 125 NaCl, 3 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 1.25 NaH_2PO4, 25 $NaHCO_3$, 10 D-glucose, pH 7.4. For electrophysiological measurements, individual slices were transferred to the recording chamber that was mounted on the stage of an upright microscope (Zeiss Axioskop; Zeiss, Thornwood, NY). ACSF was constantly exchanged by means of a gravity-driven superfusion system (flow rate, 2–3 ml/min) during experiments. All measurements were performed at room temperature, except for LTP_m recordings, which were performed at 30–32°C. Dodt infrared gradient contrast in conjunction with a contrast-enhanced CCD camera (Hamamatsu, Shizouka, Japan) were used to identify pyramidal cells in the hippocampal CA1 region.

Field EPSPs (fEPSPs) were evoked by means of a concentric bipolar electrode placed in stratum radiatum to stimulate the Schaffer collateral–commissural pathway and recorded with a glass micropipette filled with 200 mM NaCl (tip resistance, 1–2 MΩ), which was placed in CA1 stratum radiatum. Before and after theta burst stimulation (TBS), constant-current pulses (pulse width, 50 μsec) were delivered at 0.05 Hz. The strength of afferent stimulation was adjusted to evoke fEPSP amplitudes, which were ~40% of the maximum response under control conditions. The mean stimulus strength used to evoke the baseline response did not significantly vary between $M2^{+/+}$ hippocampi (0.53 ± 0.15 mA; $n = 35$ slices from 18 mice) and $M2^{-/-}$ hippocampi (0.52 ± 0.13 mA; $n = 28$ slices from 17 mice). TBS consisted of 15 bursts of 4 pulses at 100 Hz, delivered at an interburst interval of 200 msec. Field potentials were recorded and amplified using a bridge amplifier (SEC1L; npi, Tamm, Germany) in conjunction with a Digidata 1200 interface and pClamp 6 software (Axon Instruments, Foster City, CA). Extracellular signals were low-pass filtered at 1.5 kHz and sampled at 5 kHz. The slope of fEPSPs was determined using the Clampfit program of pClamp 8 software.

Whole-cell patch pipettes for EPSC recordings were filled with the following (in mM): 130 K-gluconate, 3 $MgCl_2$, 5 EGTA, 5 HEPES, 2 Na_2-ATP, 0.3 Na-GTP, pH 7.25–7.30. For recordings of IPSCs, CsCl was substituted for K-gluconate, and lidocaine *N*-ethyl bromide (5 mM) was included in the pipette solution. The electrode resistance ranged from 3 to 5 MΩ when filled with internal solution. Series resistance in the whole-cell configuration was ~10–20 MΩ, which was compensated by 70–85%. Synaptic currents were recorded at −70 mV after correcting for liquid junction potentials. Constant current pulses (pulse width, 0.1 msec) of 50–200 μA were delivered at 0.05 Hz to a stimulating electrode located in stratum radiatum to evoke synaptic responses in CA1 pyramidal cells. EPSCs and IPSCs were pharmacologically isolated by perfusing the slices with the $GABA_A$ receptor antagonist bicuculline methiodide (30 μM) and the ionotropic glutamate receptor antagonist kynurenic acid (2 mM), respectively. Whole-cell currents were filtered at 1 kHz and sampled at 10 kHz using an Axopatch 200 amplifier in conjunction with a Digidata 1200 interface and pClamp 9 software (all from Axon Instruments).

All drugs were purchased from Sigma (Deisenhofen, Germany) and added to the bathing medium at known concentration. Data are expressed as means ± SEM. Statistical comparisons of data were performed using Student's *t* test.

Results

General behavioral evaluation of $M2^{-/-}$ mice

$M2^{-/-}$ mice (10–12 weeks of age) did not differ significantly from wild-type control animals ($M2^{+/+}$) in overall health and appearance, body weight ($M2^{+/+}$, 37.4 ± 1.1 gm; $M2^{-/-}$, 38.2 ± 1.8 gm), and core body temperature ($M2^{+/+}$, 37.2 ± 0.1°C, $n = 24$; $M2^{-/-}$, 37.1 ± 0.2°C, $n = 25$). Moreover, the lack of M_2 receptors had no significant effect on spontaneous behaviors in an empty cage or on sensory-motor reflexes (eye blink, ear twitch, whisker twitch, righting reflex, and wire hang test) (Crawley and Paylor, 1997) (data not shown).

To examine spontaneous locomotor activity and response to a novel environment, $M2^{+/+}$and $M2^{-/-}$ mice were assayed in an open-field test. These studies showed that $M2^{-/-}$ mutant mice did not differ from $M2^{+/+}$ control animals in their locomotor and exploratory behavior (distance traveled in 30 min: $M2^{+/+}$, 2957 ± 309 cm, $n = 11$; $M2^{-/-}$, 3227 ± 363 cm, $n = 13$). Similarly, $M2^{-/-}$ mice performed equally well as $M2^{+/+}$ control mice in the rotarod test (data not shown), indicative of normal motor coordination.

To study whether the lack of M_2 receptors affected anxiety-related behaviors, $M2^{+/+}$ and $M2^{-/-}$ mice were subjected to the light–dark transition and elevated plus maze tests. In the light–dark transition test, $M2^{+/+}$ and $M2^{-/-}$ mice ($n = 15$ per group) showed similar numbers of transitions ($M2^{+/+}$, 11.3 ± 1.4; $M2^{-/-}$, 10.8 ± 1.5), total time spent in the lit compartment ($M2^{+/+}$, 68.2 ± 5.3 sec; $M2^{-/-}$, 60.1 ± 5.2 sec), and latency to the first transition ($M2^{+/+}$, 53 ± 14 sec; $M2^{-/-}$, 48 ± 14 sec). Likewise, $M2^{+/+}$ and $M2^{-/-}$ mice ($n = 15$ per group) did not differ significantly in the percentage of total time spent on the open arms in the elevated plus maze test ($M2^{+/+}$, 34.9 ± 6.4%; $M2^{-/-}$, 25.8 ± 5.1%) and in the number of open-arm entries ($M2^{+/+}$, 3.4 ± 0.6; $M2^{-/-}$, 3.5 ± 1.2).

Performance in the Barnes circular maze test

We next studied the performance of $M2^{+/+}$ and $M2^{-/-}$ mice in the Barnes circular maze test, a hippocampus-dependent cognitive task that requires spatial reference memory (Barnes, 1979, 1988; Bach et al., 1995). This task is similar to the Morris water maze in that both tests require an escape response. However, the Barnes maze may offer the advantage that it is less stressful and physically less taxing than the Morris water maze (Barnes, 1979, 1988; Bach et al., 1995), a significant factor given the relatively poor swimming ability of mice.

Figure 1 shows that both $M2^{+/+}$ ($n = 9$) and $M2^{-/-}$ ($n = 11$) mice learned to locate the escape hole during the course of the training period (days 1–18), as indicated by a progressive reduction in escape latencies and errors rates (errors were defined as visits to any nontarget hole). However, during the first 5 d of training, $M2^{-/-}$ mice did not perform as well as $M2^{+/+}$ mice, as indicated by a significant increase in latencies to enter the escape hole ($p < 0.05$; two-way ANOVA followed by Bonferroni's *post hoc* comparison matrix) (Fig. 1*A*). Moreover, $M2^{-/-}$ mice made

significantly more errors during this period ($p < 0.01$; two-way ANOVA followed by Bonferroni's *post hoc* comparison matrix) (Fig. 1*B*). However, on subsequent training days (days 6–18), $M2^{-/-}$ mice showed similar escape latencies and error rates as the $M2^{+/+}$ control animals, and all mice met criterion (three errors or less on five consecutive days) by the end of training (Fig. 1).

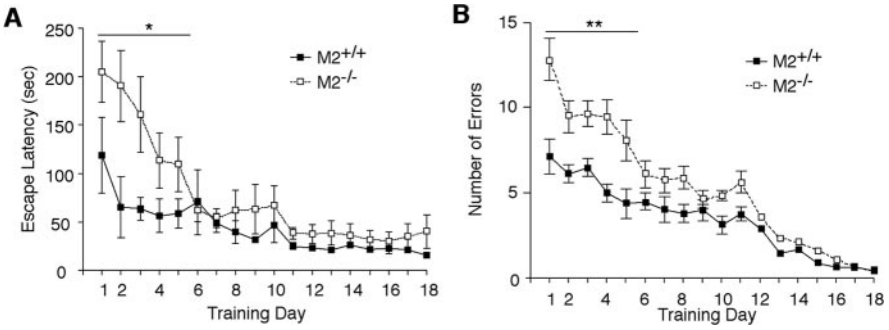

Figure 1. $M2^{-/-}$ mice show performance deficits during the first week of testing in the Barnes circular maze. *A*, Escape latencies. During training days 1–5, $M2^{-/-}$ mice took significantly more time than $M2^{+/+}$ mice to locate the escape tunnel (significantly different from $M2^{+/+}$ mice, $*p < 0.05$; two-way ANOVA, Bonferroni's *post hoc* comparison matrix). The escape latencies significantly decreased across training days in both $M2^{-/-}$ and $M2^{+/+}$ mice ($p < 0.01$; two-way ANOVA). *B*, Error rates. During days 1–5, $M2^{-/-}$ mice made significantly more errors (defined as visits to any nontarget hole) than $M2^{+/+}$ mice (significantly different from $M2^{+/+}$ mice, $**p < 0.01$; two-way ANOVA, Bonferroni's *post hoc* comparison matrix). The number of errors decreased across training days in both $M2^{-/-}$ and $M2^{+/+}$ mice ($p < 0.01$; two-way ANOVA). Values are given as means ± SEM ($M2^{+/+}$, $n = 9$; $M2^{-/-}$, $n = 11$).

During the first week of testing, $M2^{+/+}$ and $M2^{-/-}$ mice used either random or serial search strategies to locate the escape tunnel (Bach et al., 1995). Mice using a random search strategy explored many holes in an unsystematic manner, with many center crossings and perseverations (repeated visits to the same hole or two adjacent holes). In contrast, mice using a more efficient serial search strategy moved to the perimeter and then explored consecutive holes, either in a clockwise or a counterclockwise manner. Interestingly, during the first 5 d of training, a considerably smaller percentage of $M2^{-/-}$ mice (compared with $M2^{+/+}$ mice) used the more efficient serial search strategy to locate the escape tunnel (Table 1). Such differences were no longer observed during subsequent training sessions (data not shown). As described in Materials and Methods, mice were subjected to two habituation sessions on two consecutive days before the beginning of the actual test trials. The observation that a larger percentage of $M2^{+/+}$ mice (compared with $M2^{-/-}$ mice) used the more efficient serial search strategy to locate the escape tunnel at the beginning of the test trials may therefore be attributable to the pre-exposure of the mice to the maze in the habituation sessions.

Table 1. Percentage of $M2^{+/+}$ and $M2^{-/-}$ mice using a serial search strategy to locate the escape tunnel during the first 5 d of training in the Barnes circular maze

	Day				
	1	2	3	4	5
$M2^{+/+}$	33	44	67	56	78
$M2^{-/-}$	18	27	36	36	55

Barnes maze experiments were performed as described in Materials and Methods. Mice that did not use a serial search strategy used a random search strategy (Barnes, 1979; Bach et al., 1995).

To evaluate memory retention, mice were retested in the Barnes maze 1 month after the last training trial. During retention testing, $M2^{+/+}$ ($n = 9$) and $M2^{-/-}$ ($n = 11$) mice did not differ significantly in escape latencies ($M2^{+/+}$, 35.2 ± 8.7 sec; $M2^{-/-}$, 34.5 ± 7.0 sec) and in the number of errors made before finding the target hole ($M2^{+/+}$, 4.2 ± 1.3; $M2^{-/-}$, 2.5 ± 1.1).

One week after memory retention testing, the escape tunnel was moved to a new position (opposite of the original), and mice were retrained in five consecutive trials to find the new location of the escape tunnel (reversal learning). As shown in Figure 2*A*, $M2^{-/-}$ mice required approximately twice as much time as $M2^{+/+}$ mice to locate the new position of the escape hole ($p < 0.005$; *t* test). This increase in escape latencies correlated well with the significantly higher number of perseverations (defined as repeated visits to the original hole or one of the two adjacent holes) displayed by the $M2^{-/-}$ mice ($p < 0.005$; *t* test) (Fig. 2*B*).

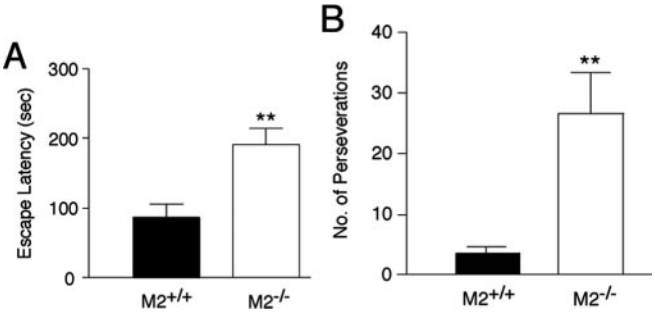

Figure 2. $M2^{-/-}$ mice are impaired in reversal learning in the Barnes circular maze. *A*, Escape latencies during reversal learning in the Barnes circular maze test. For reversal learning experiments, the escape tunnel was moved to a new position (opposite of the original; for details, see Materials and Methods). Mice were then examined for their ability to locate the new position of the escape hole. $M2^{-/-}$ mice showed significantly longer escape latencies than $M2^{+/+}$ mice ($**p < 0.005$; *t* test). *B*, Number (No.) of perseverations. $M2^{-/-}$ mice showed significantly more perseverations (defined as repeated visits to the original hole or one of the two adjacent holes) than $M2^{+/+}$ mice during reversal learning ($**p < 0.005$; *t* test). Values represent the means ± SEM of five consecutive trials ($M2^{+/+}$, $n = 9$; $M2^{-/-}$, $n = 11$).

Performance in the T-maze delayed alternation task

To examine whether the lack of M_2 receptors was associated with changes in spatial working memory, $M2^{+/+}$ ($n = 14$) and $M2^{-/-}$ ($n = 15$) mice were subjected to a T-maze delayed alternation task (Crawley, 1999). This test was chosen because previous studies had demonstrated that systemic or central administration of the non-subtype-selective muscarinic antagonist, scopolamine (Bartolini et al., 1992; Givens and Olton, 1995), or disruption of septohippocampal pathways (Jeltsch et al., 1994) reduces choice accuracy in different versions of the T-maze test.

T-maze experiments were performed with food-restricted mice, using food pellets placed at the ends of the two arms of the T-maze for food reinforcement. Mice were subjected to daily training sessions consisting of 10 consecutive test trials in which mice had to remember the location of the previously visited arm to obtain a food reward. Test trials were performed using two different delay times (intertrial intervals), 5 and 20 sec.

During training with 5 sec delay, $M2^{+/+}$ and $M2^{-/-}$ mice gradually improved their performance over the 18 d training period ($p < 0.01$ compared with day 1; two-way ANOVA followed by Bonferroni's *post hoc* comparison matrix) (Fig. 3*A*). The two groups did not differ significantly in the number of correct choices made throughout the entire training period.

To increase the difficulty of the task, the delay period was

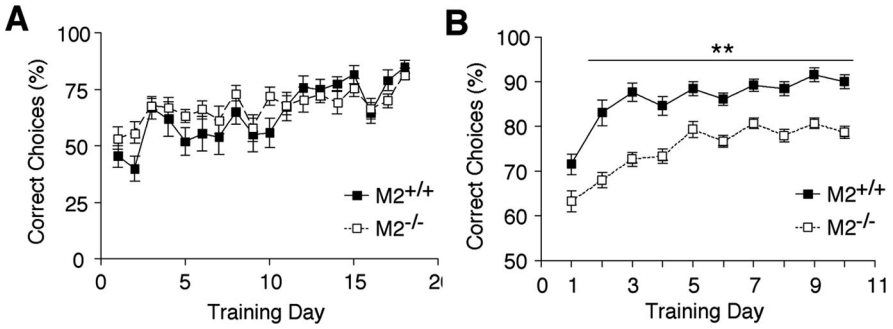

Figure 3. $M2^{-/-}$ mice show deficits in a T-maze test (spatial delayed alternation). T-maze experiments (spatial delayed alternation task) were performed as described in Materials and Methods, either with a 5 sec (*A*) or 20 sec (*B*) intertrial interval. In sessions with a 5 sec delay, there was no significant difference in the percentage of correct arm choices made by $M2^{+/+}$ and $M2^{-/-}$ mice. In contrast, in sessions with a 20 sec delay, $M2^{-/-}$ mice made significantly fewer correct choices than $M2^{+/+}$ mice (significantly different from $M2^{+/+}$ mice, $^{**}p < 0.01$; two-way ANOVA, Bonferroni's *post hoc* comparison matrix). Values represent the percentage of correct choices per session (maximum possible number of correct choices, 10). Data are given as means ± SEM ($M2^{+/+}$, $n = 14$; $M2^{-/-}$, $n = 15$).

increased from 5 to 20 sec. Under these conditions, both $M2^{+/+}$ and $M2^{-/-}$ mice were again able to gradually improve their performance during the course of the training period (Fig. 3*B*). However, on any day of training, $M2^{-/-}$ mice made significantly fewer correct choices than $M2^{+/+}$ mice ($p < 0.01$; two-way ANOVA followed by Bonferroni's *post hoc* comparison matrix) (Fig. 3*B*). The improved performance of the $M2^{+/+}$ mice in the task with 20 sec delay, compared with the task with 5 sec delay, may be attributable to a practice effect caused by the previous exposure of the mice to the T-maze in the 5 sec task (see Materials and Methods).

Muscarinic LTP is absent in hippocampi from $M2^{-/-}$ mice

We next examined whether the cognitive deficits displayed by the $M2^{-/-}$ mice were associated with changes in neuronal plasticity at the Schaffer collateral–CA1 synapse. The Schaffer collateral–commissural pathway was electrically stimulated using hippocampal slices from $M2^{+/+}$ and $M2^{-/-}$ mice, and fEPSPs were recorded in the stratum radiatum of the CA1 region. When a second stimulus was delivered 40 msec after the first stimulus, paired-pulse facilitation (expressed as slope $fEPSP_2$/slope $fEPSP_1$) was not significantly different between $M2^{+/+}$ and $M2^{-/-}$ hippocampi ($M2^{+/+}$, 1.54 ± 0.09, $n = 6$ slices from three mice; $M2^{-/-}$, 1.49 ± 0.17; $n = 6$ slices from four mice), suggesting proper functioning of this synapse in both preparations.

One possible muscarinic mechanism promoting hippocampal synaptic plasticity is the so-called muscarinic LTP (LTP_m), which is characterized by a long-term enhancement of excitatory transmission in CA1 pyramidal cells after muscarinic receptor activation (Auerbach and Segal, 1994, 1996). LTP_m does not require trains of electrical stimuli and is independent of fast GABAergic inhibition (Auerbach and Segal, 1994, 1996). To study whether LTP_m required the presence of functional M_2 receptors, we incubated $M2^{+/+}$ and $M2^{-/-}$ hippocampi with a low concentration of the muscarinic agonist, carbachol (CCh) (0.5 μM). At higher CCh concentrations, LTP_m can no longer be observed, probably because of the activation of multiple muscarinic receptors (Auerbach and Segal, 1996). In $M2^{+/+}$ preparations, CCh (0.5 μM) treatment led to a gradually developing, prominent enhancement of excitatory transmission that persisted after wash-out of CCh (Fig. 4), consistent with previous studies performed in the rat (Auerbach and Segal, 1994, 1996). The mean increase in fEPSP slopes 40 min after wash-out of CCh was 94 ± 6.8% ($n = 9$ slices from four mice). In striking contrast, CCh (0.5 μM) was unable to induce LTP_m in $M2^{-/-}$ hippocampi ($n = 9$ slices from six mice) (Fig. 4), clearly indicating that this form of synaptic plasticity is dependent on the activation of M_2 receptors.

Short-term potentiation is abolished and long-term potentiation is greatly impaired in hippocampi from $M2^{-/-}$ mice

To study muscarinic modulation of short-term potentiation (STP) and LTP at the Schaffer–CA1 synapse, we used a TBS protocol, which mimics the physiological pattern of high-frequency afferent excitatory input into CA1 pyramidal cells (Larson et al., 1986; Costa et al., 2002). As illustrated in Figure 5, TBS reliably produced long-term facilitation of excitatory transmission in $M2^{+/+}$ hippocampi. Two distinct phases could be distinguished, STP and LTP. The massive, immediate STP (Fig. 5*B*, trace 2) declined within ~10 min after TBS to reach the stable plateau of LTP (Fig. 5*B*, trace 3).

In striking contrast, TBS was unable to induce the characteristic sequence of STP–LTP in $M2^{-/-}$ hippocampal slices. The initial potentiation (STP) was entirely missing in $M2^{-/-}$ preparations (Fig. 5*B*, trace 2). Instead, the slope of fEPSPs slowly increased within the first 10 min after TBS before reaching a steady-state level. In addition to the lack of STP, LTP was significantly attenuated in $M2^{-/-}$ relative to $M2^{+/+}$ hippocampal slice preparations (Fig. 5*B*, trace 3). In $M2^{+/+}$ hippocampi, fEPSP slopes were enhanced by 56.2 ± 5.9% ($n = 9$ slices from five mice) 30 min after TBS. In contrast, $M2^{-/-}$ hippocampi displayed an increase of only 20.3 ± 4.6% ($n = 8$ slices from five mice; $p < 0.01$) at the same time point.

One possible explanation for the deficits in hippocampal plasticity displayed by the $M2^{-/-}$ mice is that TBS fails to induce STP and robust LTP unless M_2 receptors are concurrently activated by endogenously released acetylcholine. To test this hypothesis, we examined STP and LTP in $M2^{+/+}$ hippocampi superfused with the M_2 receptor-preferring antagonist, gallamine (20 μM). Strikingly, when applied in the presence of gallamine, TBS was no longer capable of inducing STP in $M2^{+/+}$ hippocampi (Fig. 6*A*). In addition, gallamine treatment led to a significant reduction in LTP (Fig. 6*A*). The slope of fEPSPs 30 min after TBS was enhanced by only 21.2 ± 3.2% in gallamine-treated $M2^{+/+}$ hippocampi ($n = 12$ slices from six mice), which was not significantly different from the small LTP remaining in $M2^{-/-}$ hippocampi (see above) (Fig. 5*B*). A second TBS applied after wash-out of gallamine produced STP and robust LTP (Fig. 6*A*, right section), indicating that transient blockade of M_2 receptors in $M2^{+/+}$ hippocampi is sufficient to mimic the diminished neuronal plasticity observed in $M2^{-/-}$ hippocampi.

During the course of these experiments, we noted that application of gallamine (20 μM) alone augmented control fEPSPs by ~25% in $M2^{+/+}$ hippocampal preparations (Fig. 6*A*). In contrast, gallamine failed to produce a similar effect in $M2^{-/-}$ hippocampi (Fig. 6*B*) ($n = 5$ slices from three mice), suggesting that ambient acetylcholine (Descarries et al., 1997) acting on M_2 muscarinic receptors dampens excitatory transmission at the Schaffer–CA1 pyramidal cell synapse under basal conditions.

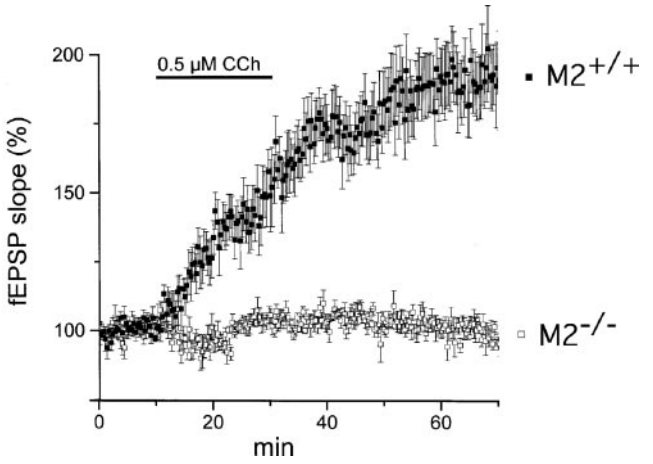

Figure 4. Carbachol-induced LTP (LTP_m) is absent in hippocampal slices from $M2^{-/-}$ mice. Superfusion of $M2^{+/+}$ hippocampal slices (filled squares; $n = 9$) with a low concentration (0.5 μM) of CCh for 20 min induced a pronounced enhancement of fEPSPs (LTP_m; filled squares; $n = 9$). In contrast, the same protocol failed to evoke LTP_m in $M2^{-/-}$ hippocampi (open squares; $n = 9$).

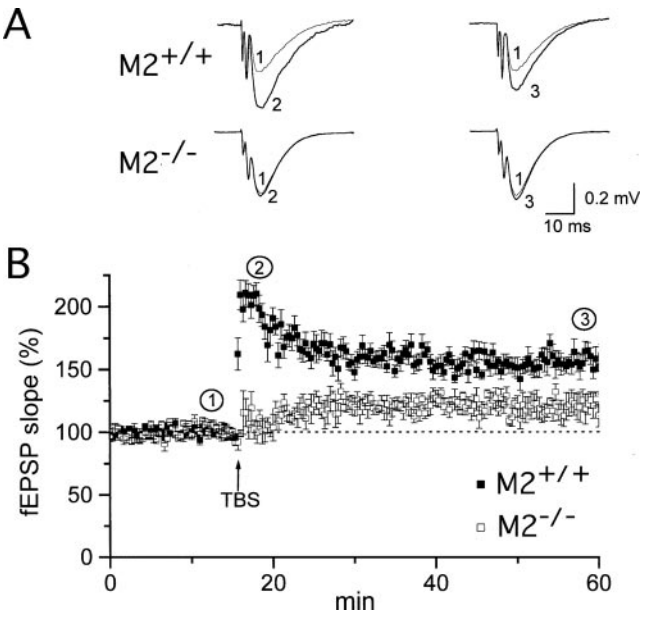

Figure 5. STP is absent, and LTP is greatly reduced in hippocampal slices from $M2^{-/-}$ mice. *A*, fEPSPs were recorded in CA1 stratum radiatum of $M2^{+/+}$ and $M2^{-/-}$ hippocampi before TBS (trace 1), in the initial phase of STP (trace 2), and after induction of LTP (trace 3). The illustrated recordings were taken at the like-numbered time points indicated in *B*. Each trace is an average of four sweeps. *B*, A comparison of TBS-induced changes in the slope of fEPSPs between $M2^{+/+}$ (filled squares) and $M2^{-/-}$ (open squares) hippocampi demonstrates the lack of STP and impaired induction of LTP in $M2^{-/-}$ mice. fEPSPs slopes were normalized to 100% before TBS. Under control conditions, fEPSP slopes did not differ between $M2^{+/+}$ (0.194 ± 0.033 mV/msec; $n = 9$) and $M2^{-/-}$ (0.188 ± 0.022 mV/msec; $n = 8$; $p > 0.7$) hippocampal preparations.

Consistent with this observation, acetylcholine has been shown to inhibit small, low-frequency EPSPs in CA1 pyramidal cells by activating postsynaptic M_2 receptors coupled to a G-protein-dependent, inwardly rectifying K^+ current (Seeger and Alzheimer, 2001). Together, these findings support the concept that M_2 receptor activity facilitates LTP of strong excitatory (behaviorally relevant) inputs at the CA1 region, while relegating small, less significant signals to background noise.

Bicuculline restores STP and robust LTP in hippocampi from $M2^{-/-}$ mice

Previous studies have shown that activation of hippocampal muscarinic receptors can disinhibit hippocampal pyramidal cells by reducing GABA release from interneurons (Ben Ari et al., 1981; Behrends and ten Bruggencate, 1993). We speculated that this effect might be mediated by M_2 receptors, offering a potential mechanism for the reduction in synaptic plasticity observed in the $M2^{-/-}$ hippocampal slices. To test this hypothesis, we measured TBS-induced STP and LTP after superfusion of $M2^{+/+}$ and $M2^{-/-}$ hippocampal slices with the $GABA_A$ receptor antagonist bicuculline (10 μM). As shown in Figure 7*B*, $GABA_A$ receptor blockade fully restored STP and allowed the development of robust LTP in $M2^{-/-}$ hippocampi ($n = 6$ slices from three mice). Although bicuculline also enhanced LTP in $M2^{+/+}$ hippocampal preparations (Fig. 7*A*) ($n = 5$ slices from three mice), the relative increase in LTP (compared with control) was significantly larger in $M2^{-/-}$ than in $M2^{+/+}$ hippocampi ($M2^{-/-}$, +33.6 ± 4.1%; $M2^{+/+}$, +20.7 ± 4.4%; $p < 0.05$) (Fig. 7*C*). The bicuculline-induced increase of fEPSPs before TBS was not significantly different between $M2^{+/+}$ and $M2^{-/-}$ hippocampi, suggesting that the pronounced LTP-enhancing effects of bicuculline observed with $M2^{-/-}$ hippocampi were not caused by nonspecific disinhibition of the slice preparation.

M_2 receptor activation controls inhibitory synapses more effectively than excitatory synapses

The fEPSP recordings suggested that M_2 receptor-mediated disinhibition of hippocampal pyramidal neurons is essential for the induction of STP and the development of robust LTP. To further corroborate this concept and to gain insight into the role of M_2 receptors in normal and high-frequency excitatory and inhibitory transmission at the synaptic level of individual neurons, we used whole-cell recordings from visually identified CA1 pyramidal cells in $M2^{+/+}$ and $M2^{-/-}$ hippocampi. After pharmacological isolation (see Materials and Methods), EPSCs or IPSCs were evoked by electrical stimulation of the Schaffer collateral–commissural pathway. We first determined the input–output (I-O) relationship for EPSCs and IPSCs by plotting the normalized current response as a function of the stimulus intensity. As shown in Figure 8*A*, the I-O curves for EPSCs were virtually indistinguishable between $M2^{+/+}$ and $M2^{-/-}$ hippocampal CA1 pyramidal cells. Also, the amplitudes of EPSCs at maximal stimulus intensity did not differ significantly between pyramidal cells from $M2^{+/+}$ (721 ± 104 pA; $n = 8$ slices from four mice) and $M2^{-/-}$ hippocampi (934 ± 204 pA; $n = 7$ slices from three mice). The I-O curves for IPSCs showed a moderate leftward shift in $M2^{-/-}$ compared with $M2^{+/+}$ hippocampi, which, however, did not reach statistical significance (Fig. 8*B*). Again, maximal IPSC responses did not differ between the two groups ($M2^{+/+}$, 1988 ± 215 pA, $n = 8$ slices from five mice; $M2^{-/-}$, 1811 ± 327 pA, $n = 8$ slices from three mice).

Because multiple muscarinic receptor subtypes are expressed in the hippocampus, we next investigated how critical M_2 receptors are in controlling excitatory and inhibitory synaptic transmission. To address this issue, we used the paired-pulse stimulation protocol shown in Figure 9*A* and *D*, using increasing concentrations of CCh, a nonselective cholinergic agonist, to obtain dose–response relationships for the muscarinic suppression of IPSCs and EPSCs in $M2^{+/+}$ and $M2^{-/-}$ hippocampi (Fig. 9*B*,*E*) ($M2^{+/+}$: IPSCs, $n = 11$ slices from seven mice; EPSCs, $n = 9$ slices from six mice; $M2^{-/-}$: IPSCs, $n = 8$ slices from six mice; EPSCs, $n = 10$ slices from five mice). In $M2^{+/+}$ hippocampi, the

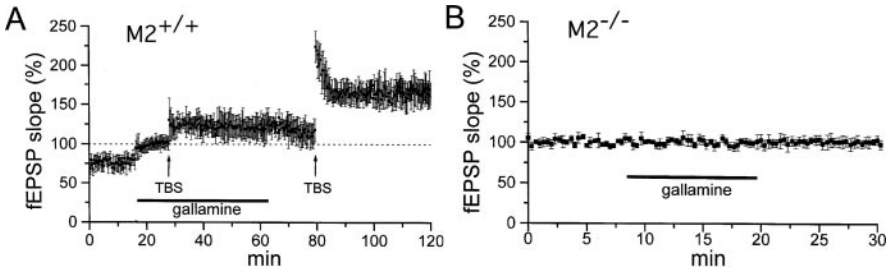

Figure 6. In $M2^{+/+}$ hippocampal slices, the M_2 receptor-preferring antagonist gallamine abrogates STP and impairs LTP but enhances control fEPSPs. *A*, In $M2^{+/+}$ hippocampal preparations, application of gallamine (20 μM) alone enhanced fEPSPs by ~25%. This effect was abolished in $M2^{-/-}$ hippocampi (*B*; $n = 5$), suggesting that low-frequency synaptic excitation of CA1 pyramidal cells is tonically inhibited by ambient acetylcholine acting on M_2 receptors. As observed with $M2^{-/-}$ hippocampal preparations, TBS failed to induce STP and robust LTP in gallamine-treated $M2^{+/+}$ hippocampi ($n = 12$). Both parameters of synaptic plasticity were reliably evoked after wash-out of gallamine.

IC_{50} value of CCh for inhibition of IPSCs (0.52 μM) was approximately one order of magnitude lower than that for inhibition of EPSCs (8.5 μM). Given the rank order of CCh potencies at muscarinic receptor subtypes ($M_2 \approx M_4 > M_3 > M_1$) (Bujo et al., 1988; McKinney et al., 1991), this observation suggests that M_2 receptors inhibit GABAergic neurotransmission more potently than glutamatergic neurotransmission. This notion was further supported by a comparison of the dose–response relationships for CCh in $M2^{+/+}$ versus $M2^{-/-}$ hippocampi. In $M2^{-/-}$ hippocampi, the efficacy of CCh to suppress IPSCs was dramatically reduced (Fig. 9*A*,*B*), whereas the shift of the dose–response curve for EPSC suppression was much less pronounced (Fig. 9*D*,*E*). Notably, CCh not only produced a potent inhibition of IPSCs, but it also reversed the paired-pulse ratio from depression (0.6 ± 0.04) to facilitation (1.2 ± 0.2; $n = 8$ slices from five mice) in $M2^{+/+}$ hippocampi. Because CCh failed to affect the paired-pulse ratio of IPSCs in $M2^{-/-}$ hippocampi (Fig. 9*A*), this effect is predicted to be mediated by the activation of M_2 receptors. Based on their strong effect on the paired-pulse ratio in $M2^{+/+}$ hippocampi, M_2 receptors are likely to control inhibitory neurotransmission primarily by interfering with GABA release at presynaptic sites.

The data described above indicated that CCh-mediated activation of M_2 receptors affected GABAergic synapses more potently than glutamatergic synapses. To examine whether this also holds true for endogenously released acetylcholine (Descarries et al., 1997), thus accounting for the impaired synaptic plasticity in $M2^{-/-}$ hippocampi, we examined short-term plasticity at inhibitory and excitatory synapses of $M2^{+/+}$ and $M2^{-/-}$ hippocampi in the absence of exogenously added muscarinic agonist. A comparison of the paired-pulse data shown in Figure 9, *A* and *D*, provided initial support for the concept that disruption of the M_2 receptor gene differentially affected short-term plasticity at inhibitory versus excitatory synapses. Although paired-pulse depression of IPSCs was significantly reduced in $M2^{-/-}$ hippocampi (Fig. 9*C*), paired-pulse facilitation of EPSPs remained unaffected (Fig. 9*F*). On the basis of this observation, we also examined the behavior of IPSCs during repetitive stimulation at theta frequency (Fig. 10*A–C*). Under control conditions, the IPSC response in $M2^{+/+}$ hippocampi was strongly depressed ($n = 15$ slices from six mice). In the presence of the M_2 receptor-preferring antagonist gallamine (20 μM; $n = 8$ slices from five mice), the amplitude of the first IPSC was significantly enhanced, and the decline of the subsequent IPSCs was substantially diminished (Fig. 10*A–C*), suggesting the involvement of M_2 receptors. In agreement with this notion, the depression of IPSCs was significantly attenuated in $M2^{-/-}$ hippocampi, compared with $M2^{+/+}$ hippocampi (Fig. 10*C*) ($n = 11$ slices from five mice), and gallamine failed to alter the response pattern to an appreciable extent in the absence of M_2 receptors (Fig. 10*A*,*B*) ($n = 5$ slices from four mice).

In contrast to IPSCs, EPSCs showed a facilitating response during repetitive stimulation ($M2^{+/+}$, $n = 10$ slices from five mice; $M2^{-/-}$, $n = 10$ slices from four mice). In $M2^{+/+}$ hippocampi, gallamine (20 μM) produced a similar enhancement of all four current responses. However, this response was absent in $M2^{-/-}$ hippocampi (Fig. 10*D*) ($M2^{+/+}$, $n = 7$ slices from five mice; $M2^{-/-}$, $n = 7$ slices from five mice). The enhancing effect of gallamine on EPSCs in $M2^{+/+}$ hippocampi as well as the lack of this effect in $M2^{-/-}$ hippocampi were mimicked by the nonselective muscarinic antagonist atro-

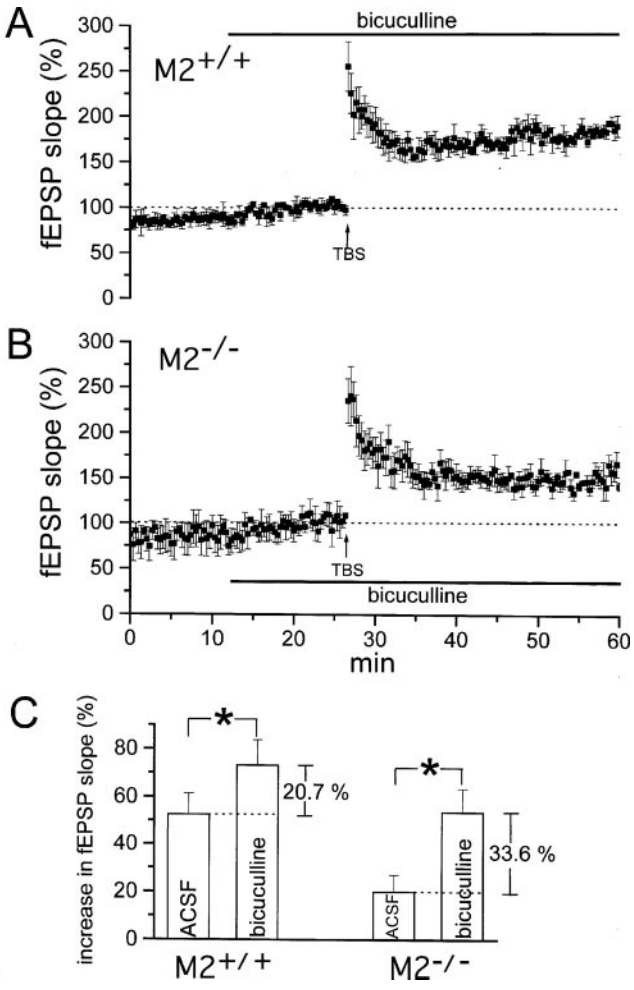

Figure 7. The $GABA_A$ receptor antagonist bicuculline restores STP and improves LTP in hippocampal slices from $M2^{-/-}$ mice. *A*, *B*, Effect of bicuculline (10 μM) in $M2^{+/+}$ (*A*) and $M2^{-/-}$ (*B*) hippocampi. $GABA_A$ receptor blockade restores STP in $M2^{-/-}$ hippocampi. *C* compares LTP (determined 30 min after TBS and expressed as percentage increase in fEPSP slopes relative to baseline) in $M2^{+/+}$ and $M2^{-/-}$ hippocampal preparations in the absence (ACSF) or presence of bicuculline. Note that the LTP-promoting action of bicuculline is more pronounced in $M2^{-/-}$ than in $M2^{+/+}$ hippocampi. *$p < 0.01$ ($M2^{+/+}$, $n = 5$; $M2^{-/-}$, $n = 6$).

pine (1–2 μM) (Fig. 10*E*) ($n = 4$ slices from three mice), consistent with the hypothesis that endogenously released acetylcholine acts via M_2 receptors to control excitatory synapses. Although M_2 receptor stimulation augmented transmission at excitatory synapses, it failed to alter the pattern of short-term plasticity at the same synapses (Fig. 10*F*). In contrast to inhibitory synapses where the absence of M_2 receptors produced a significant attenuation of synaptic depression (Fig. 10*C*), thereby disinhibiting pyramidal cells, facilitation at excitatory synapses did not vary significantly between $M2^{+/+}$ and $M2^{-/-}$ hippocampi, although the $M2^{-/-}$ preparations displayed a trend toward reduced facilitation (Fig. 10*F*).

Discussion

In the Barnes maze test, $M2^{-/-}$ mice performed significantly less well than $M2^{+/+}$ mice during the initial phase of training (days 1–5) (Fig. 1). This deficit in the initial learning phase was accompanied by differences in the application of search strategies to successfully complete the task, in that the $M2^{-/-}$ mice failed to switch as quickly as the $M2^{+/+}$ mice from using the random search strategy to a more efficient serial strategy (Table 1). The ability of a mouse to learn a new search strategy while suppressing the execution of a previously learned, but less appropriate, strategy (e.g., the subject's behavioral flexibility) significantly impacts its performance level during the acquisition phase of the Barnes maze trial.

When mice were retested in the Barnes maze 1 month after the last training session, $M2^{+/+}$ and $M2^{-/-}$ mice performed equally well, indicating that M_2 receptors are not essential for the retention of spatial memory. This finding is consistent with previous observations that scopolamine, a nonsubtype-selective muscarinic antagonist, primarily interferes with the acquisition of new information rather than with memory retention (Hagan et al., 1986; Decker et al., 1990; Anagnostaras et al., 1995).

$M2^{+/+}$ and $M2^{-/-}$ mice were then subjected to a reversal learning task, which tests the intramodal shift variety of behavioral flexibility (Ragozzino et al., 1999) and is thought to require intact function of both the hippocampus (Jarrard, 1993) and prefrontal cortex (Kesner, 2000). One week after memory retention testing (Barnes maze), $M2^{+/+}$ and $M2^{-/-}$ mice were examined for their ability to learn a new location of the escape tunnel opposite of the original (reversal learning). Under these new conditions, $M2^{-/-}$ mice performed significantly less well than the $M2^{+/+}$ control animals (Fig. 2), providing additional support for the concept that the lack of M_2 receptors causes reduced behavioral flexibility.

Several studies have shown that scopolamine treatment impairs performance in a variety of behavioral tasks requiring spatial working memory (Fibiger et al., 1991; Iversen, 1997). To assess the potential involvement of M_2 receptors in this type of memory, we subjected $M2^{+/+}$ and $M2^{-/-}$ mice to a T-maze delayed alteration task. During training with a short intertrial interval (5 sec), the $M2^{-/-}$ mice did not display significant cognitive deficits (Fig. 3*A*).

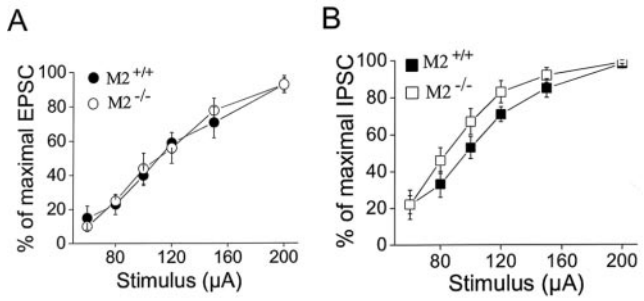

Figure 8. Input–output relationships for EPSCs (*A*) and IPSCs (*B*) in CA1 pyramidal cells of $M2^{+/+}$ and $M2^{-/-}$ hippocampi. EPSCs ($M2^{+/+}$, $n = 8$; $M2^{-/-}$, $n = 7$) and IPSCs ($M2^{+/+}$, $n = 8$; $M2^{-/-}$, $n = 8$) were normalized to their maximal amplitude. The moderate leftward shift of the I-O curve for IPSCs in $M2^{-/-}$ hippocampi did not reach statistical significance.

In contrast, when the difficulty of the task was increased by raising the delay time to 20 sec, $M2^{-/-}$ mice performed significantly less well than the $M2^{+/+}$ control mice throughout the entire testing period (Fig. 3*B*). These data indicate that M_2 receptors are involved in processes that facilitate working memory.

To examine whether the behavioral deficits observed with the $M2^{-/-}$ mice correlated with changes in neuronal plasticity, we studied STP and LTP at the Schaffer–CA1 synapse using hippocampal slices from $M2^{+/+}$ and $M2^{-/-}$ mice. Strikingly, we found that STP was abolished and LTP was greatly reduced after high-frequency stimulation of $M2^{-/-}$ hippocampi (Fig. 5). Sim-

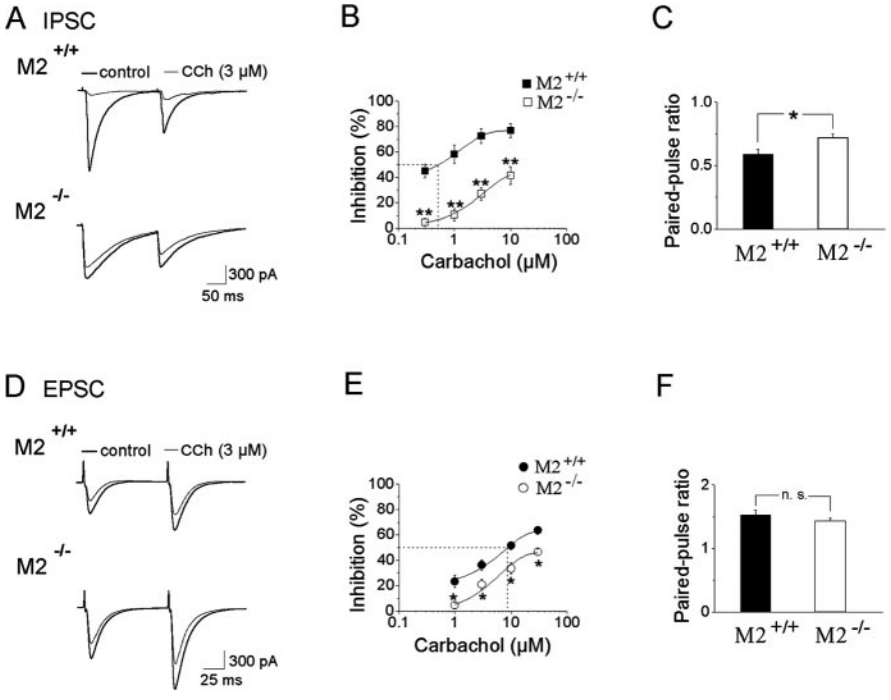

Figure 9. Effects of CCh, a nonselective cholinergic agonist, on IPSCs and EPSCs of $M2^{+/+}$ and $M2^{-/-}$ hippocampi. *A*, *D*, Synaptic current responses were evoked using a paired-pulse protocol in which IPSCs displayed paired-pulse depression, whereas EPSCs displayed paired-pulse facilitation. *B*, *E*, CCh dose dependently reduced IPSCs ($n = 11$) and EPSCs ($n = 9$) in $M2^{+/+}$ hippocampi. Note the higher potency of CCh at inhibitory versus excitatory synapses of $M2^{+/+}$ hippocampi. In $M2^{-/-}$ hippocampi, the efficacy of CCh to suppress synaptic responses was much more attenuated for IPSCs ($n = 10$) than for EPSCs ($n = 8$). Dose–response curves depict the relative inhibition of the first synaptic response during paired-pulse stimulation. Each data point is the average of five to nine measurements. *C*, *F*, The strong paired-pulse depression of IPSCs found in $M2^{+/+}$ mice (ratio, 0.59 ± 0.04; $n = 11$) was significantly alleviated in $M2^{-/-}$ hippocampi (ratio, 0.72 ± 0.03; $n = 10$), whereas paired-pulse facilitation of EPSCs did not vary between $M2^{+/+}$ (ratio, 1.53 ± 0.08; $n = 9$) and $M2^{-/-}$ hippocampi (ratio, 1.43 ± 0.05; $n = 8$). $^*p < 0.05$; $^{**}p < 0.01$. NS, Not significantly different.

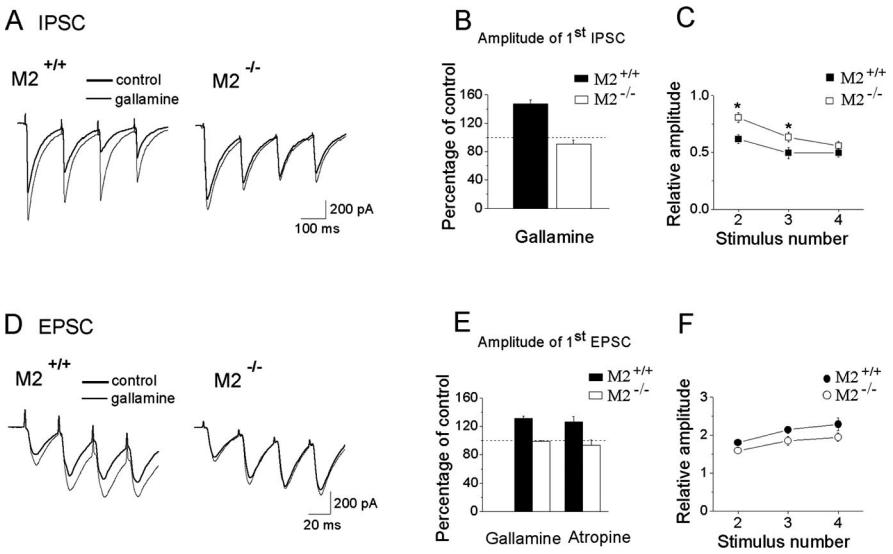

Figure 10. Response pattern of IPSCs and EPSCs during repetitive stimulation in M2 $^{+/+}$ and M2 $^{-/-}$ hippocampi. *A*, The strong depression of IPSCs in M2 $^{+/+}$ hippocampi (left; $n = 15$) was substantially attenuated by the M_2 receptor-preferring antagonist gallamine (20 μM; $n = 8$). In M2 $^{-/-}$ hippocampi, the IPSC response displayed much less depression (right; $n = 11$), and gallamine was rendered ineffective ($n = 6$). *B*, The histogram summarizes the experiments shown in *A* and compares the effect of gallamine on the amplitudes of the first IPSC in a stimulus train. *C*, To compare quantitatively the degree of depression between M2 $^{+/+}$ ($n = 15$) and M2 $^{-/-}$ hippocampi ($n = 11$), the amplitudes of the second to fourth IPSCs were normalized to that of the first IPSC. *D*, Facilitation of EPSCs in M2 $^{+/+}$ (left side) and M2 $^{-/-}$ hippocampi (right side) in the absence (M2 $^{+/+}$, $n = 10$; M2 $^{-/-}$, $n = 10$) and presence of 20 μM gallamine (M2 $^{+/+}$, $n = 7$; M2 $^{-/-}$, $n = 8$). *E*, The histogram summarizes the effects of 20 μM gallamine and 1–2 μM atropine ($n = 4$) on the amplitude of the first EPSC in a stimulus train. *F*, Quantitative comparison of EPSC facilitation between M2 $^{+/+}$ ($n = 10$) and M2 $^{-/-}$ hippocampi ($n = 10$) showed a trend toward less facilitation in M2 $^{-/-}$ preparations, which, however, did not reach statistical significance. *$p < 0.05$.

ilar deficits were observed when M2 $^{+/+}$ hippocampal slices were incubated with the M_2 receptor-preferring antagonist, gallamine (20 μM) (Fig. 6*A*). These data indicate that M_2 receptor activity is essential for the generation of STP and robust LTP at the Schaffer–CA1 synapse. Moreover, these results suggest that endogenously released acetylcholine regulates hippocampal plasticity primarily via activation of M_2 receptors. In contrast to the findings reported here, LTP was little affected in hippocampal slices (Schaffer–CA1 synapse) from M1 $^{-/-}$ mice (Anagnostaras et al., 2003), consistent with the key role of the M_2 receptor subtype in mediating this activity.

Strikingly, treatment of M2 $^{-/-}$ hippocampi with the $GABA_A$ receptor antagonist bicuculline (10 μM) fully restored STP and significantly increased LTP caused by high-frequency electrical stimulation (Fig. 7*B*). A likely explanation for this finding is that presynaptic M_2 receptors mediate suppression of GABAergic ($GABA_A$ receptor mediated) inhibition of CA1 neurons in the M2 $^{+/+}$ hippocampus. This concept is supported by previous observations indicating that M_2 receptors are present on GABA-containing nerve terminals throughout the hippocampus (Rouse et al., 1997; Hajos et al., 1998) and that activation of hippocampal muscarinic receptors inhibits GABA release from hippocampal interneurons (Ben Ari et al., 1981; Behrends and ten Bruggencate, 1993). Together, these findings are consistent with a model in which induction of STP and LTP requires the activity-dependent stimulation of presynaptic M_2 heteroceptors suppressing the GABA-mediated inhibition of CA1 pyramidal neurons.

This concept was further corroborated by a series of whole-cell recordings from CA1 pyramidal neurons of M2 $^{+/+}$ and M2 $^{-/-}$ hippocampi, in which we studied the behavior of pharmacologically isolated IPSCs and EPSCs during single and repetitive electrical stimulation. Our data indicate that endogenously released acetylcholine acts mainly on M_2 receptors to control synaptic transmission at both inhibitory and excitatory synapses. However, we found that the lack of M_2 receptors did not affect synaptic excitation and inhibition equally but had a more pronounced effect on IPSCs than on EPSCs, particularly during repetitive stimulation. This conclusion is based on the following observations obtained with M2 $^{-/-}$ hippocampi. First, the suppressing action of CCh was much more compromised at inhibitory than at excitatory synapses (Fig. 9*B*,*E*). Second, paired-pulse inhibition of IPSCs was significantly attenuated, whereas paired-pulse facilitation of EPSCs remained unaffected (Fig. 9*C*,*F*). Third, the strong depression of IPSCs during trains of stimuli was significantly diminished, whereas the facilitation of EPSCs was not altered (Fig. 10*C*,*F*). Together, these findings strongly support the concept that the lack of STP and robust LTP in M2 $^{-/-}$ hippocampi results, at least in part, from excessive GABA release, keeping the depolarization of pyramidal cells below the range required to initiate the molecular machinery underlying synaptic plasticity.

In addition to regulating the strength of excitatory and inhibitory transmission by presynaptic mechanisms, acetylcholine also uses postsynaptic mechanisms to modulate synaptic plasticity (Auerbach and Segal, 1994, 1996). In the present study, a low concentration of the cholinergic agonist, CCh (0.5 μM), was able to induce LTP_m in M2 $^{+/+}$ hippocampal slices. Strikingly, this activity was totally abolished in M2 $^{-/-}$ mice (Fig. 4), providing unambiguous evidence that the development of LTP_m depends on the activation of hippocampal M_2 receptors. Although our results do not provide direct evidence for a postsynaptic localization of the M_2 receptors involved in mediating LTP_m, electrophysiological (Auerbach and Segal, 1996; Seeger and Alzheimer, 2001) and *in situ* mRNA hybridization studies (Vilaro et al., 1992) indicate that M_2 receptors are expressed by CA1 pyramidal cells.

The electrophysiological data suggest the possibility that the deficits in behavioral flexibility and working memory observed in M2 $^{-/-}$ mice are caused, at least in part, by impaired hippocampal plasticity. Interestingly, a similar phenotype (abnormalities in both behavioral flexibility and LTP) has been reported for mutant mice lacking TrkB receptors in the forebrain (Minichiello et al., 1999; Vyssotski et al., 2002). Previous studies suggest that the prefrontal cortex plays a key role in behavioral flexibility and working memory (Ragozzino et al., 1999; De Bruin et al., 2000; Granon and Poucet, 2000; Kesner, 2000). However, our findings raise the possibility that M_2 receptor-mediated modulation of hippocampal neuronal activity may also be involved in these processes. Consistent with this concept, a large body of evidence suggests the existence of complex functional interactions between the hippocampus and the prefrontal cortex in cognition and memory (Laroche et al., 2000). Moreover, studies with forebrain-specific calcineurin knock-out mice recently revealed a good correlation between impaired hippocampus-dependent working memory and deficits in hippocampal synaptic plasticity (Zeng et al., 2001).

Interestingly, Anagnostaras et al. (2003) recently reported that $M1^{-/-}$ mice also display selective cognitive impairments. Whereas $M1^{-/-}$ mice showed normal or enhanced memory for tasks that involved matching-to-sample problems, they were severely impaired in nonmatching-to-sample working memory and consolidation (Anagnostaras et al., 2003). These authors therefore proposed that M_1 muscarinic receptors play a role in cognitive processes that require interactions between the hippocampus and cortex (Anagnostaras et al., 2003).

M_2 receptors located on cholinergic nerve endings in the hippocampus and cerebral cortex are known to mediate autoinhibition of acetylcholine release (Quirion et al., 1995; Kitaichi et al., 1999; Zhang et al., 2002). This finding has prompted the proposal that pharmacological blockade of central inhibitory M_2 autoreceptors may represent a useful approach to enhance cholinergic neurotransmission in patients suffering from Alzheimer's disease (Quirion et al., 1989, 1995; Carey et al., 2001). However, the results of the present study clearly indicate that complete blockade of M_2 receptors may interfere with proper hippocampal plasticity and certain cognitive tasks.

Consistent with the role of presynaptic M_2 autoreceptors in regulating hippocampal ACh release, Tzavara et al. (2003) recently reported that $M2^{-/-}$ mice showed significant changes in pharmacologically and physiologically evoked acetylcholine release in the hippocampus. These neurochemical changes were accompanied by performance deficits in a passive avoidance test, suggesting that improper regulation of synaptic acetylcholine release may also contribute to the cognitive deficits caused by the lack of M_2 receptors.

In conclusion, our data show that M_2 receptors are required for intact working memory, behavioral flexibility, and hippocampal synaptic plasticity. Because reduced muscarinic cholinergic neurotransmission represents a key factor leading to impaired cognition associated with Alzheimer's disease and old age (Bartus et al., 1982; Coyle et al., 1983), these results should be of considerable therapeutic interest.

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