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Learning and Memory Impairments in a Congenic C57BL/6 Strain of Mice That Lacks the M_2 Muscarinic Acetylcholine Receptor Subtype

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Abstract

The neurotransmitter acetylcholine is an important modulator of cognitive functions including attention, learning, and memory. The actions of acetylcholine are mediated by five distinct muscarinic acetylcholine receptor subtypes (M_1-M_5). The lack of drugs with a high degree of selectivity for these subtypes has impeded the determination of which subtypes mediate which components of cholinergic neurotransmission relevant to cognitive abilities. The present study examined the behavioral functions of the M_2 muscarinic receptor subtype by utilizing congenic C57BL/6 mice possessing a null-mutation in the M_2 muscarinic receptor gene ($M_2^{-/-}$ mice). Comprehensive assessment of general health and neurological function found no major differences between $M_2^{-/-}$ and wild-type ($M_2^{+/+}$) mice. In tests of learning and memory, $M_2^{-/-}$ mice were impaired in the acquisition (trials to criterion), but not the retention (72 hr) of a passive avoidance task. In a novel open field, $M_2^{-/-}$ mice were impaired in between-sessions, but not within-session habituation. In a holeboard test of spatial memory, $M_2^{-/-}$ mice committed more errors in working memory than $M_2^{+/+}$ mice. Reference memory did not differ between the genotypes. $M_2^{-/-}$ mice showed no impairments in either cued or contextual fear conditioning. These findings replicate and extend earlier findings in a hybrid strain and solidify the interpretation that the M_2 receptor plays a critical role in specific components of cognitive abilities.

Keywords

acetylcholine; muscarinic; memory; knockout; M_2; passive avoidance; habituation; holeboard

Introduction

Many clinically important functions of the neurotransmitter acetylcholine (ACh) are mediated by muscarinic acetylcholine receptors [8,25,49]. Muscarinic receptor subtypes have been

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implicated in components of attentional processes, learning, and memory [26]. The role of muscarinic receptors in learning and memory is of particular interest because cholinergic dysfunction plays a major role in neurodegenerative disorders such as Alzheimer's disease [4,10,51].

There are five molecularly distinct muscarinic receptor subtypes (M_1-M_5) [9], but little is known about the specific physiologic roles of each subtype because of a lack of subtype-selective ligands. Furthermore, most organs, tissues, and cells express two or more muscarinic receptor subtypes, making it difficult to determine the role of each individual subtype [30,47, 52]. In mice, studies using the null mutation strategy (gene knockout) to study individual receptor subtypes provide a way to address these issues [48–50].

Previous studies using the null mutation strategy have demonstrated that the M_2 muscarinic receptor subtype facilitates learning and memory. Mice homozygous for a null mutation in the M_2 receptor gene ($M_2^{-/-}$ mice) showed impaired performance in a passive avoidance task relative to their wild-type littermates ($M_2^{+/+}$ mice) indicating a learning and memory deficit [45]. $M_2^{-/-}$ mice were also impaired in a simple form of learning, between-sessions habituation to the open field [14]. In another study, using the Barnes circular maze and the T maze delayed alternation task, $M_2^{-/-}$ mice were impaired in spatial learning and behavioral flexibility [41].

These behavioral findings were obtained in mice with a hybrid genetic background (129J1/CF1;50/50%) leaving open the questions of whether the observed phenotypes depend on genetic background and whether flanking genes from the embryonic stem cell donor strain contribute to the phenotypes [5,12,18,53]. In the present study we addressed these questions by generating a congenic strain of $M_2^{-/-}$ mice through the backcrossing of heterozygotes ($M_2^{+/-}$ mice) into the C57BL/6NTac strain for 10 successive generations. $M_2^{-/-}$ mice and $M_2^{+/+}$ mice (littermate controls) were then compared in a battery of learning and memory tasks. The learning and memory tests included passive avoidance to criterion, within session habituation to the open field, between session habituation to the open field, cued and contextual fear conditioning, and spatial memory in the holeboard task. These studies were undertaken because knowledge of the precise functions of muscarinic receptors is essential for the development of new therapeutic approaches to diseases involving memory and cognition.

Materials and Methods

Mice

$M_2^{-/-}$ mice were generated on a mixed genetic background as previously described [19]. Heterozygous ($M_2^{+/-}$) mice were backcrossed for 10 generations into the C57BL/6NTac background (Taconic Farms, Germantown, NY). Two batches of mice were produced by breeding heterozygous ($M_2^{+/-}$) mice at the National Institute of Diabetes and Digestive and Kidney Diseases (Bethesda, MD). Mouse genotyping was performed by PCR analysis of mouse tail DNA. Batch 1 (males and females) was used to assess whether the null mutation affected the general health and neurological function of the mice. Batch 2 (males only) was shipped at 10 months of age from the National Institute of Diabetes and Digestive and Kidney Disease to the Drake University College of Pharmacy and Health Sciences where behavioral assessment was conducted at 12–17 months of age. The test order for Batch 2 is indicated by the order of description in the methods. The time interval between behavioral tests is provided at the beginning of the description of each test. In all procedures measures were taken to minimize pain and discomfort of the mice, and all procedures were approved by the Institutional Animal Care and Use Committee at Drake University.

Assessment of General Health and Neurological Function

Measures of general health and neurological function were performed as previously described [11]. Briefly, general health was determined by assessment of fur condition, whisker condition, body and limb tone, and observation of home cage behavior. Neurological reflexes assessed were trunk curl, forepaw reaching, eye-blink, pinna twitch, vibrissae response, toe pinch response, and the righting reflex. Behavioral reactivity was assessed by attempted escape during petting, struggling or vocalization during handling, and biting of a wooden dowel. Empty cage behavior was assessed by placing each subject in a clean, empty cage for 3 min, and noting the occurrence of freezing, wild running, stereotypies, exploration, and grooming. Olfactory ability was assessed by measuring the latency to find a buried peanut, as previously described. All subjective assessments were done by an experimenter who was uninformed of the subjects' genotypes.

Open Field Activity and Within Session Habituation

An automated open field apparatus was used to assess spontaneous motor activity and within session habituation to a novel environment. The open field was a square arena (40 × 40 × 35 cm; Med Associates, St. Albans, VT) with clear Plexiglas walls and floor, evenly illuminated by white light (~ 700 lux). Each subject (males, $M_2^{+/+}$, n = 23; $M_2^{-/-}$, n = 21) was placed in the center of the open field and allowed to explore for 60 min. The arena was traversed by 32 infrared beams emitted from each of two sides of the arena (16 per side) such that the beams formed a grid of equally sized squares. As the mouse moved about in the arena, the automated computer software (Activity Monitor, version 5.8, Med Associates, St. Albans, VT) counted beam breaks, termed ambulatory counts, as a measure of horizontal activity. Beam breaks were counted as ambulatory counts whenever 3 beams were broken and the interval between breaks was less than 500 msec. Within-session habituation was measured as the decrease in horizontal activity across the duration of the test. A second array of infrared beams, situated above those that assessed horizontal activity, detected the vertical activity (rearing) of the mouse. The apparatus was cleaned with 70% ethanol between subjects.

Passive Avoidance

Two to four days after open field testing, passive avoidance learning was assessed in male $M_2^{+/+}$ mice (n = 23) and $M_2^{-/-}$ mice (n = 20) in a computer-controlled Gemini shuttle-box (San Diego Instruments, San Diego, CA) that was divided into two equal chambers by a guillotine door. Training began by placing the subject into the chamber on the right and allowing free exploration for 1 min. At the end of this exploration period, the chamber was illuminated (6,340 lux) and the guillotine door was opened. Latency to pass through the doorway and to enter the other chamber, which remained darkened (3 lux), was recorded by the computer. Upon entering the darkened chamber, the guillotine door closed and a mild footshock (2 sec, 0.75 mA) was delivered. The mouse was then removed from the darkened chamber after 10 sec and placed back into the illuminated chamber. This sequence was repeated until the mouse remained in the illuminated chamber for 300 sec. The number of trials needed to reach this criterion was taken as the measure of task acquisition. Approximately 72 hrs after task acquisition, retention of passive avoidance was assessed by placing each mouse in the illuminated chamber as before. Latency to enter the darkened chamber was taken as the measure of task retention.

Between Sessions Habituation to the Open Field

Approximately 2 months after passive avoidance training, between sessions habituation to a novel environment was assessed. Male mice ($M_2^{+/+}$, n = 20; $M_2^{-/-}$, n = 20) were placed in the automated open field described above for a duration of 15 min. After an intersession interval of 4 hrs, the mice were placed in the apparatus again for 15 min. During both trials, horizontal

activity was recorded as before. To create a novel environment from the previous exposure to the open field box, the top half of the box was "wall papered" with white bench paper and was cleaned with orange-scented cleaner (Fantastik Orange Action, S.C, Johnson, Racine, WI) between subjects rather than ethanol. The percent reduction in horizontal activity during the entire second exposure to the apparatus relative to the entire first exposure served as a measure of between sessions habituation.

Cued and Contextual Fear Conditioning

Approximately 3 weeks after assessment of between-sessions habituation, cued and contextual fear conditioning was conducted in an automated conditioning chamber (32 cm × 25 cm × 23 cm, Med Associates, St. Albans, VT) equipped with a digital video camera interfaced to a PC installed with commercially available software (Video Freeze, version 1.12.0.0, Med Associates, St. Albans, VT) that uses a pixel-based method for determining the occurrence of freezing behavior, the species-specific conditioned response produced by fear conditioning protocols. The conditioning chamber was housed in a wooden, sound attenuating cubicle (64 cm × 76 cm × 42 cm). The chamber was illuminated by a fluorescent light mounted on the back wall of the sound-attenuating cubicle (1,800 lux). The user-determined settings for the determination of freezing behavior were: observation interval of 10 sec, observation duration of 0.5 sec, and a motion threshold of 20 arbitrary units. These settings were found in pilot studies in our laboratory to reliably correlate with human observation of freezing behavior ($r = 0.944$).

For fear conditioning training, male subjects ($M_2^{+/+}$, $n = 23$; $M_2^{-/-}$, $n = 21$) were placed in the chamber and presented with two white noise (conditioned stimulus (CS), 90 dB) and footshock (unconditioned stimulus (US), 0.75 mA) pairings. The footshock was delivered through the floor which was in the form of parallel bars with a diameter of 3.2 mm.. Each pairing was preceded and followed by a 2 min exploration period. The CS-US pairings were comprised of 30 sec of white noise and 1 sec of footshock that overlapped and co-terminated with the CS.

Twenty-four and 48 hrs after training, the mice were tested for contextual and cued fear conditioning, respectively. For contextual conditioning, the mice were individually removed from their home cages and placed in the conditioning chamber under environmental conditions identical to those of the training day. No stimuli were presented to the mice during the contextual conditioning test. Freezing behavior was recorded by the computer software for 5 min and served as the measure of the strength of association between the shock and the training environment.

In the cued fear conditioning test, mice were individually removed from their home cages and placed in the conditioning chambers. To isolate cued conditioning from contextual conditioning, the environmental context was altered by replacing the parallel bar floor with a grid-mesh floor made of smaller bars and a white, rounded plastic insert was placed along the walls in order to change the overall appearance of the chamber. Furthermore, the chambers were cleaned with an orange-scented cleaning solution rather than the usual 70% ethanol between subjects in order to alter the olfactory stimuli in the environment. Each mouse was placed in the altered-context environment for 6 min. The first 3 min established the baseline exploration in the absence of any stimuli. During the last 3 min, the CS white noise was presented. In order to analyze the effect of cue onset freezing behavior during the last minute of cue offset and the first minute of cue onset were compared. The presence or absence of freezing behavior was recorded by computer software and served as the measure of the strength of the learned association between the auditory cue and the footshock.

Holeboard Task of Spatial Memory

Approximately two months after fear conditioning, a food-motivated holeboard task was used to assess spatial learning and memory [27]. The apparatus consisted of an open field arena (40 × 40 × 35 cm; Med Associates, St. Albans, VT) with a floor insert that consisted of 16 evenly spaced holes (diameter of 1.3 cm, depth of 9 cm). An array of infrared beams was used to detect nosepokes (entries) into the holes. Extra-maze spatial cues consisted of cables, hardware, and the experimenter who always sat in the same location. The apparatus was illuminated at a level of approximately 700 lux.

Prior to being trained in this task, male mice ($M_2^{+/+}$, n = 16; $M_2^{-/-}$, n = 15) were singly housed and placed on a food restriction schedule in which their body weights were maintained at 80–85% of their free-feeding body weights. The first three days of the task consisted of acclimation and shaping sessions in which all the holes were baited with a food pellet and the mouse was allowed to freely explore the arena. The habituation session ended when the mouse had obtained all of the food rewards or 15 min had expired. Training of the mice in the task began on the fourth day. In the training trials, 4 randomly chosen holes (holes number 1, 8, 10, and 15 with the top left hole being #1 and bottom right being #16) were baited with a 20 mg pellet (Bio-Serve, Frenchtown, NJ). The 12 unbaited holes and 4 baited holes also contained a 45-mg pellet (changed daily) that was located beneath a wire grid and thus inaccessible to the mice. By setting up the baited and unbaited holes in this manner, the mice could not use olfaction to locate the baited holes and were required to learn their location relative to spatial cues. Training days consisted of 3 trials per day with each trial being separated by an interval of approximately 2.5 min. Trials continued until all 4 baits were obtained or 3 min had expired. The apparatus was cleaned with 70% ethanol between subjects. Mice were run in the task for 19 days, but only data from the first six days is shown because performance did not significantly improve after that time (asymptotic performance).

The performance measures in the holeboard task were working memory errors and reference memory errors. Working memory errors were defined as any re-entry into a baited or unbaited hole. Reference memory errors were defined as any first-time entry into an unbaited hole.

Statistical Analysis

All passive avoidance measurements were analyzed by t-test. Ambulatory counts in the open field were analyzed by two-way repeated measures analysis of variance (ANOVA) using 5-min time bin and genotype as the factors. For between-sessions habituation to the open field, percent reduction in ambulatory activity during the entire second session relative to the entire first session was analyzed by t-test. Freezing behavior during training and during the test of cued conditioning was analyzed by two-way repeated measures ANOVA using genotype and stage of session (pre CS-US pairing or post CS-US pairing) as the factors. Freezing behavior during the contextual conditioning test was analyzed by t-test. For cued conditioning, freezing behavior during the last minute of the cue off condition and first minute of cue on condition were analyzed by two-way repeated measures ANOVA using genotype and cue state as the factors. Working and reference memory errors in the holeboard task were analyzed by two-way repeated measures ANOVA using genotype and day as the factors. The threshold for significance for all tests was $p < 0.05$, and all analyses were conducting using SigmaStat (version 3.10).

Results

General Health and Neurological Function

Table 1 summarizes the results of the assessment of general health and neurological function. $M_2^{-/-}$ mice were generally indistinguishable from $M_2^{+/+}$ mice on all measures indicating that

the null mutation did not preclude testing of performance in more sophisticated behavioral domains such as learning and memory. Although the mice were older than those used in previous behavioral studies of M_2 knockout mice (12–17 months vs. 3–6 months), we found no grossly apparent, age-related characteristics that would limit our studies.

Open Field Motor Activity

$M_2^{-/-}$ mice and $M_2^{+/+}$ mice did not differ in their motor activity levels in an open field nor in their habituation to the open field within the test session, as indicated by significantly decreased motor activity across a 60 min test session (Figure 1a; two-way repeated measures ANOVA, main effect of time bin, $F_{(11, 442)} = 44.69$, $p < 0.001$). The genotypes also did not differ in vertical activity (rearing; Figure 1b).

Passive Avoidance

In the first trial of passive avoidance training to criterion, $M_2^{+/+}$ mice and $M_2^{-/-}$ mice entered the darkened chamber with similar latencies (Figure 2a) indicating that there was no confounding effect of genotype on motor activity in this task. $M_2^{-/-}$ mice were impaired in their ability to learn the passive avoidance as indicated by significantly lower latencies (Figure 2b; $t = 2.29$, $df = 41$, $p = 0.03$) than $M_2^{+/+}$ mice to enter the dark on the second trial. Furthermore, an acquisition deficit was indicated by the $M_2^{-/-}$ requiring significantly more training trials than $M_2^{+/+}$ mice to obtain the criterion of remaining in the illuminated chamber for 300 sec (Figure 2c; $t = -2.77$, $df = 41$, $p = 0.008$). The passive avoidance deficit in $M_2^{-/-}$ mice was limited to acquisition as shown by the 72-hr retention test (Figure 2d) in which there was no effect of genotype.

Between Session Habituation to Open Field

In the test of between session habituation to a novel open field, there was no effect of genotype on ambulatory counts during either the first or second 15-min exposure to the open field ($M_2^{+/+}$ first exposure mean = 720.10 ± 92.08; $M_2^{+/+}$ second exposure mean = 463.80 ± 71.41; $M_2^{-/-}$ first exposure mean = 602.65 ± 65.49; $M_2^{-/-}$ second exposure mean = 468.65 ± 56.21), replicating the finding described above. However, during the second exposure (Figure 3), $M_2^{-/-}$ mice decreased their motor activity significantly less (approx. 20%) than $M_2^{+/+}$ mice (approx. 40%; $t = 2.46$, $df = 38$, $p = 0.02$). These data indicate decreased between session habituation to the novel environment in $M_2^{-/-}$ mice suggesting an impairment in their recognition memory of the novel environment.

Cued and Contextual Fear Conditioning

During fear conditioning training (Figure 4a), both genotypes increased freezing subsequent to the CS-US pairings (two-way repeated measures ANOVA, $F_{(1, 42)} = 30.81$, $p < 0.001$), but the genotypes did not differ in their degree of freezing either prior or subsequent to the CS-US pairings. There was not a significant interaction between genotype and phase of training. During testing of contextual conditioning, the genotypes did not differ in the amount of freezing exhibited in the training context (Figure 4b). During testing of cued conditioning, both genotypes increased their freezing behavior during the first minute of cue onset relative to the last minute of the cue off condition (Figure 4c; two-way repeated measures ANOVA, $F_{(1, 42)} = 24.91$, $p < 0.01$) indicating that the mice associated the auditory cue with footshock. There was no effect of genotype on freezing either during the cue off or cue on phases of the experiment, and there was no significant interaction between genotype and state of the cue.

Holeboard Task of Spatial Memory

In the autoshaping stage of the holeboard task, $M_2^{-/-}$ mice and $M_2^{+/+}$ mice showed equivalent propensity to explore the holes (Figure 5a) and consume the pellets. When spatial memory was

taxed by baiting only 4 of the 16 holes, $M_2^{-/-}$ made significantly more working memory errors (two-way repeated measures ANOVA, $F_{(1, 29)} = 8.04$, $p = 0.008$) than $M_2^{+/+}$ mice (Figure 5b). Both genotypes decreased their working memory errors with training ($F_{(5, 130)} = 6.89$, $p < 0.001$), and there was not a significant interaction between genotype and day of training. The genotypes did not differ in terms of reference memory errors (Figure 5c) with both genotypes decreasing their reference memory errors with training ($F_{(5, 130)} = 3.10$, $p = 0.01$). There was not a significant interaction between genotype and day.

Discussion

The principal finding of the current study is that congenic C57BL/6TacN mice possessing a null mutation in the gene for the M_2 receptor have impaired performance in several learning and memory tests. These impairments included passive avoidance acquisition, recognition of an open field, and spatial working memory. However, these mice were not impaired in cued or contextual fear conditioning. $M_2^{-/-}$ mice were not different from $M_2^{+/+}$ mice in general health and neurological function, a finding that minimizes the likelihood that the differential behavioral phenotype is a false positive for mnemonic dysfunction.

In our passive avoidance experiment hyperactivity is one potential genotype effect that could lead to a false positive finding for memory impairment. Several of our measurements dismiss this interpretation. Within the passive avoidance experiment itself, there was no genotype effect on either first trial or 72 hr latency which argues against general hyperactivity. Further, spontaneous motor activity in the open field did not differ between the genotypes.

Genotype effects on exploratory activity can also confound the holeboard task. The measures of re-entries for working memory and entries into unbaited holes for reference memory assume that experimental and control mice do not differ in their general propensity to explore the holes in the floor. We dealt with this issue by confirming that during the pre-training days (autoshaping), $M_2^{-/-}$ mice and $M_2^{+/+}$ mice made the same number of hole entries. The difference between the genotypes arose only when working memory was taxed by baiting only four holes. Moreover, the lack of a genotype effect on reference memory argues against a confound caused by a general increase in exploratory behavior.

Despite observing a genotype effect on passive avoidance acquisition, no effect was observed on contextual or cued fear conditioning. We favor a cautious interpretation of our fear conditioning data because the same subjects were used in both the passive avoidance and fear conditioning experiments. Interestingly, the level of pre-cue freezing in the test of cued fear was relatively elevated in both genotypes. These data suggest that experience in passive avoidance may have sensitized the mice to exhibit generalized fear in subsequent shock-motivated paradigms. We are not aware of any controlled studies suggesting that passive avoidance testing influences subsequent testing in fear conditioning, but the possibility of an interaction between the paradigms should be considered when interpreting the present fear conditioning data.

In light of the fact that genetic deletions of muscarinic receptor subtypes other than M_2 can impact behavior [2,33,43], one must consider the possibility that the cognitive impairments reported here are not due to the absence of M_2 directly, but may be due to changes in the expression of other muscarinic receptors. We do not favor this interpretation of the present data because knockout mice of the various individual muscarinic receptor subtypes have not shown changes in protein expression levels of the other receptors in hippocampus, cortex, striatum, and other brain regions [19,20,33,54]. Although changes in expression of other muscarinic receptors seems to be an unlikely cause of the present findings, it remains a possibility that alterations in signal transduction pathways of other muscarinic receptors may

be playing a role. Further, as is the case for all life-long gene knockouts, undetected developmental effects of the loss of M_2 may contribute to the phenotype.

The learning and memory deficits in our C57BL/6NTac congenic line replicate the findings of previous studies of a non-backcrossed, hybrid background line of $M_2^{-/-}$ mice. Similar to the spatial working memory impairment reported in the present study, Seeger *et al.* [41] showed that $M_2^{-/-}$ mice with a genetic background of 50% 129/J1 and 50% CF1 had spatial working memory deficits in the T-maze delayed alternation test. This same hybrid line was also shown to have impaired passive avoidance learning [45] and diminished between sessions habituation to an open field [14]. All of these findings are similar to the phenotypes in the present report.

The replication of the behavioral phenotype of $M_2^{-/-}$ mice in our backcrossed line is important for two reasons. Firstly, it is now well-understood that the phenotype produced by a genetic manipulation can vary depending on the genetic background in which the mutation is expressed [23,38,42,44]. Based on the two background strains studied so far, there is no strain-dependent variation in penetrance for the genetic deletion of M_2 receptors. Secondly, a differential phenotype can conceivably result from "flanking" genes that are located near the null mutation in the genome and are polymorphic between the embryonic stem cell donor and background strains [5,12,18,53]. This situation can cause a false positive finding in which the phenotypic difference between mutants and wild-types is erroneously attributed to the null mutation. The use of a congenic background strain produced by repeated backcrossing, as was done in the present study, has been suggested as a means of addressing this issue [3]. These backcrosses serve to reduce the size of the flanking region through crossing-over events and thus reduce the number of genes that differ between the mutants and controls. Nevertheless, it must be kept in mind that the size of the flanking region is reduced asymptotically with each successive backcross such that even after 12 backcrosses, the flanking region can represent 1% of the genome [12,18] - an amount that would be 300 genes in a 30,000 gene genome. Hence, in the present study we have only reduced, but not eliminated [40] the likelihood of the behavioral phenotype of $M_2^{-/-}$ mice being due to flanking genes.

The role of M_2 receptors in learning and memory was first examined pharmacologically using M_2 receptor-preferring antagonists. The rationale for these studies was based on the premise the M_2 receptor functions as an inhibitory autoreceptor on cholinergic terminals of the hippocampus and neocortex [55]. Thus, in situations such as Alzheimer's disease in which cholinergic deficiency is associated with cognitive impairment, M_2 receptor antagonists may prove therapeutically useful by increasing ACh release in the hippocampus and neocortex. Pharmacological studies stemming from this line of reasoning have shown that in aged animals that have impaired cognition and impaired cholinergic function, M_2 receptor-preferring antagonists increase ACh release and improve cognitive performance [36,37,46]. Similarly, M_2 receptor-preferring antagonists reverse scopolamine-induced cognitive deficits in young animals [7,28,36,46]. Based on these data, it appears that blockade of M_2 receptors offers a way of fine-tuning a disrupted cholinergic system by restoring cholinergic activity to some optimal level for cognitive function. Nevertheless, all of these pharmacological studies are limited by the fact that the available M_2 receptor antagonists have limited subtype selectivity [6,15,16,22,28,29,32].

Similar to the pharmacological studies, data from $M_2^{-/-}$ mice have provided evidence that the M_2 receptor functions as an inhibitory autoreceptor that regulates evoked acetylcholine release. Tzavera *et al.* [45] showed that stimulus-evoked release of ACh is increased in the hippocampus of $M_2^{-/-}$ mice while the scopolamine-evoked increase in ACh release is diminished. However, counter to what might be expected based on the behavioral pharmacological studies, the increase in ACh levels was associated with impaired passive avoidance learning [45]. Furthermore, $M_2^{-/-}$ mice are impaired in a number of other rodent memory tasks. In addition,

despite the fact that pharmacological blockade of M_2 receptors and genetic deletion of M_2 receptors seem to have a similar effect on ACh release, their respective impacts on learning and memory are in opposition.

The pattern of contradictory behavioral results from pharmacological and genetic studies of M_2 receptors function suggests that there is an optimal level of cholinergic activity for cognition. Thus, elevating ACh release through pharmacological antagonism of M_2 receptors is beneficial because it restores ACh release to this optimal level after cholinergic signaling has been decreased by aging [36,37,46], drugs (e.g. scopolamine) [7,28,36,46], or disease (e.g. AD). However, when cholinergic function has not been lowered by some deleterious process, the loss of M_2 receptors by genetic deletion leads to a dysregulated ACh release that is in excess of the optimum and is detrimental to cognitive function. Dysregulation of ACh release would be expected to be deleterious to cognition because ACh normally has a strong temporal association with the detection of novel or behaviorally-significant stimuli [1,13,24,34,35]. This stimulus-bound character of ACh release is thought to be critical to its role in detecting and processing stimuli [39]. Thus, when ACh release is excessive and not reliably associated with behaviorally relevant stimuli, one would expect to find, as reported here, impairments in tasks such as between-sessions habituation that require learning about novel stimuli.

Complicating the interpretation of pharmacological and genetic studies of M_2 function is the fact that M_2 is not exclusively an inhibitory autoreceptor, but is also found on GABAergic neurons of the basal forebrain [31], and on GABAergic interneurons of Ammon's horn [17, 21]. Expression of M_2 by hippocampal interneurons places this receptor in a location where it may be involved in the plasticity of Schaffer-CA1 synapses. Indeed, Seeger *et al.* [41] showed that short-term potentiation was abolished and long-term potentiation was diminished in Schaffer-CA1 synapses in $M_2^{-/-}$ mice. Given the role of CA1 pyramidal cells in spatial memory, this disruption in plasticity likely underlies the impaired spatial working memory of $M_2^{-/-}$ mice reported here and elsewhere [41].

In summary, mice possessing a null mutation in the gene encoding the M_2 receptor are impaired in the acquisition of passive avoidance, between-sessions habituation to a novel open field, and spatial working memory in a holeboard task. These deficits were demonstrated in a C57BL/6NTac congenic strain. This replication and extension of previous findings from a non-backcrossed hybrid line shows that the behavioral phenotype of $M_2^{-/-}$ mice does not vary between two different genetic backgrounds. These findings are in contrast with a number of behavioral pharmacological studies, underscoring the complexity of M_2 receptor function. Further studies that address this complexity by using inducible and anatomically-restricted null mutations will be necessary to inform the development of therapeutics that target cholinergic dysfunction in diseases such as Alzheimer's disease.

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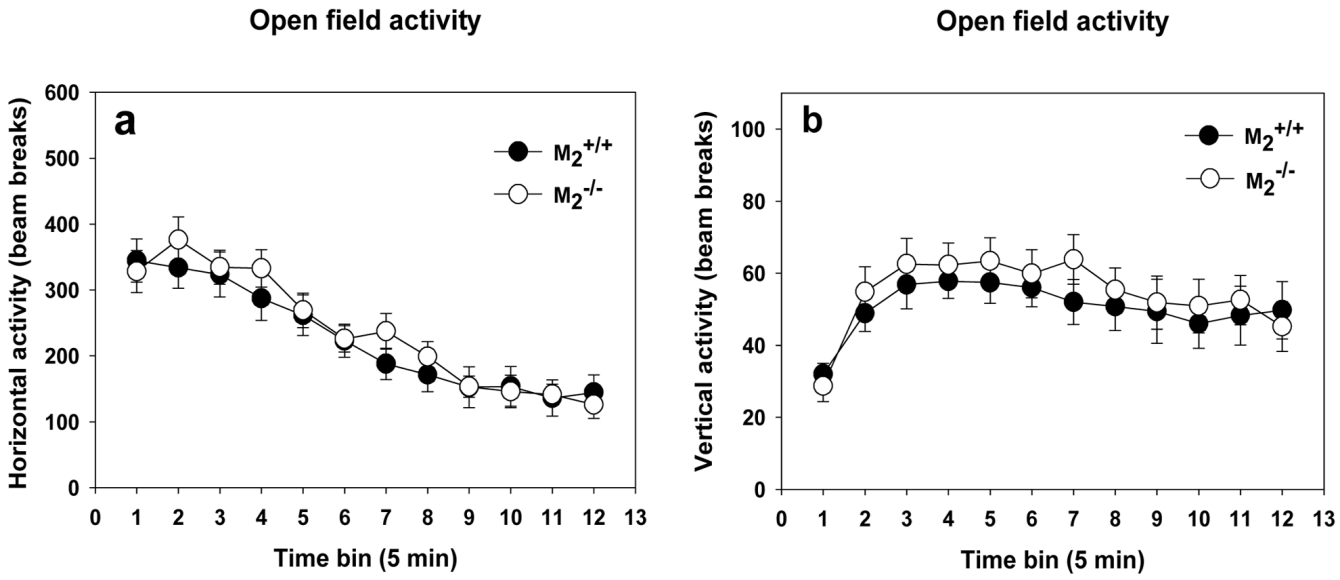

Figure 1.

Spontaneous Motor Activity and Within Session Habituation. (a) There was no difference between $M_2^{+/+}$ (n = 23) and $M_2^{-/-}$ (n = 21) mice in spontaneous motor activity in a novel environment. The genotypes also did not differ in their habituation to the novel environment as indicated by a decrease in motor activity with time. (b) There was no difference between $M_2^{+/+}$ and $M_2^{-/-}$ mice in vertical activity (rearing) in a novel environment.

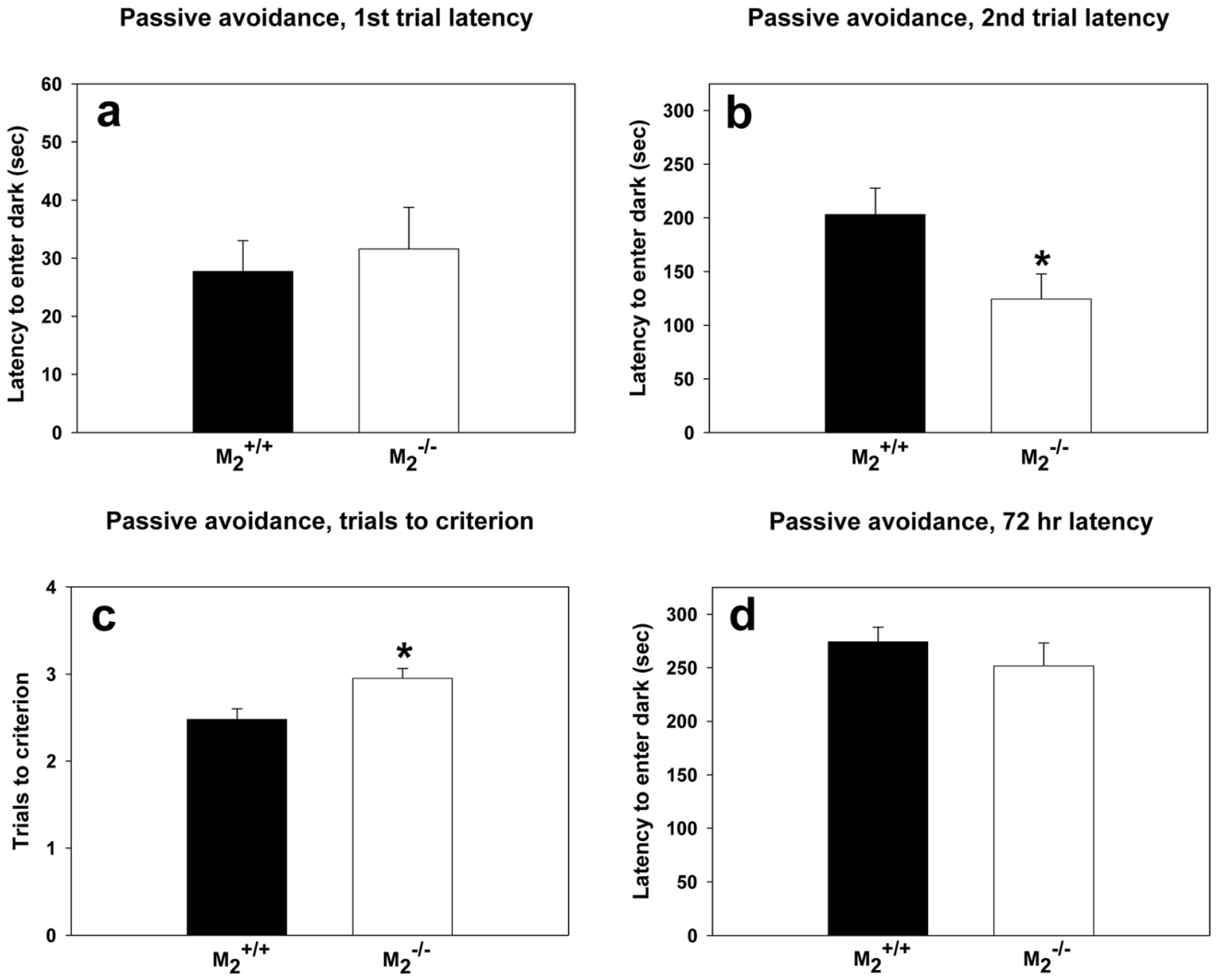

Figure 2.

Passive Avoidance Trials to Criterion. (a) There was no difference between the genotypes in latency to enter the dark chamber on the first training trial. (b) As indicated by the asterisk, on the second training trial, $M2^{-/-}$ mice (n = 20) had significantly lower latencies (t = 2.29, df = 41, p = 0.03) than $M2^{+/+}$ mice (n = 23) to enter the dark chamber. (c) As indicated by the asterisk, $M2^{-/-}$ mice required significantly more trials (t = −2.77, df = 41, p =0.008) than $M2^{+/+}$ mice to reach the criterion of remaining in the lit chamber for 300 sec. (d) There was no effect of genotype on latency to enter the dark chamber in the 72 hr retention trial.

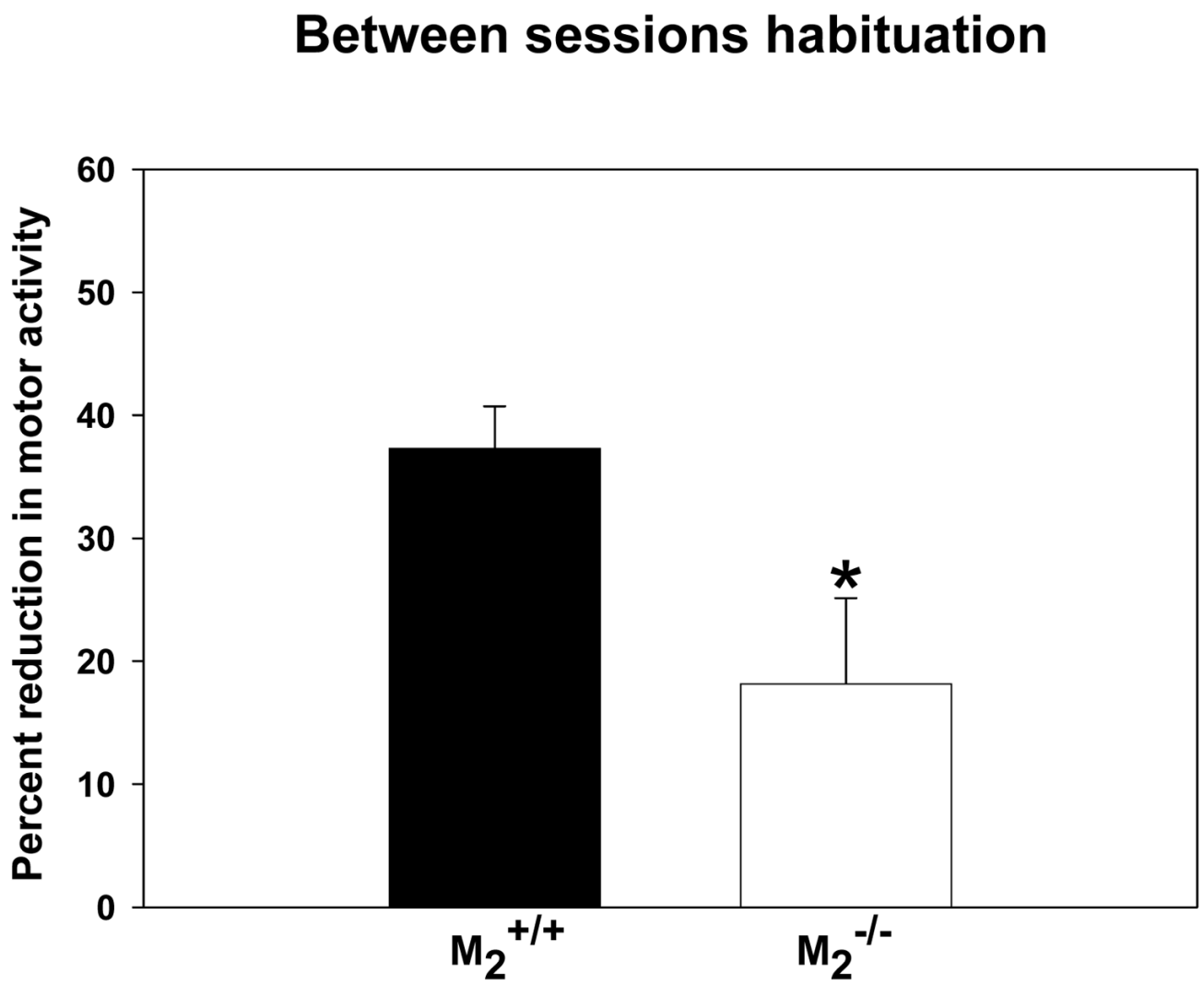

Figure 3.
Between Sessions Habituation. As indicated by the asterisk, $M_2^{-/-}$ (n = 20) mice exhibited a significantly smaller (t = 2.46, df = 38, p = 0.02) degree of habituation (as measured by a decrease in motor activity) than $M_2^{+/+}$ (n = 20) mice during the second exposure (4 hr interval) to a novel environment.

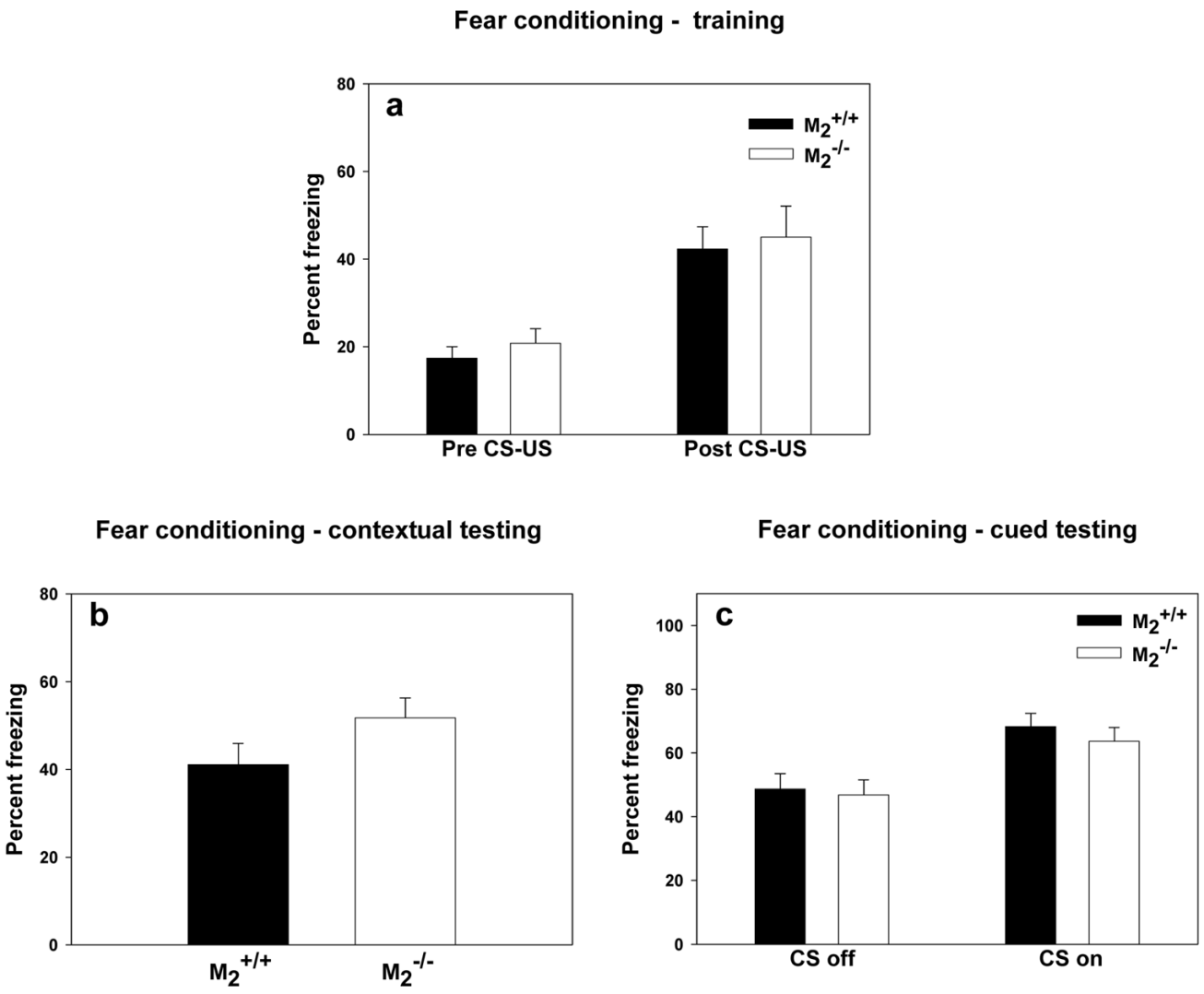

Figure 4.

Cued and Contextual Fear Conditioning. (a) During training freezing behavior did not differ by genotype ($M_2^{+/+}$, n = 23; $M_2^{-/-}$, n = 21) prior or subsequent to cue-shock pairings. (b) During testing for contextual conditioning, there was no effect of genotype on freezing. (c) During testing for cued conditioning, freezing was significantly greater during the first minute of "cue on" than during the last minute of "cue off" ($p < 0.01$), but there was no effect of genotype on freezing.

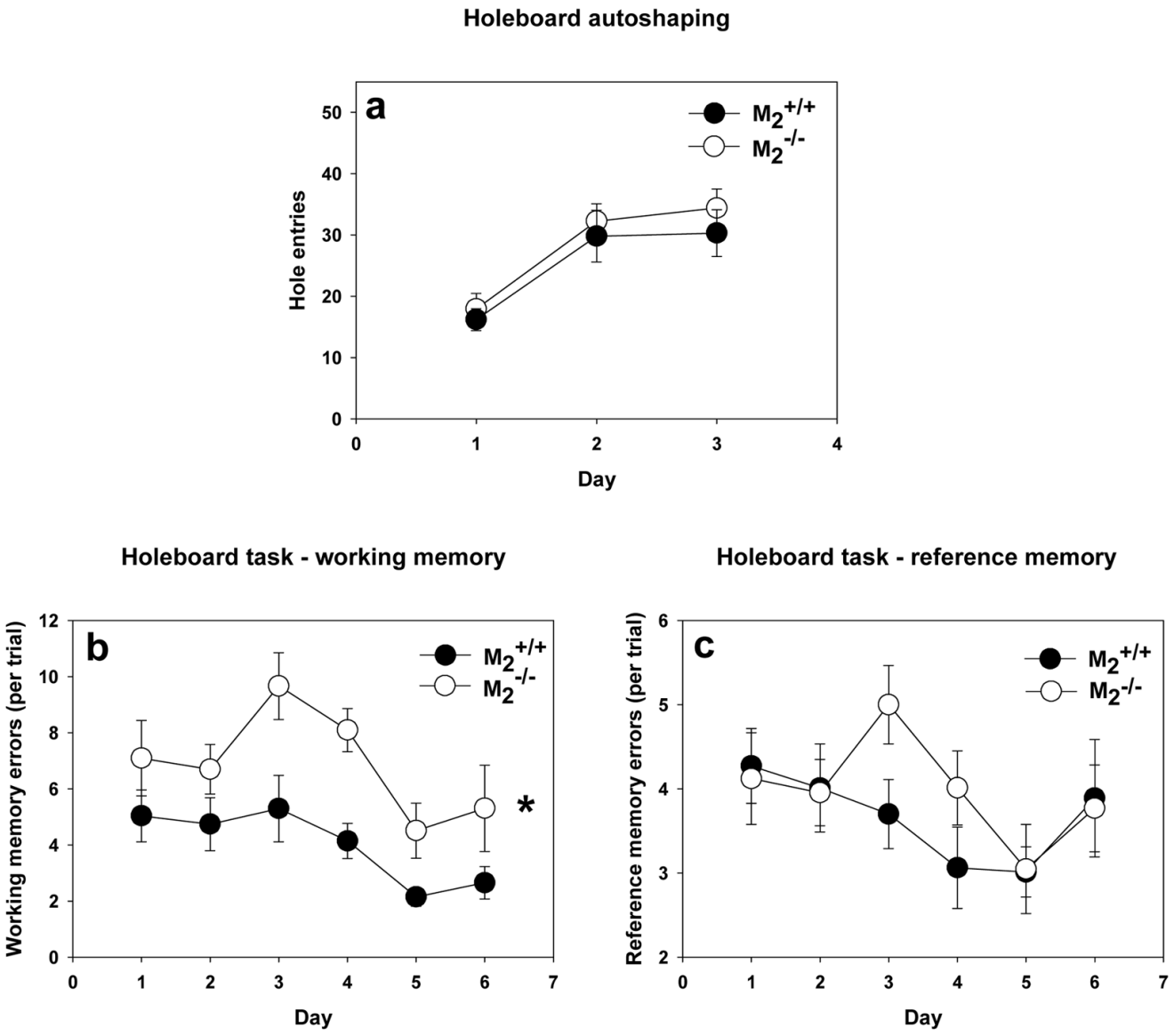

Figure 5.

Holeboard Task of Spatial Memory. (a) During autoshaping trials, the $M_2^{-/-}$ mice (n = 15) and $M_2^{+/+}$ mice (n = 16) did not differ in their propensity to explore the holes. (b) As indicated by the asterisk, during training in the holeboard task, $M_2^{-/-}$ mice made a significantly greater number of working memory errors than $M_2^{+/+}$ mice ($F_{(1, 29)} = 8.04$, p =0.008). (c) During training in the holeboard task, $M_2^{-/-}$ and $M_2^{+/+}$ made a similar number of reference memory errors.

Table 1

Assessment of general health and neurological function in Batch 1

	$M2^{+/+}$, male (n=9)	$M2^{-/-}$, male (n=11)	$M2^{+/+}$, female (n=12)	$M2^{-/-}$, female (n=15)
General health				
Fur condition (3 point scale)	2.5	3.0	3.0	3.0
Bald patches (%)	0	0	0	0
Piloerection (%)	0	0	0	0
Body tone (3 point scale)	2.0	2.0	2.0	2.0
Limb tone (3 point scale)	2.0	2.0	2.0	2.0
Motoric abilities				
Positional passivity (%)	0	0	0	0
Trunk curl (%)	100	100	100	100
Reflexes				
Forepaw reaching (%)	100	100	100	100
Righting reflex (%)	100	100	100	100
Corneal (%)	100	100	100	100
Pinna (%)	100	100	100	100
Vibrissae (%)	100	100	100	100
Toe pinch (%)	100	46	92	60
Reactivity				
Petting escape (%)	100	100	100	100
Dowel biting (3point scale)	1.2	1.0	1.5	1.1
Empty cage behavior				
Transfer freezing (%)	0	0	0	0
Wild running (%)	0	0	0	0
Stereotypies (%)	0	0	0	0
Exploration (3 point scale)	2.0	2.0	1.8	2.0
Grooming (3 point scale)	1.0	1.0	1.2	1.3