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Increased drinking in mutant mice with truncated M5 muscarinic receptor genes

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Abstract

The rarest and least understood of the muscarinic receptors is the M5 subtype. Recombinant methods were used to create mutant mice with a deletion in the third intracellular loop of the M5 receptor gene. Salivation induced by the nonselective muscarinic agonist pilocarpine (1 mg/kg sc) was reduced in homozygous mutants from 15 to 60 min after injection as compared with wild-type mice. After 18-h food and water deprivation, drinking was increased in homozygous mutants, but feeding was not increased. The mutant and wild-type mice had similar responses in tests of open-field exploration, seizures induced by pilocarpine (300 mg/kg) or hypothermia induced by pilocarpine (1–3 mg/kg). These results indicate that M5 muscarinic receptors are important for fluid intake and suggest that M5 receptors are involved in slow secretory processes.

Keywords: Acetylcholine; Dry mouth; Prandial; Locomotion; Salivation; Seizures

1. Introduction

Muscarinic receptors are found in high concentrations in many visceral organs and widely throughout the central nervous system (Levey, 1993; Vilaro et al., 1994). Five muscarinic receptor genes have been identified in human and rats (Bonner et al., 1987, 1988; Kubo et al., 1986; Liao et al., 1989; Peralta et al., 1987). Protein and mRNA products of these genes have been localized throughout the viscera and brain of rats and mice (Flynn et al., 1997; Hohmann et al., 1995; Levey, 1993; Levey et al., 1991; Vilaro et al., 1990, 1994; Weiner et al., 1990). How the five muscarinic receptor subtypes contribute to the many central and peripheral effects of muscarinic drugs is an active area of research (Gomeza et al., 1999a,b; Hamilton et al., 1997; Levine and Birdsall, 1997; Levine et al., 1999). Methods for deleting a single gene in mice provide a way to assess which subtypes are responsible for each effect of muscarinic agents. For example, the M1 receptor is needed for seizures induced by pilocarpine (Hamilton et al., 1997). The M2 receptor is needed for the antinociceptive effect of oxotremorine (Gomeza et al., 1999a,b).

Of the five muscarinic receptor subtypes, the M5 receptor is found in the lowest concentrations in the brain and visceral organs (Flynn et al., 1997; Levey, 1993; Reever et al., 1997). The M5 receptor has been the hardest muscarinic subtype to study for several reasons: (1) No selective ligands for the M5 receptor have been found. (2) Antibodies for the M5 receptor have not labeled rodent M5 receptors adequately for precise anatomical analysis unlike M1–M4 antibodies (Levey et al., 1991; Wolfe and Yasuda, 1995; Yasuda et al., 1993). (3) No tissue has been found where M5 receptors are in higher concentrations than all other muscarinic receptors (Flynn et al., 1997). Therefore, no pharmacologically identified M5 subtype is known. For these reasons, genetic methods are required to study the functions of the M5 receptor directly at the present time (Flynn et al., 1997; Yeomans et al., 1999, 2000).

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The highest concentration of M5 receptors known is in the submaxillary glands, where the concentration is still less than 20% of the muscarinic receptors found (Flynn et al., 1997). On the other hand, M5 mRNA, but not M1–M4 mRNA, is found near dopamine cells of the midbrain (Vilaro et al., 1994; Weiner et al., 1990), suggesting an important role for M5 receptors in muscarinic stimulation of dopamine neurons (Forster and Blaha, 2000; Gronier and Rasmussen, 1998; Lacey et al., 1990; Yeomans et al., 2000, 2001). The salivary glands are important because muscarinic agonists, such as pilocarpine, induce salivation at low doses (Iwabuchi et al., 1994; Murai et al., 1995). Many therapeutic drugs have antimuscarinic effects, leading to uncomfortable dry mouth and thirst.

2. Methods

2.1. Mutants

Mice that were mutant for the M5 muscarinic receptor gene were created using recombinant methods (Nagy et al., 1993). DNA for the rat M5 receptor gene (obtained courtesy of N. Buckley) was used to search a mouse cDNA library. An M5 receptor gene candidate was mapped with restriction enzymes (Fig. 1). A large region of this gene (0.5 kb) was deleted in the section of the gene that codes for the third intracellular loop (i3) of the protein. According to the rat M5 receptor gene sequence and the restriction enzymes used by us, the deletion should stretch from bases 753 to 1256 (Bonner et al., 1988; Levine and Birdsall, 1999), a length of 503 bases. Our map of the mouse gene is consistent with this location and this deletion length. This precise deletion site would result in a reading frame change and loss of the M5 receptor from AA251 and AA531.

To create a targeting vector, a neomycin resistance gene (neo) was spliced into the gene to replace the deleted segment as a positive selection factor, with a thymidine kinase gene (tk) placed externally as a negative selection factor (Fig. 1). The targeting vector (17 kb) was inserted into embryonic stem (ES) cells from mice of the 129SvJ strain by electroporation. The resulting ES cells were selected for homologous recombination using G418 and GANC, and the surviving ES cells were tested with Southern blot methods.

2.2. Locomotor activity

The initial behavioral study was designed to test the health and behavioral vigor of the mice. Mice were placed individually in square-bottom, open-field chambers ($43 \times 43 \times 30$ cm; Med Associates, Catalog no. ENV-515) and their activity was monitored continuously by computer-controlled sensors for 60 min. The mice were housed from birth under reversed day/night conditions so that testing occurred during the night part of their light cycle in dim light conditions. The test was the first exposure of the mice to the apparatus. The 12 mice tested (four mice of each genotype) were all between 51 and 54 days old on the day of testing.

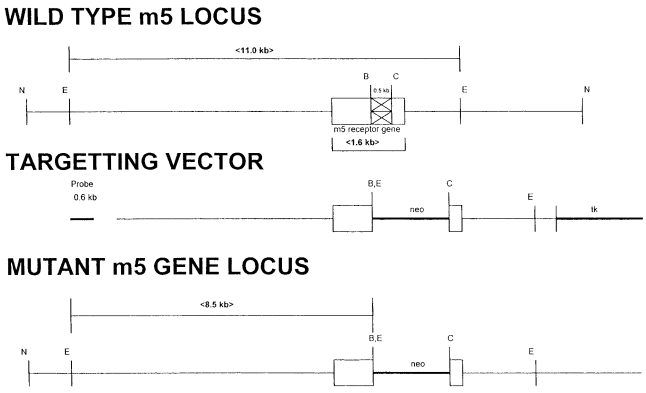

Fig. 1. Map of the M5 receptor gene and vicinity showing the wild-type form (above), targeting vector (middle) and mutant form (below). The restriction enzymes used were N = *Not*I, E = *Eco*RV, B = *Bgl*II and A = *Bam*HI. The selection factors used were neo = neomycin resistance gene and tk = thymidine kinase gene.

2.3. Salivation

The second set of studies tested the responses of mice to a muscarinic agonist (Gomeza et al., 1999a,b; Hamilton et al., 1997). Pilocarpine, a nonselective direct muscarinic agonist, induces salivation at low doses (Iwabuchi et al., 1994; Murai et al., 1995). This peripheral effect of pilocarpine is blocked by NMS (1 mg/kg ip), which does not cross the blood–brain barrier.

Mice were anesthetized with urethane (1.5 g/kg ip) and 35 min later were injected with pilocarpine (1.0 mg/kg sc). The 40–110-day-old mice (five mice in each group, matched for age) were weighed and placed on a 15-cm diameter circle of filter paper, allowing observation of external saliva secretions. As soon as drops were observed from the mouth, a preweighed cotton ball was placed in the mouth for 3–6 min. The time of removal of each cotton ball was recorded to calculate the amount of saliva secreted in each 3-min period (for the first 30 min) or each 5-min period (from 30 to 60 min).

2.4. Temperature- and pilocarpine-induced hypothermia

Mice with altered M2 receptor genes show reduced responses to muscarinic agonists, including lack of bradycardia in response to the cholinergic agonist carbachol, reduced antinociceptive response to the nonselective muscarinic agonist oxotremorine and reduced tremor and hypothermia in response to oxotremorine (Gomeza et al., 1999a). We measured temperature changes induced by the muscarinic agonist pilocarpine (0, 1 or 3 mg/kg sc) in another group of mice under urethane anesthesia (1.5 mg/kg ip

35 min before pilocarpine). Core temperature was monitored with a rectal probe for 120 min in age-matched sets of homozygous and wild-type mice (six mice in each group, age range 2–8 months old, at each dose).

2.5. Seizures induced by pilocarpine

Mice with altered M1 muscarinic receptor genes show reduced seizures in response to pilocarpine at doses of 200 and 300 mg/kg (Hamilton et al., 1997). Seizures are a test of the overall excitability of the cerebral cortex and limbic system and have been studied extensively using pilocarpine (Maslanski et al., 1994). Although M1 receptors are the most common muscarinic receptor in the forebrain, low levels of M5-selective binding and M5 mRNA are found in the cerebral cortex and hippocampus (Flynn et al., 1997; Levey, 1993).

For this study, five mice were tested in each genotype group, and the groups were matched for mass (all aged 6–7 months old). In order to prevent the peripheral effects of pilocarpine from affecting the results, NMS (1.0 mg/kg ip) was administered 10 min before pilocarpine (300 mg/kg ip). Seizure intensity was rated by a trained observer blind to the genotype of the mice using the following five-point rating scale: 0 = nothing; 1 = tail twitch; 2 = running with tail twitching or body biting; 3 = intermittent seizure activity; 4 = tonic (continuous) seizure activity; 5 = death.

2.6. Drinking and feeding

To study spontaneous, nondeprived drinking and feeding, mice were housed individually in plastic home cages for at least 3 days before testing on a reverse 12:12 light cycle. Each chamber had continuously available food and water. Total food and water intake was then monitored for 3 days by weighing food cups and water bottles each day at 10 AM (just before the time of lights out). The 12 mice studied were all 58–60 days old on the day of the first tests. The mean mass of the homozygous mutants was 27.9 g, of the heterozygous mutants was 32.0 g and of the wild-type mice was 30.2 g.

In the second test, drinking and eating were observed in these same 12 adult mice in their home cages after 18 h of food and water deprivation. The normal diet of the mice was Purina Rat Chow, available at all times. For 3 days before deprivation, the mice were exposed to the food to be tested in individual food cups in their home cages. In this test, both dry chow and sucrose were given as the food. All food and water was then removed at 4 PM of the last day and then replaced in the home cages at 10 AM the following morning (just before the time of lights out on a reverse 12:12 dark/light cycle). Total food and water intake was measured for 2 h after food and water replacement by weighing food and water cups before and after the 2-h period.

The methods used in these experiments were approved by Animal Care Committees of the University of Toronto following the guidelines of the Canadian Council on Animal Care.

3. Results

3.1. Genetics

Using internal and external probes for Southern blots, bands were observed near 6, 8.5 and 11 kb in several of the mutant ES cell lines, as compared with bands at 6 and 11 kb in wild-type ES cell lines (Fig. 2, left lanes). Three ES cell lines showing homologous recombination were aggregated and introduced into blastocysts from CD-1 mice. These embryos were placed in pseudopregnant females to produce chimeric offspring. Chimeric males were bred to produce one line of heterozygous offspring (F1). The F2 progeny, genotyped using tail DNA, showed Southern blot bands at 6, 8.5 and 11 (heterozygous for the mutation), 6 and 8.5 (homozygous) or 6 and 11 kb (wild type; Fig. 2, right lanes).

3.2. Health, growth and locomotor activity

A higher survival rate was found for the homozygous mutant mice than expected by Mendelian ratios, indicating better survival for mutant than wild-type mice. Homozygous mutant mice had normal birth mass. However, by the time of weaning (23 days old), they were 5–10 g smaller on average than wild-type mice for both sexes. Mutant mice had slightly larger litter sizes on average than wild-type mice (8.8 mice/litter ± 1.2 for homozygous mutants versus 6.2 mice/litter ± 0.7 for wild-type mice; $n = 28$ litters for both groups). Although this is not significantly different (t test, $P = .053$), it may be a factor in the mass difference at the time of weaning. By the time of testing, mutant mice had recovered to a mean of only 2–3 g lighter than wild-type mice of the same age.

The mutant mice had no apparent defects in health, motor function or behavioral vigor. For example, exploration of a

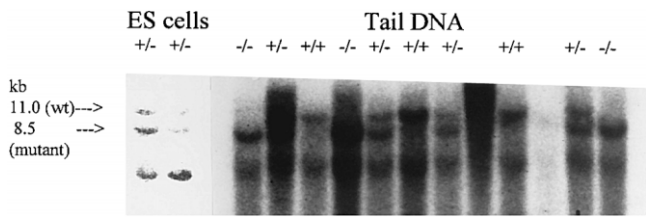

Fig. 2. Southern blots of DNA from embryonic stem cells (left two lanes) and tails (right lanes). Bands at 11.0 and 8.5 kb are marked by arrows, and the genotype is shown above or below each lane.

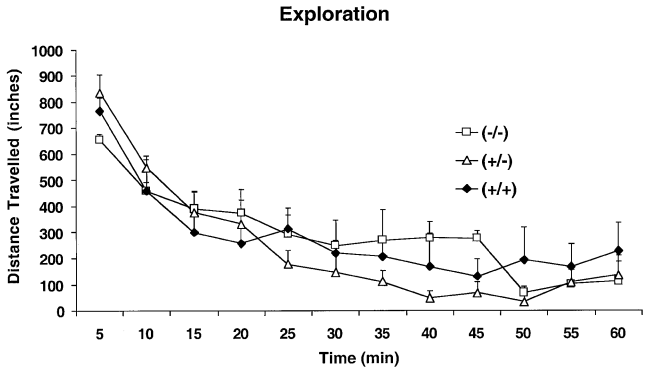

Fig. 3. Open-field exploration of a novel environment for 60 min. Distance traveled in each 5-min bin is shown for the three genotype groups. No significant differences were found. Error bars show standard errors of the mean in this and all subsequent figures.

novel open-field environment for 60 min was not significantly different in the three groups of mice [Fig. 3; $F(2,9) = 0.257$, n.s.].

3.3. Anatomy

No gross anatomical abnormalities in Nissl-stained brains or salivary glands of mutant mice were observed.

3.4. Salivation induced by pilocarpine

As shown in Fig. 4, profuse salivation was observed in wild-type controls a few minutes after pilocarpine administration then continued steadily, but less profusely, for at least 60 min. Homozygous mutants showed vigorous salivation in the first 15 min after pilocarpine administration but showed lesser salivation than wild-type mice from 18 to 60 min after injection. Wild-type mice showed 22% more total salivation than mutants (t test, $P < .01$).

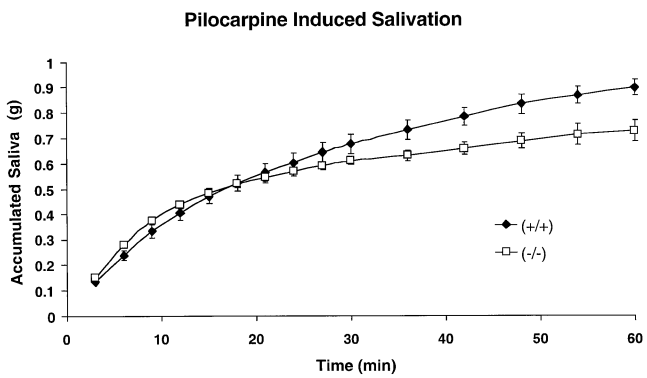

Fig. 4. Salivation induced by the muscarinic agonist pilocarpine (1 mg/kg). The cumulative amount of saliva collected in urethane-anesthetized mice is shown for each 3-min time bin for the first 30 min then each 5-min time bin from 30 to 60 min. Mutants differed from wild-type mice in total salivation.

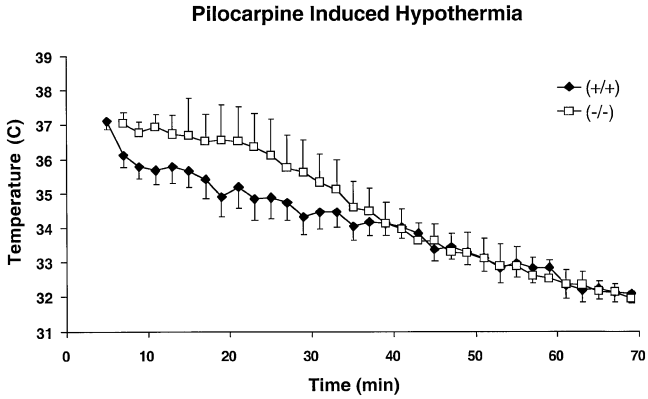

Fig. 5. Temperature changes induced by pilocarpine (3 mg/kg) in mice anesthetized with urethane.

3.5. Hypothermia induced by pilocarpine

Pilocarpine reduced temperature in a dose-dependent manner in both mutant and wild-type groups. There was no change in temperature when a sham injection was made, only a 1 °C reduction in temperature in both mutants and wild-type mice at the 1-mg/kg dose (data not shown), but a reduction of over 5 °C from 37 to below 32 °C in both groups at the 3-mg/kg dose (Fig. 5). No significant differences between mutant and wild-type groups were found at any time for 1- and 3-mg/kg doses.

3.6. Seizures induced by pilocarpine

Mice in all three groups showed intense seizure activity in response to 300-mg/kg pilocarpine (Fig. 6). The effectiveness of the NMS pretreatment on the peripheral effects of pilocarpine was observed by the lack of salivation and tearing in mice of all genotypes following pilocarpine administration. There were no significant differences in seizure intensity at any time frame within the 60-min test ($P < .05$).

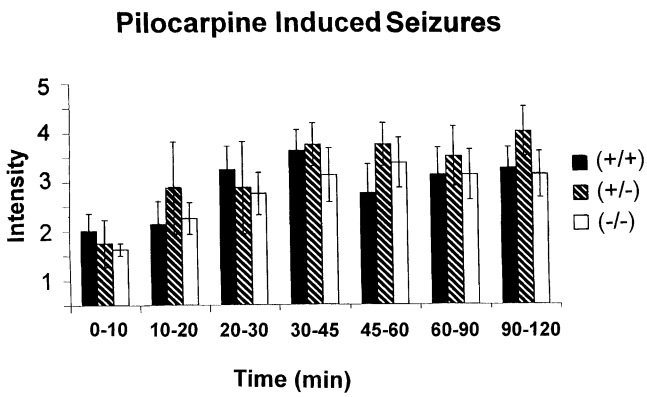

Fig. 6. Seizure levels induced by pilocarpine (300 mg/kg) in mice pretreated with *N*-methyl-scopolamine. No significant differences were found between groups.

3.7. Drinking and feeding

In the free feeding and drinking test, homozygous M5 mutant mice were found to have total food and water intake, expressed as a percentage of body weight, of slightly above wild-type controls with heterozygous mice in between [$F(2,9) = 1.93$ and 2.68, respectively, $P = .199$ and .122; Fig. 7].

After depriving the mice of dry food and water for 18 h, homozygous mutants drank over twice as much water per unit body mass as wild-type controls [$F(2,9) = 13.82$, $P = .002$] and heterozygous mutants (Fig. 8, top). During the same 2-h test period, there were no significant differences in dry food (chow and sucrose) intake between mutants and wild-type mice [$F(2,9) = 1.3$, $P = .319$; Fig. 8, bottom].

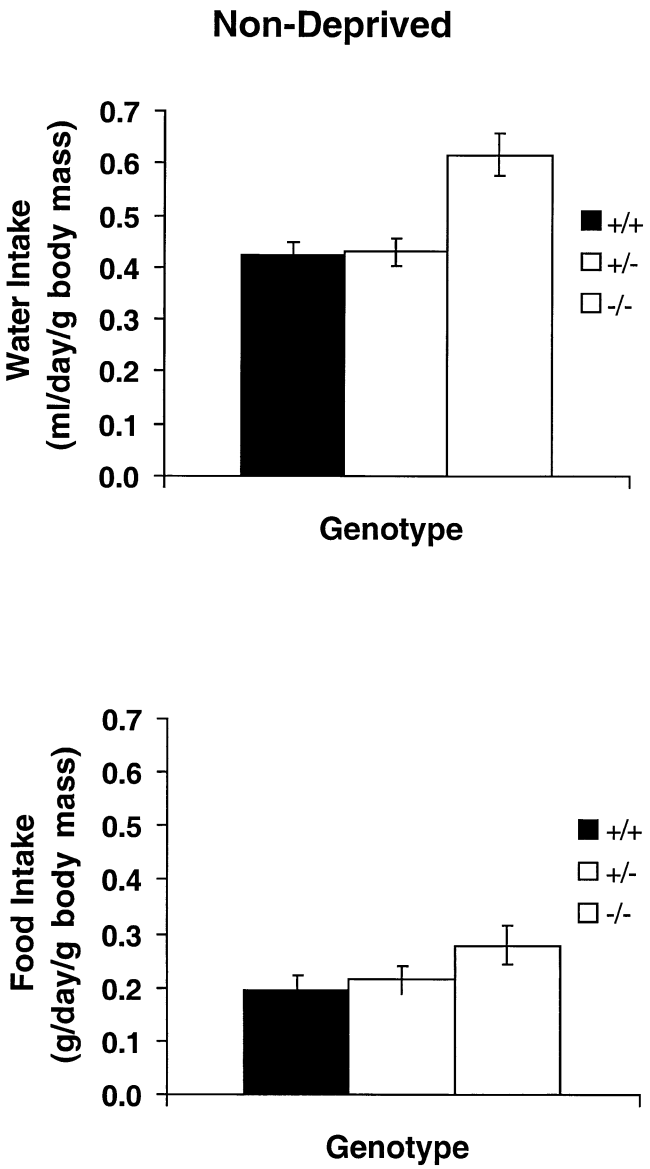

Fig. 7. Total drinking and feeding each day in free-feeding mice without deprivation. No significant differences were found.

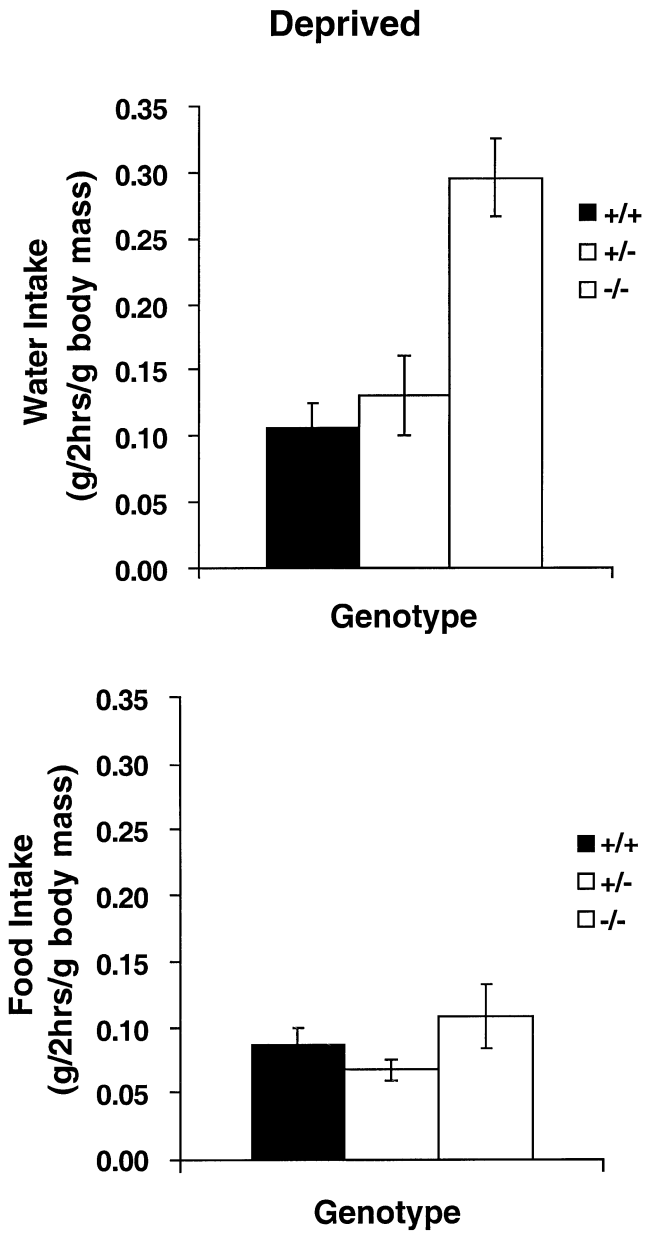

Fig. 8. Total drinking in a 2-h test period after 18-h food and water deprivation (above). Total feeding in a 2-h test period after 18-h food and water deprivation (above) in the same test. Food consisted of chow and sucrose. Significant differences are shown by stars.

4. General discussion

Mice with mutant M5 receptor genes showed greatly increased water intake after food and water deprivation conditions and slightly reduced salivation in response to pilocarpine. Seizure responses to high doses of pilocarpine were similar to those of wild-type mice, as were locomotor responses in an open field.

4.1. Salivation induced by pilocarpine

The decreased secretion in mutant mice 15–60 min following pilocarpine injection indicates that M5 receptors are not important for the majority of salivation but only for the delayed salivation. Accordingly, intact M5 muscarinic receptors are important only for the sustained salivation that occurs 15–60 min after pilocarpine administration. The rapid salivation that occurs in the first 15 min does not depend on M5 receptors, consistent with previous data that pharmacologically defined M3 receptors and genetically defined M3 or M4 receptors are most important for the salivation response (Iwabuchi et al., 1994; Levey et al., 1991; Matsui et al., 2000).

Salivation induced by the much higher 300-mg/kg dose of pilocarpine was still blocked by NMS in both M5 mutants groups or wild-type mice. This supports the idea that pilocarpine-induced salivation results entirely from actions on peripheral muscarinic receptors in all three genotypes. The deficit in sustained salivation in M5 mutants might result from mutation of M5 receptors in the salivary glands or secondarily from changes in other peripheral organs.

Recently, sustained secretion of dopamine observed in the nucleus accumbens of these M5 mutant mice was found to be totally lost 10–40 min following mesopontine stimulation (Forster et al., in press). Furthermore, M5 receptors in vitro show much slower binding to, and dissociation from, NMS than do any other muscarinic subtypes (Ferrari-DiLeo et al., 1994). Therefore, M5 receptors may provide slower neural and secretory effects than all other muscarinic receptors (Yeomans et al., 2001). This conclusion may explain why M5 receptors are often colocalized with other cholinergic receptors that provide faster responses.

4.2. Seizures and hypothermia induced by pilocarpine

By contrast, pilocarpine was still effective in inducing seizures in the central nervous system in all three groups and in reducing temperature in homozygous mutants. Therefore, unlike M1 mutants, M5 mutants showed normal seizures in response to pilocarpine. Unlike M2 mutants, the M5 mutation did not alter the hypothermic effect of pilocarpine. These results strongly support the general conclusion that the five different muscarinic receptors mediate clearly different functions in both the periphery and in the central nervous system, even where the receptors are colocalized (Levine et al., 2001).

4.3. Drinking and feeding

M3 mutant mice exhibit reduced feeding and body fat (Yamada et al., 2001). M5 mutant mice in the present study, however, showed significantly increased drinking relative to wild-type mice when the mice were deprived of food and water for 18 h. The mechanisms for this increased feeding and drinking are not yet determined. The evidence that only homozygous mutants showed a reliable difference from wild-type mice suggests that the trait is recessive.

Previous work indicates that complete loss of salivation, by salivary duct ligation or by lesions to brain centers, results in (1) massive increases (by three to five times) in drinking at the time of feeding (“prandial drinking”) and (2) a reduction in feeding shortly after salivation loss, resulting in a loss of body mass followed later by gradual recovery in feeding and body mass along with continued increases in prandial drinking (Kissileff, 1969; Kissileff and Epstein, 1969; Ramos et al., 1988; Stricker, 1979).

In the present data, water intake was higher in homozygous mutants than wild-type mice in deprivation conditions. Furthermore, the salivation deficit in M5 mutant mice in response to pilocarpine is only about 20%. This suggests that the hyperdipsia seen in M5 mutants does not result mainly from prandial drinking resulting from inadequate salivation.

One possible experiential explanation for the increased hyperdipsia after deprivation is that M5 mutant mice were raised in larger litters before weaning, resulting in lower body mass at the time of weaning. Consequently, mutants may have been previously exposed to more milk deprivation and so might respond more strongly to the deprivation challenge than wild-type mice that came from smaller litters and so were not as deprived.

Whether the M5 receptor mutation leads to increased drinking by central or peripheral factors is not yet clear. Centrally administered muscarinic agonists increase drinking (Schiavone et al., 1987), and muscarinic antagonists decrease angiotensin II-induced drinking (Lee et al., 1996). Therefore, the M5 contribution to drinking is opposite to the central effect of muscarinic receptors. By contrast, increased thirst is observed, along with dry mouth and throat, in patients administered low doses of antimuscarinic drugs (e.g., 1-mg atropine; Shrader and Greenblatt, 1971; Weiner, 1985). The present results suggest that the contribution of M5 muscarinic receptors to this effect may be clinically relevant.

5. Conclusions

M5 receptor mutation resulted in hyperdipsia following food and water deprivation. M5 mutants also showed decreased sustained salivation in response to pilocarpine. These changes in salivation and drinking suggest further studies of the involvement of M5 receptors in gland secretions, respiratory secretions and other tissues involved in water regulation, such as the kidneys and bladder.

Acknowledgments

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