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Melanocortin-5 Receptor Deficiency Reduces a Pheromonal Signal for Aggression in Male Mice

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Abstract

Mice lacking the melanocortin-5 receptor (MC5R) exhibit decreased sensitivity to the stimulatory effects of systemic melanocortin injections on aggressive behavior. Because the pheromone-producing preputial gland expresses the MC5R, we tested the hypothesis that decreases in preputial pheromones underlie the behavioral deficit. Here we show that MC5R deficiency decreases preputial and urine levels of the sex pheromones, alpha- and beta-farnesene, relative to wild-type mice. We also demonstrate that farnesenes potently stimulate aggression in mice. Moreover, farnesene-stimulated aggression is reduced in MC5R-deficient mice, relative to wild-type mice. Our results suggest that activation of the MC5R promotes aggression by increasing farnesene signaling.

Key words: aggression, farnesene, gas chromatography–mass spectrometry, gene knockout, melanocortin-5 receptor, pheromones

Introduction

In male mice, social dominance is acquired during aggressive encounters that are influenced by pheromonal signals. The male rodent preputial gland releases aggression-promoting pheromones to the urine (Jones and Nowell, 1973; Novotny *et al.*, 1985; Ingersoll *et al.*, 1986). Additionally, preputial hypertrophy in dominant males (Hucklebridge *et al.*, 1972) and the correlation between induced preputial atrophy and subordinate behavior (Brain *et al.*, 1991) suggest that preputial pheromones may promote the acquisition and maintenance of social status. However, these relationships are poorly understood.

Preputial activity depends on the circulating melanocortin, alpha-melanocyte-stimulating hormone (α-MSH) (Cooper *et al.*, 1975; Ebling *et al.*, 1975), and chronic melanocortin administration stimulates preputial lipogenesis, hypertrophy, hyperplasia, and pheromone secretion in rodents (Ozegovic and Milkovic, 1972; Thody and Shuster, 1975; C. Morgan and R.D. Cone, submitted for publication). In this regard, melanocortins act synergistically with androgens (Cooper *et al.*, 1975; Ebling *et al.*, 1975; Thody and Shuster, 1975). Therefore, endogenous melanocortins may stimulate biosynthesis of aggression-promoting pheromones in the preputial gland.

Of the five known melanocortin receptors (MC1R–MC5R) (Cone *et al.*, 1996), the MC5R is most abundantly expressed in the preputial gland (Chen *et al.*, 1997; van der Kraan *et al.*, 1998). Melanocortin injection releases into the urine of male mice an unidentified chemical signal that stimulates attacking behavior from test mice when swabbed onto stimulus mice (Nowell and Wouters, 1975; Nowell *et al.*, 1980), and we and others have shown that MC5R deficiency increases the release of this signal (Caldwell *et al.*, 2001; Caldwell and Lepri, 2002; Morgan *et al.*, 2004). We have also shown that MC5R knockout (KO) mice exhibit reduced aggression and elevated defense toward wild-type (WT) opponents (Morgan *et al.*, 2004), and that these mutant mice are less sensitive to melanocortin stimulation of aggression and preputial growth (C. Morgan and R.D. Cone, submitted for publication). In the present study, we tested the hypothesis that decreased preputial pheromones contribute to the decreased aggressive responses of MC5R KO mice.

Materials and methods

Animals and housing

Male wild-type (WT) and MC5R knockout (KO) mice on a C57BL/J6 genetic background (Chen *et al.*, 1997) were maintained in group-housed conditions (3–5/cage). Room temperature was maintained at 22 ± 2°C on a standard light-dark cycle (12 h light: 12 h darkness; lights on from 06.00 to 18.00 h) with food and water provided *ad libitum*. At 8 weeks of age, mice were individually housed for at least 4 weeks prior to behavioral testing. Mice were housed in filter-top cages within a Maxi-Miser System (Thoren Caging Systems; Hazelton, PA) that provides positive ventilation and air exhaust from each cage. During handling, individual cages of mice were transferred to a fume hood supplied with negative ventilation. Thus, the exchange of olfactory signals between mice was minimized. All animal experiments were approved by the Institutional Animal Care and Use Committee.

Surgical procedures

Preputialectomy (PPX)

Mice were anesthetized with an intraperitoneal injection of 100 mg/kg ketamine and 10 mg/kg xylazine. A transverse incision was made through the skin of the lower abdominal region. Subcutaneous fat and connective tissue were removed, and the preputial gland was excised at the base and placed on dry ice until weighed.

Gonadectomy (GDX)

The supplier (Taconic Farms; Germantown, NY) performed the gonadectomies. Surgically altered mice were individually housed (following PPX) or socially housed (following GDX), and permitted at least three weeks for post surgical recovery, before being used in behavioral tests.

Behavior

Test mice were paired with GDX stimulus mice in neutral cages with fresh bedding during the light phase. Prior to testing, the tail the stimulus mouse was marked with a black marker to distinguish it. Encounters lasting 5 min were videotaped and behaviors were scored from the videotapes by trained observers who were blind to the treatments. Aggressive behavior consisted of offensive (biting and striking) and dominant (dominant posturing, chasing, and tail rattling) behaviors. Each mouse was scored for each occurrence of these behaviors.

Gas chromatography–mass spectrometry

Analysis by gas chromatography coupled to mass spectrometry (GC–MS) of volatile compounds was performed as previously described (Novotny *et al.*, 1974). Preputial extracts or pooled 10 ml aliquots of urine from wild-type and MC5R-deficient mice were homogenized in 1 ml of distilled water, 200 mg of ammonium sulfate and 4 µl of internal standard (200 p.p.m. 7-tridecanone in methanol). Samples were extracted at 50°C with purified helium at 70 ml/min for 30 min. The upper phase was passed through a water-chilled condenser then a pre-column packed with 4 mg of Tenax GC adsorbent (Applied Science Laboratories' State College, PA). Compounds trapped on the pre-column were desorbed in the heated injection port (250°C) of a Finnegan MAT Magnum GC/MS Instrument (San Jose, CA) and trapped in a liquid nitrogen-cooled capillary column. The analytical column was a 30 mm × 0.25 mm (ID) DB-5 (J & W Scientific, Folsum, CA). Helium was used as the carrier at an inlet pressure of 12 PSI. The oven temperature was maintained at 30°C for 5 min, and elevated to 200°C at a rate of 2°C/min. Transfer line temperature was 290°C. Peak areas of compounds were normalized to peak area of the internal standard.

Farnesene treatment

Mice were permitted to inhale a 250 or 500 p.p.m. solution of α- and β-farnesene or vehicle (distilled water) from a cotton swab for 1 min in their home cages. The test animals were maintained in their home cages for 60 min before behavioral testing.

Statistical analyses

Data were analyzed with SigmaStat 2.0 statistical software (SPSS, Inc. Chicago, IL). Because behavioral data from farnesene-stimulated mice (Figure 2B) were not normally distributed or contained unequal variance, the Mann-Whitney rank sum test was used. All other data were analyzed using Student's *t*-test. GC–MS data were log-transformed before statistical analysis.

Results

GC–MS analysis of preputial extracts revealed smaller peaks corresponding to the sesquiterpenes, α-farnesene and β-farnesene, as well as an unidentified sesquiterpene and ethyl nonanoate in the KO mice, relative to WT littermates (Figure 1A, upper panel). Statistical analysis of log-transformed data, by Student's *t*-test, confirmed that in WT mice the levels of α-farnesene, β-farnesene, uncharacterized sesquiterpene and ethyl nonanoate exceeded those of the KO mice by 39, 27, 207 and 185%, respectively (Figure 1B, upper panel).

GC–MS analysis of pooled urine samples revealed reduced peaks corresponding to farnesenes in the KO mice (Figure 1B, lower panel), relative to WT mice. Quantitative analysis of the urine data suggested that in WT mice the levels of α- and β-farnesene exceeded those of KO mice by as much as 62 and 92%, respectively (Figure 1B, lower panel). Sample pooling (10 ml from 8–10 mice per treatment group) prevented statistical analysis. Under our experimental conditions, the uncharacterized sesquiterpene and ethyl nonanoate were not detected.

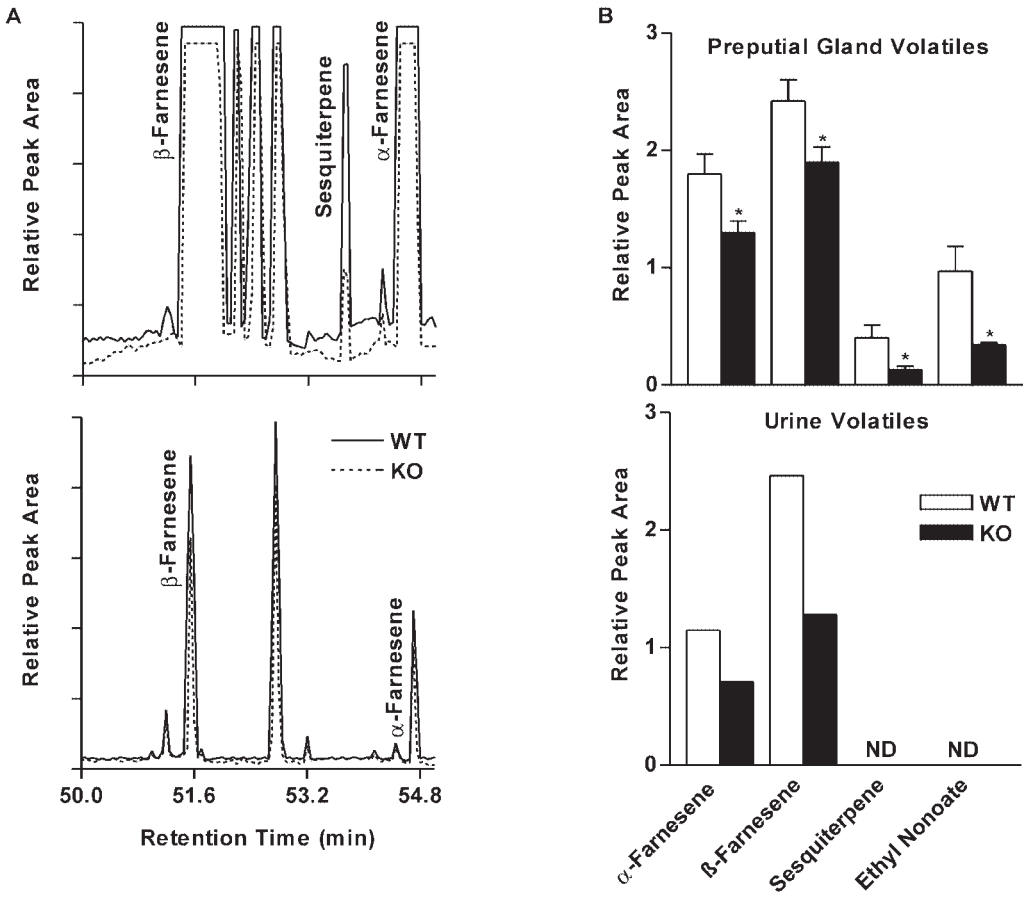

Figure 1 Gas chromatography-mass spectrometry analysis of volatile compounds of preputial origin. **(A)** Upper panel: partial chromatogram of volatile compounds extracted from the preputial gland of a wild-type (WT) mouse and an MC5R-deficient (KO) mouse. Peaks corresponding to sex pheromones (α- and β-farnesene) and an uncharacterized, but structurally related compound (a sesquiterpene) are shown. Lower panel: partial chromatogram of volatile compounds extracted from the urine of a WT mouse and a KO mouse. Peaks corresponding to α- and β-farnesene are shown. Preputial and urine peaks for ethyl nonanoate are not shown. **(B)** Upper panel: preputial levels of α- and β-farnesene, the uncharacterized sesquiterpene, and ethyl nonanoate were reduced in KO mice ($n = 8$), relative to WT mice ($n = 10$). Lower panel: urine levels of α- and β-farnesene were reduced in a 10 ml sample pooled from eight KO mice, relative to a 10 ml sample pooled from 10 WT mice. The uncharacterized sesquiterpene and ethyl nonanoate were not detected (ND) under these experimental conditions. Means (± SEM) are shown (*$P < 0.05$).

To determine the effects of farnesenes on aggression in WT and KO mice, we tested vehicle-treated and farnesene-treated mice against stimulus mice. We also tested preputial-ectomized (PPX) mice because we have shown previously that PPX and KO mice are insensitive to stimulatory effects of melanocortins on aggression (C. Morgan and R.D. Cone, submitted for publication). Sixty min following a one-minute inhalation of a low (250 p.p.m.) dose, farnesenes increased aggression 18-fold in WT mice (Figure 2A). Analysis by Student's *t*-test revealed that farnesene-stimulated aggression was reduced in the KO mice ($P < 0.05$) and PPX mice ($P < 0.01$), relative to the WT mice.

Analysis by Mann–Whitney rank sum test revealed that 60 min following exposure to a high (500 p.p.m.) dose of farnesenes, WT and KO mice displayed similar increases in aggression (Figure 2B). The aggression-stimulating effects of the high-dose farnesene treatment were transient, as the levels of aggressive behavior in WT and KO mice returned to basal 7 and 14 days, respectively, after single exposures (Figure 2B).

Discussion

The results of the present study demonstrate that MC5R deficiency in mice reduces preputial gland and urine levels of α- and β-farnesene. In addition to their actions as sex pheromones (Ma *et al.*, 1999; Novotny *et al.*, 1999), we show that farnesenes are potent aggression-promoting pheromones. Although statistical analysis was not performed on the urine data because of pooling, we collectively analyzed by GC–MS samples from 8–10 mice per genotype. Because the reduction in urine farnesenes (and other compounds) was accompanied by decreases in preputial farnesenes, we conclude that decreased biosynthesis and secretion occur in MC5R KO mice. Therefore, the reduction of endogenous

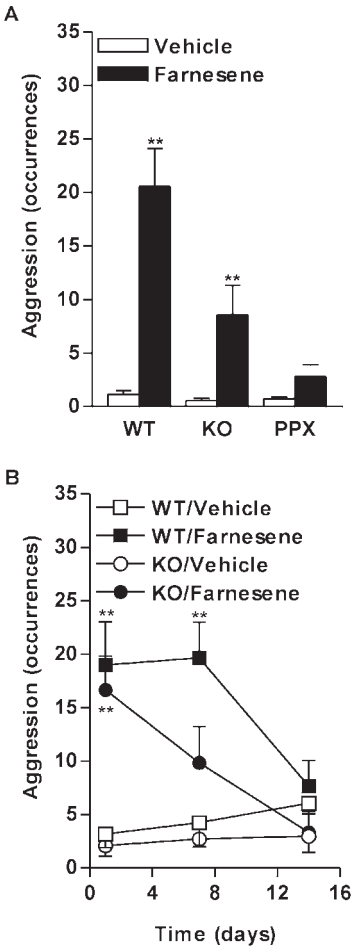

Figure 2 Farnesene effects on aggressive behavior. **(A)** Brief inhalation of 250 p.p.m. α- and β-farnesene for 1 min in the home cage stimulated aggression in wild-type (WT), MC5R-deficient (KO), but not preputial-ectomized (PPX), mice during 5 min interactions with stimulus mice. **(B)** On day 1, brief inhalation of 500 p.p.m. farnesenes stimulated the aggressive ebehavior of WT and KO mice toward stimulus mice. WT mice, but not KO mice, exhibited farnesene-stimulated aggression on day 7. WT and KO mice exhibited basal levels of aggression on day 14. Means (± SEM) are shown ($n = 6$; $^{**}P < 0.01$).

farnesene levels may contribute to the reduction of aggressive behavior in the mutant mice toward WT opponents (Morgan *et al.*, 2004).

At a low dose, farnesenes stimulate less aggression in the KO mice, relative to WT mice. The diminished response might be partly due to reductions in the levels of preputial and urine farnesenes of the KO mice. Consistent with this idea, farnesene-stimulated aggression is markedly reduced in PPX mice. The preputial gland is the only exocrine gland known to secrete aggression-promoting pheromones downstream of the urinary bladder (Ingersoll *et al.*, 1986), and bladder urine does not contain farnesenes (Novotny *et al.*, 1990). Therefore, our finding that farnesenes stimulate aggression confirms that the preputial gland is the principal source of farnesenes in the urine. Thus, the responsiveness of WT, KO, and PPX mice to exogenous farnesenes correlated with their endogenous farnesene levels.

Inhalation of a high dose of farnesenes transiently increases aggression similarly in WT and KO mice. This finding demonstrates that, at the high dose, farnesenes are sufficient to overcome behavioral defects in the KO mice. Moreover, it suggests that a fifty-percent difference in exogenous farnesene concentration (500 versus 250 p.p.m.) is detectable and has important behavioral consequences for the KO mice. Presumably, prolonged decreases in self-exposure to farnesenes might negatively affect responsiveness to these same pheromones. Therefore, it is plausible that the observed deficits in urine farnesenes contribute to the decrease of farnesene-stimulated aggression in the mutant mice.

Even at the high dose, farnesene-stimulated aggression diminished more rapidly in the KO mice, relative to WT mice. The reduced farnesene release might have contributed to the more rapid return to basal levels of aggressive behavior in the KO mice. Additionally, because the MC5R is thought to be expressed in the olfactory bulbs and brain (Griffon *et al.*, 1994; Chen *et al.*, 1997), reduced farnesene responsiveness in the KO mice might have been due to decreased olfactory detection and central processing of the farnesene signal.

It is unlikely that the KO mice exhibit a universal defect in pheromone detection. Untreated MC5R KO mice in homotypic pairs do not exhibit decreased aggression (Morgan *et al.*, 2004), demonstrating that the behavioral responses of these mice are context-dependent. Furthermore, MC5R-deficiency does not reduce responsiveness to an aggression-promoting pheromonal signal that is released by melanocortin injection (C. Morgan and R.D. Cone, submitted for publication). Future studies should be able to determine the specific role of the MC5R in farnesene release and farnesene processing.

Taken together, the requirement for long-term preputial stimulation in melanocortin-stimulated aggression and preputial hypertrophy (C. Morgan and R.D. Cone, submitted for publication), and abundant preputial MC5R expression (Chen *et al.*, 1997; van der Kraan *et al.*, 1998), suggest that MC5R-deficiency may reduce biosynthesis and secretion of preputial pheromones that stimulate aggression. This view is supported by the present finding that MC5R-deficient mice exhibit deficits in farnesenes and other volatile compounds that originate in the preputial gland and are secreted into the urine.

Farnesenes are male pheromones that induce estrus in female mice (Ma *et al.*, 1999), and they are aversive olfactory signals that discourage territorial urine marking in male mice (Jemiolo *et al.*, 1992). Furthermore, the preputial gland is the only known source of farnesenes in the urine of male mice (Ingersoll *et al.*, 1986; Novotny *et al.*, 1990). Therefore, MC5R-deficiency and preputialectomy appear to produce a common physiological defect (i.e. reduced endogenous

farnesene levels), as well as a common behavioral defect (i.e. reduced aggression).

We have shown previously that melanocortin-stimulated aggression, following repeated systemic injection, requires preputial hypertrophy, and that MC5R KO mice are deficient in the preputial and behavioral responses to melanocortin treatment (C. Morgan and R.D. Cone, submitted for publication). We have also shown previously that following surgical removal of the testes preputial atrophy accompanies decreased aggressive behavior in WT mice (C. Morgan and R.D. Cone, submitted for publication). Our present findings suggest that the behavioral insensitivity to melanocortin treatment might be due to deficits in a pheromonal pathway involving preputial farnesenes. Because melanocortin treatment, in the previous study did not involve the exchange of male pheromonal signals between opponents, it is plausible that the farnesene signal promotes aggression in a self-stimulatory manner.

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