



# Histamine Released from Epidermal Keratinocytes Plays a Role in $\alpha$ -Melanocyte–Stimulating Hormone-Induced Itching in Mice

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Sunburn, wound repair, and chronic renal failure with hemodialysis are usually accompanied by both pigmentation and itching. Proopiomelanocortin-derived  $\alpha$ -melanocyte–stimulating hormone ( $\alpha$ -MSH) is produced in response to external stimuli, such as UV irradiation, and is involved in cutaneous pigmentation. However, it is unclear whether  $\alpha$ -MSH is also involved in the itching. We therefore investigated whether  $\alpha$ -MSH elicited itch-related responses in mice. We found that an intradermal injection of  $\alpha$ -MSH induced hind-paw scratching, an itch-related response, in mice. The  $\alpha$ -MSH–induced scratching was inhibited by the  $\mu$ -opioid receptor antagonist naltrexone and the H<sub>1</sub> histamine receptor antagonist terfenadine. In mast cell-deficient mice,  $\alpha$ -MSH also elicited scratching, which was inhibited by terfenadine. The immunoreactivity for L-histidine decarboxylase, a key enzyme required for the production of histamine, histamine, and the melanocortin 1 and 5 receptors were shown in not only mast cells but also keratinocytes in murine skin. In addition to the expression of L-histidine decarboxylase and melanocortin 1 and 5 receptors, the mouse keratinocyte cell lines (Pam212) also showed immunoreactivity for L-histidine decarboxylase, histamine, and melanocortin 1 and 5 receptors. The application of  $\alpha$ -MSH induced the release of histamine from Pam212 cells. These findings indicate that  $\alpha$ -MSH may play an important role in the itching associated with pigmented cutaneous lesions and that the histamine released from keratinocytes is involved in this  $\alpha$ -MSH–induced itching. (*Am J Pathol* 2015, ■: 1–8; <http://dx.doi.org/10.1016/j.ajpath.2015.07.015>)

Q6 Itch is an unpleasant sensation associated with the immediate desire to scratch, thereby making the cutaneous symptoms worse. Several skin and general conditions are associated with both pigmentation and itch. In addition, itch associated with sunburn is widely recognized to be the result of an inflammatory reaction to UV irradiation, which increases the amount of itch and erythema, after which the healing process results in later skin pigmentation.<sup>1</sup> Gilchrist et al<sup>2</sup> reported that the histamine level rises immediately after the onset of UV-induced erythema. Itch and cutaneous pigmentation also occur in association with chronic wounds and hypertrophic scars. Paul<sup>3</sup> noted that wound-related itch is more frequently observed in patients with severe wounds. Itch may also be caused by dry skin or serious internal diseases, such as chronic renal failure that requires hemodialysis, which is usually accompanied by cutaneous pigmentation. Taken

together, it is still unclear if the underlying mechanisms of itch in these pruritic diseases involve the interaction between cutaneous pigmentation and itch.

$\alpha$ -Melanocyte–stimulating hormone ( $\alpha$ -MSH) is one of the neuropeptides that is generated through the cleavage of a precursor protein called proopiomelanocortin, which is produced through the production of corticotropin-releasing hormone, also known as corticotropin-releasing factor, after various stressors, such as UV irradiation.<sup>4–7</sup>  $\alpha$ -MSH receptors are known as melanocortin receptors (MC1R, MC3R, MC4R, and MC5R).<sup>4</sup> These receptors belong to the

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G protein-coupled receptor family, and the activation of these receptors increases the production of cAMP.<sup>8</sup> In addition, it was also reported that  $\alpha$ -MSH increases intracellular-free  $\text{Ca}^{2+}$  concentration in human embryonic kidney cells that express these melanocortin receptors.<sup>9</sup> In the skin, MC1R and MC5R are mainly expressed.<sup>10</sup>  $\alpha$ -MSH produced by external stimuli, such as UV irradiation, induces cutaneous pigmentation through the activation of MC1R.<sup>4,6,7,10,11</sup>  $\alpha$ -MSH is also increased in plasma of patients with chronic hemodialysis<sup>12</sup> and in epidermal keratinocytes during cutaneous wound repair.<sup>13</sup> However, no previous reports indicate whether  $\alpha$ -MSH is also involved in the itching that results from these factors.

Here, we investigated whether  $\alpha$ -MSH elicited itch in mice. Furthermore, we performed a series of experiments to elucidate the mechanism underlying the development of  $\alpha$ -MSH-induced itch, focusing on the involvement of histamine, which is known to play an important role in the pathogenesis of itch.

## Materials and Methods

### Animals

Male ICR mice (4 to 9 weeks old), mast cell-deficient WBB6F1  $W/W^u$  (6 to 9 weeks old), and the normal littermates (WBB6F1<sup>+/+</sup>, 6 to 9 weeks old) were used in this study. These mice were purchased from Japan SLC (Hamamatsu, Japan). They were housed in a room with controlled temperature (21°C to 23°C), humidity (45% to 65%), and light (7:00 AM to 7:00 PM) conditions. Food and water were available *ad libitum*. Procedures for animal experiments were approved by the Committee for Animal Experiments at the University of Toyama.

### Cell Culture and siRNA Treatment

The murine epidermal cell line (Pam212) was cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100  $\mu\text{g}/\text{mL}$  streptomycin under standard cell culture conditions (37°C, 5%  $\text{CO}_2$  in a humidified incubator).

In a part of the experiment, siRNA-treated cells were used. siRNAs as a control, MC1R and MC5R, were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). These siRNAs were transfected with Lipofectamin RNAi MAX reagent (Life Technologies, Carlsbad, CA). The transfection was performed according to the manufacturer's protocol (Life Technologies).

### Agents

$\alpha$ -MSH was purchased from Peptide Institute, Inc. (Osaka, Japan). For *in vivo* experiments,  $\alpha$ -MSH was dissolved in physiologic saline and was injected intradermally in a volume of 50  $\mu\text{L}$  into the rostral skin of mice. For *in vitro* experiments,

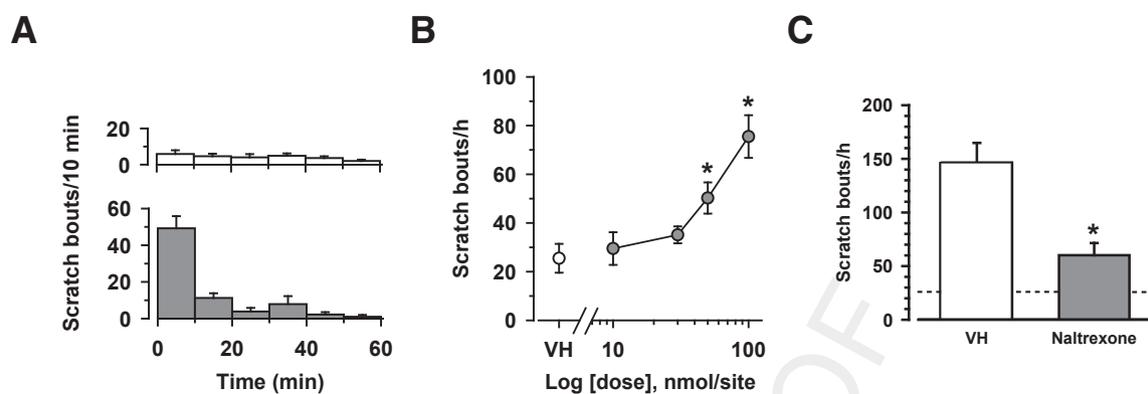
$\alpha$ -MSH was dissolved in Opti-MEM (Thermo Fisher Scientific Inc., Waltham, MA). Naltrexone hydrochloride (Sigma-Aldrich, St. Louis, MO) was dissolved in physiologic saline and injected subcutaneously 15 minutes before  $\alpha$ -MSH injection. Terfenadine (Sigma-Aldrich) was suspended in 0.5% sodium carboxy methylcellulose (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and was administered 30 minutes before  $\alpha$ -MSH injection. SQ 22,536 (Tocris Bioscience, Bristol, UK) and EGTA (Dojindo Laboratories, Kumamoto, Japan) were dissolved in dimethyl sulfoxide and diluted with Opti-MEM (final concentration dimethyl sulfoxide, 0.1%). These agents were treated 30 minutes before the application of  $\alpha$ -MSH.

### Behavioral Experiments

The day before the experiment, the hair was clipped over the rostral part of the mouse back. Before behavioral observation, the animals were placed individually in an acrylic cage composed of four compartments (13  $\times$  9  $\times$  35 cm) for at least 1 hour for acclimation. Immediately after intradermal injection, mice were put back into the same cells, and their behaviors were recorded with the use of a digital video camera (HDC-TM25; Panasonic Co., Osaka, Japan) for 1 hour with personnel kept out of the observation room. Playback of the digital recording allowed for counting of injection site scratching by the hind paw. The series of movements scratched several times for about 1 second were considered as one bout of scratching.<sup>14</sup>

### Immunostaining

Under anesthesia with pentobarbital (80 mg/kg, intraperitoneal), mice were transcardially perfused with phosphate-buffered saline (PBS) and then 4% paraformaldehyde. The skin of the rostral back was isolated, postfixed with 4% paraformaldehyde, and immersed in 30% sucrose solution for 2 days. The tissue was embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek Co., Ltd., Tokyo, Japan) and kept at  $-80^\circ\text{C}$  until use. The frozen samples were sectioned at 20  $\mu\text{m}$  with a cryostat (Leica, Wetzlar, Germany). In the keratinocyte cell line Pam212, cells cultured on glass-bottomed dishes were washed twice with PBS and fixed with 4% paraformaldehyde. After being washed three times with PBS, the sections or cells were treated with 0.3% Triton X-100 in PBS and then with 0.25% fetal bovine serum to block nonspecific immunoglobulin binding. The sections or cells were treated with the first antibodies at a dilution of 1:100 at 4°C overnight; the antibodies used were rabbit antibodies against histidine decarboxylase (HDC; Santa Cruz Biotechnology Inc.) and histamine (Abcam, Cambridge, UK), and goat antibodies against MC1R, MC5R, and mast cell protease 5 (Santa Cruz Biotechnology Inc.). After washing, the preparations were incubated with Alexa Fluor 555-conjugated anti-goat IgG (Life Technologies) for 1 hour at room temperature. The sections and cells were rinsed in PBS after each treatment. Finally, the sections and cells were counterstained with DAPI. Immunofluorescence was visualized with the use of a laser



**Figure 1** Scratching after intradermal injections of  $\alpha$ -MSH in ICR mice and effects of naltrexone. Hind-paw scratching of the injection site was counted for 1 hour after intradermal injection of  $\alpha$ -MSH or the VH1. **A:** Time course of scratching after VH1 (upper panel) and  $\alpha$ -MSH (100 nmol/L per site; lower panel) injections. **B:** Dose-response curve for the scratching effects of  $\alpha$ -MSH and VH1. **C:** Naltrexone hydrochloride (1 mg/kg) or VH was injected subcutaneously 15 minutes before  $\alpha$ -MSH (100 nmol/L per site) injection. The dotted line represents the average value of scratching bouts after intradermal injection of VH1. Data are expressed as means  $\pm$  SEM.  $n = 2$  independent experiments (**B**);  $n = 8$  animals (**C**). \* $P < 0.05$  versus VH (**B**, Holm-Sidak multiple comparisons; **C**, Student's  $t$ -test).  $\alpha$ -MSH,  $\alpha$ -melanocyte-stimulating hormone; VH, vehicle.

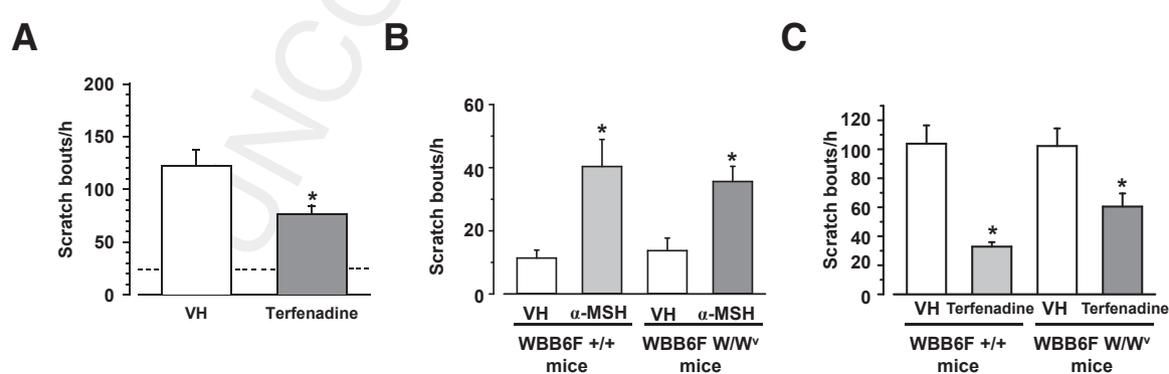
scanning confocal microscope (Leica Microsystems, Tokyo, Japan) or a fluorescent microscope (Olympus Co., Tokyo, Japan). After scanning, the slide glass was washed with PBS. The skin section was stained with toluidine blue and observed with the use of light microscope (Olympus Co.).

For a portion of the immunostaining, we used the antibody preabsorbed with the antigen peptides as a negative control. The antigen peptides for HDC, MC1R, and MC5R were purchased from Santa Cruz Biotechnology, Inc. The preparation of the antibody preabsorbed with the antigen peptides was performed according to the manufacturer's protocol (Santa Cruz Biotechnology, Inc.).

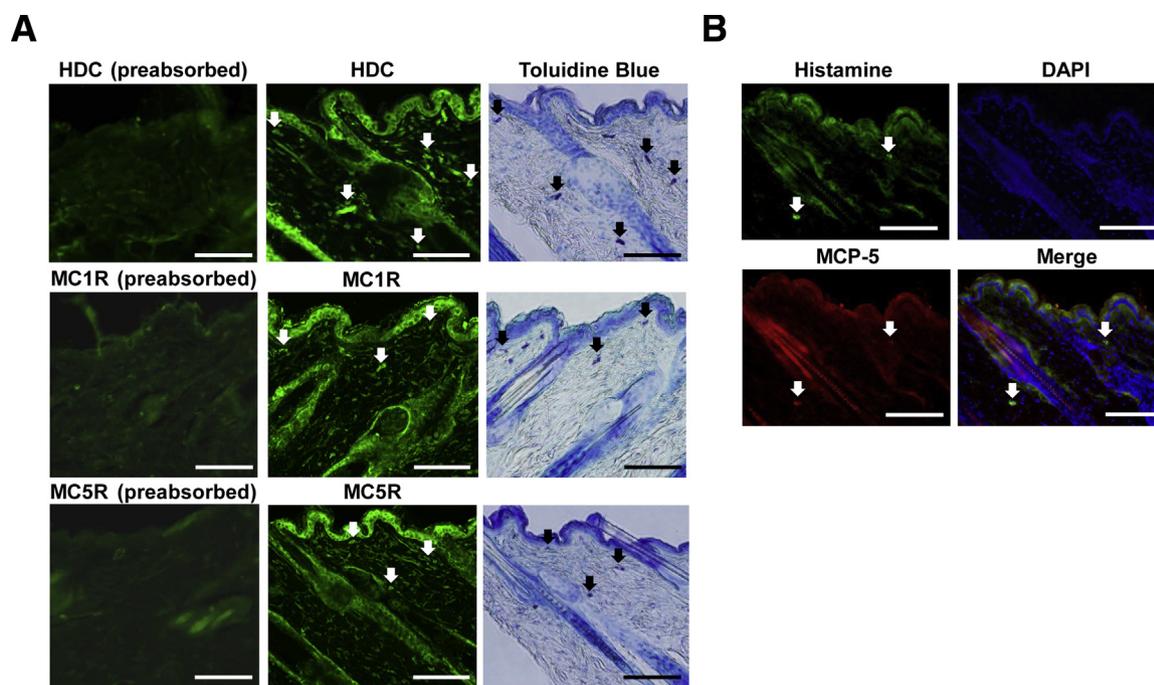
## RT-PCR

Total RNA was extracted from cultured Pam212 cells. The cell samples were lysed with the TRIzol reagent (Invitrogen) for RNA preparation. Total RNA (0.4  $\mu$ g/sample)

was used for cDNA synthesis with oligo (dT)<sub>16</sub> primers and reverse transcriptase (Reverscript III; Wako Pure Chemical Industries Ltd.). cDNA was amplified with the use of the following primers: MC1R, 5'-GCCAC-ATGTTACGAGAGC-3' (forward) and 5'-AGTTACC-TTTCTCTGGCCC-3' (reverse); MC5R, 5'-AAATCC-GATGCCAAGAAGTG-3' (forward) and 5'-GGTAGCG-CAAGGCATAGAAG-3' (reverse); HDC, 5'-AGCACAA-GCTGTGTCCTTT-3' (forward) and 5'-GTGGATCAG-AAGACCCTGT-3' (reverse). Glyceraldehyde 3-phosphate dehydrogenase was used as a positive control. The primers used for glyceraldehyde 3-phosphate dehydrogenase were 5'-ACCCAGAAGACTGTGGAT-3' (forward) and 5'-TCG-TTGAGGGCAATGCCA-3' (reverse). The cycling conditions were 5 minutes at 94°C, 35 cycles of 30 seconds at 94°C, 30 seconds at 58°C, and 30 seconds at 72°C, and 7 minutes at 72°C. After PCR, the amplified products were analyzed by 2% agarose gel electrophoresis.



**Figure 2** Effect of terfenadine and mast-cell deficiency on  $\alpha$ -MSH-induced scratching. Terfenadine (30 mg/kg) or VH1 was injected orally 30 minutes before  $\alpha$ -MSH (100 nmol/L per site) injection. Hind-paw scratching of the injection site was counted for 1 hour after intradermal injection of  $\alpha$ -MSH or VH2. **A:** Effect of terfenadine on  $\alpha$ -MSH-induced scratching in ICR mice. The dotted line represents the average value of scratching bouts after intradermal injection of VH2. **B:** Effect of mast-cell deficiency on  $\alpha$ -MSH-induced scratching in mast cell-deficient WBB6F1-W/W<sup>m</sup> mice and in normal littermates (WBB6F1<sup>+/+</sup>). **C:** Effect of terfenadine on  $\alpha$ -MSH-induced scratching in WBB6F1-W/W<sup>m</sup> and WBB6F1<sup>+/+</sup> mice. Data are expressed as means  $\pm$  SEM.  $n = 7$  to 8 animals. \* $P < 0.05$  versus VH1 or VH2 (**A**, Student's  $t$ -test; **B** and **C**, Holm-Sidak multiple comparisons).  $\alpha$ -MSH,  $\alpha$ -melanocyte-stimulating hormone; VH, vehicle.



**Figure 3** Cutaneous distribution of HDC, melanocortin receptors (MC1R and MC5R), and histamine in ICR mice. **A: Center panels** show HDC and melanocortin receptors (MC1R and MC5R) immunoreactivity in the skin of the ICR mice. **Left panels** show the images obtained with the antibody preabsorbed with the antigens for HDC, MC1R, and MC5R (negative control). **Right panels** show the light microscopy images for toluidine blue staining. **B:** Expression of histamine (green) and mast cell protease 5 (red). **Arrow** shows mast cell. Scale bar = 100  $\mu\text{m}$ . HDC, histidine decarboxylase; MC1R, melanocortin 1 receptor; MC5R, melanocortin 5 receptor.

### Western Blot Analysis

Proteins were extracted from cultured Pam212 cells with a lysis buffer [20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 1% NP-40, 10% glycerol, 1 mM phenylmethyl sulfonyl fluoride, 10  $\mu\text{g}/\text{mL}$  aprotinin, and 1  $\mu\text{g}/\text{mL}$  leupeptin]. The protein lysates were denatured at 95°C for 5 minutes and were applied to an SDS-polyacrylamide gel for electrophoresis and transferred to nitrocellulose membranes. After blocking with 1% skim milk in PBS that contained 0.1% Tween 20, the membrane was reacted with goat polyclonal anti-HDC, anti-MC1R, anti-MC5R, and anti- $\beta$ -actin antibodies (dilution 1:1000 each), respectively, overnight at 4°C. After washing with PBS that contained 0.1% Tween 20, the membranes were incubated with horseradish peroxidase-labeled donkey anti-goat IgG antibody (dilution 1:1000; Bethyl Laboratories, Inc., Montgomery, TX) for 2 hours at room temperature. These membranes were then scanned with the lumino image analyzer Image Quant LAS-4000 (Fujifilm, Tokyo, Japan).

### Measurement of Histamine Released from Murine Cell Line Pam212

$\alpha$ -MSH (200  $\mu\text{mol}/\text{L}$ ) was dissolved in Opti-MEM, as a reaction medium, and applied to the cells. Five and 10 minutes later, the reaction medium was collected. In the experiments that used the cells treated with siRNA, SQ 22,536, or EGTA,

the reaction medium was collected 5 minutes after  $\alpha$ -MSH application. The concentration of the released histamine was measured with the histamine enzyme immunoassay kit (Bertin Pharma, Montigny-le-Bretonneux, France) according to the manufacturer's recommendation. The protein of Pam212 cells was extracted by the application of 1% Triton X-100, and the concentration was measured by using protein assay reagent (Bio-Rad, Hercules, CA). The released histamine concentration in the reaction medium was normalized to the protein concentration of the Pam212 cells.

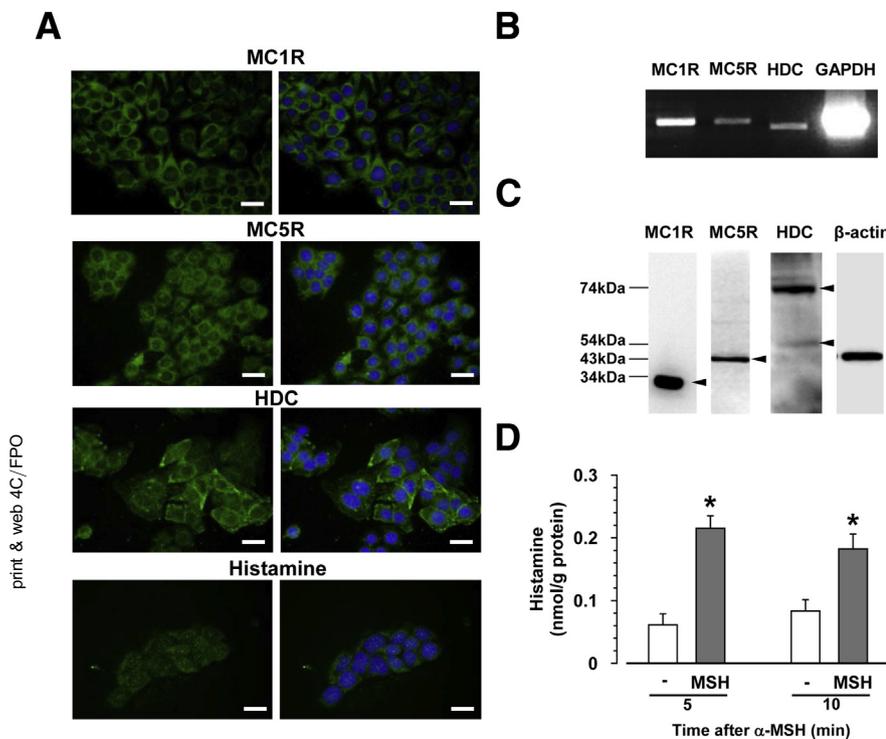
### Statistical Analysis

All values are expressed as the means  $\pm$  SEM of the respective test or control group. Statistical significance was evaluated with either Student's *t*-test or Holm-Šidák multiple comparisons.  $P < 0.05$  was considered significant.

## Results

### Behavioral Effects of $\alpha$ -MSH

An intradermal injection of  $\alpha$ -MSH (100 nmol/L per site) induced marked scratching of the injected site by the hind paws compared with the vehicle. The effect peaked in the initial 10-minute period and almost completely subsided within 60 minutes (Figure 1A). The administration of  $\alpha$ -MSH at intradermal doses of 10 to 100 nmol per site elicited scratching in a dose-dependent manner (Figure 1B). In the



**Figure 4** Expression of HDC, MC1R, MC5R, and histamine in murine keratinocyte cell line Pam212 and release of histamine after  $\alpha$ -MSH treatment. **A:** HDC, MC1R, MC5R, and histamine immunoreactivity in Pam212 cells. **B** and **C:** RT-PCR and a Western blot analysis show the expression of HDC, MC1R, and MC5R in the Pam212 cells. The **arrowhead** indicates the protein levels of MC1R (34 kDa), MC5R (43 kDa), and HDC (74 and 54 kDa). **D:** Release of histamine after 200  $\mu$ mol/L  $\alpha$ -MSH treatment. Data are expressed as means  $\pm$  SEM.  $n = 2$  independent experiments (**B–D**);  $n = 6$  wells (**D**). \* $P < 0.05$  versus culture medium only (Holm–Sidak multiple comparisons). Scale bar = 20  $\mu$ m. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HDC, histidine decarboxylase; MC1R, melanocortin 1 receptor; MC5R, melanocortin 5 receptor;  $\alpha$ -MSH,  $\alpha$ -melanocyte-stimulating hormone.

following *in vivo* experiments, a dose of 100 nmol per site of  $\alpha$ -MSH was used.

#### Effects of Various Agents on the $\alpha$ -MSH-Induced Scratching

Subcutaneous pretreatment with 1 mg/kg selective  $\mu$ -opioid receptor antagonist, naltrexone hydrochloride,<sup>15</sup> inhibited the  $\alpha$ -MSH-induced scratching (Figure 1C). Oral pretreatment with 30 mg/kg H<sub>1</sub> histamine receptor antagonist, terfenadine,<sup>16</sup> also suppressed the  $\alpha$ -MSH-induced scratching (Figure 2A).

#### Effects of Mast Cell Deficiency on the $\alpha$ -MSH-Induced Scratching

An intradermal injection of  $\alpha$ -MSH elicited significant scratching in both mast cell-deficient mice (WBB6F1 *W/W<sup>n</sup>*) and their normal littermates (WBB6F1 *+/+*), compared with vehicle-injected mice (Figure 2B). The number of scratches was almost the same between these mice. Interestingly, the administration of 30 mg/kg H<sub>1</sub> histamine receptor antagonist terfenadine significantly inhibited the  $\alpha$ -MSH-induced scratching in both mast cell-deficient mice (WBB6F1 *W/W<sup>n</sup>*) and their normal littermates (WBB6F1 *+/+*), compared with vehicle-treated mice (Figure 2C).

#### Distribution of HDC, MC1R, MC5R, and Histamine in Mouse Skin

Immunohistochemical staining showed that HDC, MC1R, and MC5R were mainly expressed in both epidermal keratinocytes and dermal cells stained with toluidine blue (Figure 3A).

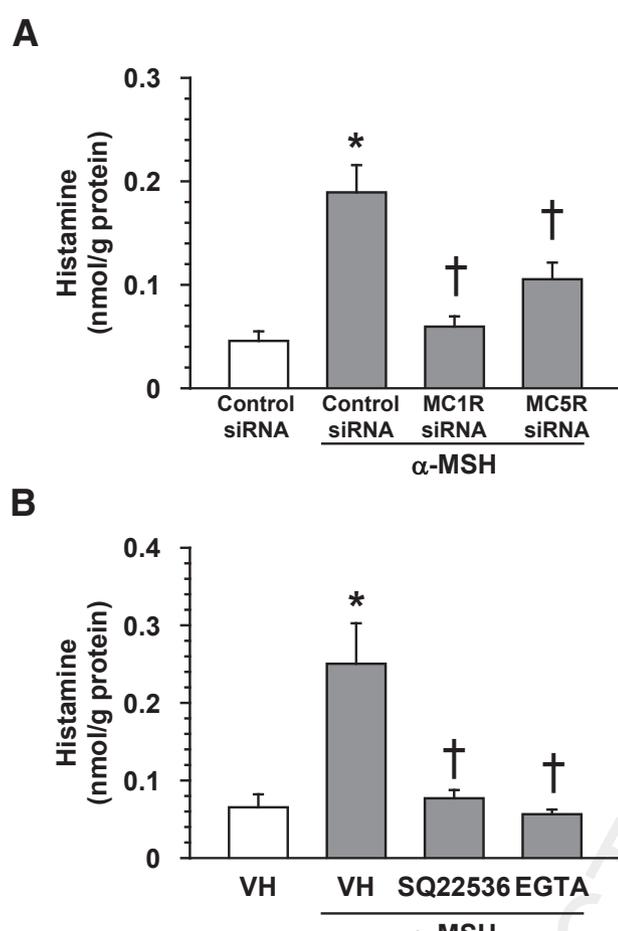
Histamine was similarly expressed in both epidermal keratinocytes and dermal cells seen by the immunoreactivity of mast cell protease 5 (Figure 3B).

#### Expression of HDC, MC1R, MC5R, and Histamine in the Mouse Keratinocyte Cell Line Pam212 Cells

Pam212 cells showed immunoreactivity for HDC, MC1R, MC5R, and histamine (Figure 4A). In addition, RT-PCR and Western blot analysis also showed the expression of HDC, MC1R, and MC5R in Pam212 cells (Figure 4, B and C).

#### Release of Histamine from Pam212 Cells Stimulated with $\alpha$ -MSH

In the present *in vivo* study, an intradermal injection of  $\alpha$ -MSH (100 nmol/50  $\mu$ L = 2 mmol/L) elicited scratching (Figure 1A). However,  $\alpha$ -MSH (10 nmol/50  $\mu$ L = 200  $\mu$ mol/L) led to a slight, but not significant, increase in scratching, compared with the vehicle-injected group (Figure 1B). Because  $\alpha$ -MSH acts directly on the cells *in vitro*,  $\alpha$ -MSH was administered at a final concentration of 200  $\mu$ mol/L in these cell culture studies. Because  $\alpha$ -MSH-induced scratching was observed mainly during the initial 10-minute period, the release of histamine was measured for 10 minutes after  $\alpha$ -MSH stimulation. Treatment with 200  $\mu$ mol/L  $\alpha$ -MSH significantly increased the concentration of histamine in the culture medium 5 and 10 minutes after the application of  $\alpha$ -MSH, compared with the medium of cells treated without  $\alpha$ -MSH (Figure 4D). The effect peaked after the initial 5-minute period (Figure 4D).



**Figure 5** Effects of siRNA on MC1R, MC5R, and cell signaling-regulated agents. **A:**  $\alpha$ -MSH (200  $\mu$ mol/L) was applied to Pam212 cells treated with siRNA for MC1R or MC5R or nonspecific control siRNA. **B:** SQ 22,536 (100  $\mu$ mol/L), EGTA (1 mmol/L), or VH (0.1% dimethyl sulfoxide) was treated 30 minutes before 200  $\mu$ mol/L  $\alpha$ -MSH application. The amount of histamine in the reaction medium was measured 5 minutes after 200  $\mu$ mol/L  $\alpha$ -MSH application with the use of an enzyme immunoassay kit and was normalized to the amount of protein in the cells. Data are expressed as means  $\pm$  SEM.  $n = 6$  wells. \* $P < 0.05$  versus the reaction medium that contained the Pam212 cells treated with control siRNA or VH in the Pam212 cells; † $P < 0.05$  versus the reaction medium applied with  $\alpha$ -MSH in the Pam212 cells treated with control siRNA or VH (Holm–Šidák multiple comparisons). MC1R, melanocortin 1 receptor; MC5R, melanocortin 5 receptor;  $\alpha$ -MSH,  $\alpha$ -melanocyte-stimulating hormone; VH, vehicle.

$\alpha$ -MSH did not elicit the release of histamine in the cells [F5] treated with MC1R or MC5R siRNA (Figure 5A and Supplemental Figure S1). In addition, 100  $\mu$ mol/L adenylyl cyclase inhibitor SQ 22,536<sup>17</sup> (Supplemental Figure S2<sup>18</sup>) and 1 mmol/L calcium chelator EGTA<sup>19</sup> also inhibited the release of histamine induced by  $\alpha$ -MSH (Figure 5B).

## Discussion

Intradermal injections of  $\alpha$ -MSH into the rostral part of the skin elicited hind-paw scratching of the injection site in mice. The  $\alpha$ -MSH-induced scratching was inhibited by treatment with the  $\mu$ -opioid receptor antagonist naltrexone. It

was reported that  $\mu$ -opioid receptor antagonists inhibit scratching induced by pruritogens<sup>15,20–22</sup> and dermatoses in rodents<sup>16,23–25</sup> and itching and scratching in humans with pruritic diseases.<sup>26–29</sup> However,  $\mu$ -opioid receptor antagonists attenuate itch-related but not pain-related behavior.<sup>30–33</sup> Taking into account these findings in humans and rodents, our results showing that the action of  $\alpha$ -MSH was inhibited by  $\mu$ -opioid receptor antagonists are consistent with the idea that  $\alpha$ -MSH-induced scratching is because of pruritogenic, but not algogenic, stimulation of the treated skin.

In this study, we also found that  $\alpha$ -MSH-induced scratching was inhibited partially, but significantly, by treatment with a H<sub>1</sub> histamine receptor antagonist terfenadine at a dose that almost completely inhibited the histamine-induced scratching,<sup>16</sup> suggesting that histamine is involved in  $\alpha$ -MSH-induced scratching.  $\alpha$ -MSH is involved in the pigmentation due to sunburn,<sup>10,11</sup> hemodialysis,<sup>12</sup> and wound repair.<sup>13</sup> Antihistamines are effective for treating pruritus in the patients with the above-mentioned causes of pigmentation.<sup>1,34,35</sup> Therefore, Q9 these reports support our findings.

It is well known that histamine is mainly produced by the mast cells in skin. However, in this study,  $\alpha$ -MSH elicited scratching in both mast cell-deficient mice and their normal littermates. Interestingly, terfenadine inhibited the  $\alpha$ -MSH-induced scratching in both the mast cell-deficient mice and normal littermates. Taken together, these findings suggest that histamine is involved in the  $\alpha$ -MSH-induced scratching, and mast cells may not contribute to the release of histamine involved in this scratching.

HDC is a key enzyme in the biosynthesis of histamine.<sup>36</sup> In this study, we observed the immunoreactivity to HDC and histamine not only in mast cells but also keratinocytes in mouse skin. In addition, the mouse keratinocyte cell line, Pam212, also showed the immunoreactivity to HDC and histamine. Human epidermal cells and keratinocytes also express HDC.<sup>37,38</sup> We also detected low (54 kDa)- and high (74 kDa)-molecular weight protein bands of HDC in the Pam212 cells; 74-kDa HDC is a precursor protein that exhibits a low enzyme activity. Furthermore, 74-kDa HDC is post-translationally cleaved to a 53- to 55-kDa species, and histamine is synthesized mainly by a 53- to 55-kDa HDC.<sup>36</sup> Treatment with  $\alpha$ -MSH induced histamine release from Pam212 cells. Thus, keratinocytes may play an important role in the production of histamine, and this appears to be related to the  $\alpha$ -MSH-induced scratching.

In mast cells, histamine is stored in granules and is released by several types of stimuli (such as immune reactions).<sup>36</sup> Macrophages also express HDC and spontaneously release histamine without it being stored intracellularly (these cells do not contain histamine-storing granules).<sup>36,39</sup> Because keratinocytes also do not have histamine-storing granules, the histamine would be released immediately after its biosynthesis. In this study, keratinocytes slightly produced histamine. A recent report has shown histamine induces proliferation in keratinocytes through H<sub>4</sub> histamine

receptors.<sup>40</sup> Thus, in a normal skin condition, histamine is involved in the proliferation in keratinocyte as one of the actions. However, overproduction of histamine induced by several stimulations, such as  $\alpha$ -MSH (present study) and surfactant,<sup>37</sup> may be involved in the induction of itching.

The application of  $\alpha$ -MSH induced the release of histamine from the mouse keratinocytes cell line Pam212 in this study. Pam212 cells also expressed MC1R and MC5R receptors. The cells treated with siRNA for MC1R and MC5R showed no release of histamine after stimulation with  $\alpha$ -MSH, suggesting that at least MC1R and MC5R are involved in  $\alpha$ -MSH-induced histamine release. The mechanism underlying the production of histamine after the stimulation with  $\alpha$ -MSH is still unclear. Miyazaki et al<sup>41</sup> have shown that the activity of HDC was increased by  $N^6, O^2$ -dibutyryl cAMP plus  $Ca^{2+}$  ionophore A23187. The activation of MC1R and MC5R receptors by  $\alpha$ -MSH increases both the production of cAMP<sup>8</sup> and the intracellular-free  $Ca^{2+}$  concentration.<sup>9</sup> Here,  $\alpha$ -MSH-induced histamine release was inhibited by the adenylyl cyclase inhibitor, which suppressed cAMP production and a calcium chelator. Therefore, our findings suggest that increased cAMP (Supplemental Figure S2) and intracellular calcium levels may be involved in the production of histamine through melanocortin receptors.

In the present study, the treatment with a  $H_1$  histamine receptor antagonist did not completely inhibit the  $\alpha$ -MSH-induced scratching. The cutaneous distribution of melanocortin receptors (MC1R, MC3R, MC4R, and MC5R) for  $\alpha$ -MSH is not fully understood. It is known that in the mouse dorsal root ganglia, MC1R, MC3R, and MC4R, but not MC5R, are expressed,<sup>42</sup> suggesting that MC1R, MC3R, and MC4R are presented on primary afferents. Thus,  $\alpha$ -MSH may act directly via primary afferents to induce itch. Recently, it was reported that there are two main types of itch-related primary afferents;  $H_1$  histamine receptor-expressing neurons and mas-related G-coupled protein receptor A3-expressing neurons.<sup>43</sup> A future study should be performed to determine the distribution of melanocortin receptors in these primary afferents.

## Conclusion

In conclusion,  $\alpha$ -MSH is an itch mediator, and the histamine released from keratinocytes, but not mast cells, may be involved in this  $\alpha$ -MSH-induced itching.

## Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2015.07.015>.

## References

- Han A, Maibach HI: Management of acute sunburn. *Am J Clin Dermatol* 2004, 5:39–47
- Gilchrest BA, Soter NA, Stoff JS, Mihm MC Jr: The human sunburn reaction: histologic and biological studies. *Am Acad Dermatol* 1981, 5: 411–422
- Paul J: Characteristics of chronic wounds that itch. *Adv Skin Wound Care* 2013, 26:320–332
- Slominski A, Tobin DJ, Shibahara S, Wortsman J: Melanin pigmentation in mammalian skin and its hormonal regulation. *Physiol Rev* 2004, 84:1155–1228
- Slominski A, Wortsman J, Luger T, Paus R, Solomon S: Corticotropin releasing hormone and proopiomelanocortin involvement in the cutaneous response to stress. *Physiol Rev* 2000, 80:979–1020
- Slominski AT, Zmijewski MA, Zbytek B, Tobin DJ, Theoharides TC, Rivier J: Key role of CRF in the skin stress response system. *Endocr Rev* 2013, 34:827–884
- Slominski AT, Zmijewski MA, Skobowiat C, Zbytek B, Slominski RM, Stekete JD: Sensing the environment: regulation of local and global homeostasis by the skin's neuroendocrine system. *Adv Anat Embryol Cell Biol* 2012, 212:v. vii, 1–115
- Cooray SN, Clark AJ: Melanocortin receptors and their accessory proteins. *Mol Cell Endocrinol* 2011, 331:215–221
- Mountjoy KG, Kong PL, Taylor JA, Willard DH, Wilkison WO: Melanocortin receptor-mediated mobilization of intracellular free calcium in HEK293 cells. *Physiol Genomics* 2011, 5:11–19
- Böhm M, Luger TA, Tobin DJ, García-Borrón JC: Melanocortin receptor ligands: new horizons for skin biology and clinical dermatology. *J Invest Dermatol* 2006, 126:1966–1975
- Oren M, Bartek J: The sunny side of p53. *Cell* 2007, 128:826–828
- Airaghi L, Garofalo L, Cutuli MG, Delgado R, Carlin A, Demitri MT, Badalamenti S, Graziani G, Lipton JM, Catania A: Plasma concentrations of alpha-melanocyte-stimulating hormone are elevated in patients on chronic haemodialysis. *Nephrol Dial Transplant* 2000, 15:1212–1216
- Muffley LA, Zhu KQ, Engrav LH, Gibran NS, Hocking AM: Spatial and temporal localization of the melanocortin 1 receptor and its ligand  $\alpha$ -melanocyte-stimulating hormone during cutaneous wound repair. *J Histochem Cytochem* 2011, 59:278–288
- Kuraishi Y, Nagasawa T, Hayashi K, Satoh M: Scratching behavior induced by pruritogenic but not algescogenic agents in mice. *Eur J Pharmacol* 1995, 275:229–233
- Andoh T, Kuwazono T, Lee JB, Kuraishi Y: Gastrin-releasing peptide induces itch-related responses through mast cell degranulation in mice. *Peptides* 2011, 32:2098–2103
- Ohtsuka E, Kawai S, Ichikawa T, Nojima H, Kitagawa K, Shirai Y, Kamimura K, Kuraishi Y: Roles of mast cells and histamine in mosquito bite-induced allergic itch-associated responses in mice. *Jpn J Pharmacol* 2001, 86:97–105
- Turcato S, Clapp LH: Effects of the adenylyl cyclase inhibitor SQ22536 on iloprost-induced vasorelaxation and cyclic AMP elevation in isolated guinea-pig aorta. *Br J Pharmacol* 1999, 126:845–847
- Andoh T, Kuraishi Y: Antipruritic mechanisms of topical E6005, a phosphodiesterase 4 inhibitor: inhibition of responses to proteinase-activated receptor 2 stimulation mediated by increase in intracellular cyclic AMP. *J Dermatol Sci* 2014, 76:206–213
- Sun FC, Shyu HY, Lee MS, Lee MS, Lai YK: Involvement of calcium-mediated reactive oxygen species in inductive GRP78 expression by geldanamycin in 9L rat brain tumor cells. *Int J Mol Sci* 2013, 14: 19169–19185
- Andoh T, Nagasawa T, Satoh M, Kuraishi Y: Substance P induction of itch-associated response mediated by cutaneous NK1 tachykinin receptors in mice. *J Pharmacol Exp Ther* 1998, 286:1140–1145
- Andoh T, Saito A, Kuraishi Y: Leukotriene B(4) mediates sphingosylphosphorylcholine-induced itch-associated responses in mouse skin. *J Invest Dermatol* 2009, 129:2854–2860
- Yamaguchi T, Nagasawa T, Satoh M, Kuraishi Y: Itch-associated response induced by intradermal serotonin through 5-HT2 receptors in mice. *Neurosci Res* 1999, 35:77–83
- Yamaguchi T, Maekawa T, Nishikawa Y, Nojima H, Kaneko M, Kawakita T, Miyamoto T, Kuraishi Y: Characterization of itch-associated

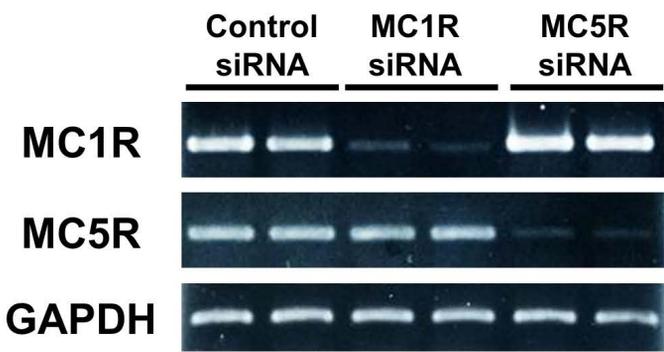
- 869 responses of NC mice with mite-induced chronic dermatitis. *J Dermatol* 2001, 25:20–28
- 870
- 871 24. Miyamoto T, Nojima H, Shinkado T, Nakahashi T, Kuraishi Y: Itch-
- 872 associated response induced by experimental dry skin in mice. *Jpn J*
- 873 *Pharmacol* 2002, 88:285–292
- 874 25. Fujii M, Nabe T, Tomozawa J, Kohno S: Involvement of skin barrier
- 875 dysfunction in itch-related scratching in special diet-fed hairless mice.
- 876 *Eur J Pharmacol* 2006, 530:152–156
- 877 26. Bernstein JE, Swift R: Relief of intractable pruritus with naloxone.
- 878 *Arch Dermatol* 1979, 115:1366–1367
- 879 27. Monroe EW: Efficacy and safety of nalmefene in patients with severe
- 880 pruritus caused by chronic urticaria and atopic dermatitis. *J Am Acad*
- 881 *Dermatol* 1989, 21:135–136
- 882 28. Bergasa NV, Alling DW, Talbot TL, Swain MG, Yurdaydin C,
- 883 Turner ML, Schmitt JM, Walker EC, Jones EA: Effects of naloxone
- 884 infusions in patients with the pruritus of cholestasis. A double-blind,
- 885 randomized, controlled trial. *Ann Intern Med* 1995, 123:161–167
- 886 29. Bergasa NV, Talbot TL, Alling DW, Schmitt JM, Walker EC,
- 887 Baker BL, Korenman JC, Park Y, Hoofnagle JH, Jones EA: A
- 888 controlled trial of naloxone infusions for the pruritus of chronic
- 889 cholestasis. *Gastroenterology* 1992, 102:544–549
- 890 30. Maekawa T, Yamaguchi-Miyamoto T, Nojima H, Kuraishi Y: Effects
- 891 of naltrexone on spontaneous itch-associated responses in NC mice
- 892 with chronic dermatitis. *Jpn J Pharmacol* 2002, 90:193–196
- 893 31. Nojima H, Simons CT, Cuellar JM, Carstens MI, Moore JA,
- 894 Carstens E: Opioid modulation of scratching and spinal c-fos expres-
- 895 sion evoked by intradermal serotonin. *J Neurosci* 2003, 23:
- 896 10784–10790
- 897 32. Akiyama T, Carstens MI, Carstens E: Differential itch- and pain-
- 898 related behavioral responses and  $\mu$ -opioid modulation in mice. *Acta*
- 899 *Derm Venereol* 2010, 90:575–581
- 900 33. Gotoh Y, Andoh T, Kuraishi Y: Noradrenergic regulation of itch
- 901 transmission in the spinal cord mediated by  $\alpha$ -adrenoceptors. *Neuro-*
- 902 *pharmacology* 2011, 61:825–831
- 903
- 904
- 905
- 906
- 907
- 908
- 909
- 910
- 911
- 912
- 913
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- 919
- 920
- 921
- 922
- 923
- 924
- 925
- 926
- 927
- 928
- 929
- 930
- 931 34. Matsui C, Ida M, Hamada M, Morohashi M, Hasegawa M: Effects of
- 932 azelastine on pruritus and plasma histamine levels in hemodialysis
- 933 patients. *Int J Dermatol* 1994, 33:868–871
- 934 35. Matheson JD, Clayton J, Muller MJ: The reduction of itch during burn
- 935 wound healing. *J Burn Care Rehabil* 2001, 22:76–81
- 936 36. Ichikawa A, Sugimoto Y, Tanaka S: Molecular biology of histidine
- 937 decarboxylase and prostaglandin receptors. *Proc Jpn Acad Ser B Phys*
- 938 *Biol Sci* 2010, 86:848–866
- 939 37. Inami Y, Andoh T, Sasaki A, Kuraishi Y: Topical surfactant-induced
- 940 pruritus: involvement of histamine released from epidermal keratino-
- 941 cytes. *J Pharmacol Exp Ther* 2013, 344:459–466
- 942 38. Gutowska-Owsiak D, Greenwald L, Watson C, Selvakumar TA,
- 943 Wang X, Ogg GS: The histamine-synthesizing enzyme histidine
- 944 decarboxylase is upregulated by keratinocytes in atopic skin. *Br J*
- 945 *Dermatol* 2014, 171:771–778
- 946 39. Hirasawa N, Murakami A, Ohuchi K: Expression of 74-kDa
- 947 histidine decarboxylase protein in a macrophage-like cell line
- 948 RAW 264.7 and inhibition by dexamethasone. *Eur J Pharmacol*
- 949 2001, 418:23–28
- 950 40. Glatzer F, Gschwandtner M, Ehling S, Rossbach K, Janik K, Klos A,
- 951 Bäumer W, Kietzmann M, Werfel T, Gutzmer R: Histamine induces
- 952 proliferation in keratinocytes from patients with atopic dermatitis
- 953 through the histamine 4 receptor. *J Allergy Clin Immunol* 2013, 132:
- 954 1358–1367
- 955 41. Miyazaki T, Ohgoh M, Ohmori E, Yamamoto J, Emoto S,
- 956 Yatsunami K, Ichikawa A: Synergistic effects of cyclic AMP and
- 957  $Ca^{2+}$  ionophore A23187 on de novo synthesis of histidine decar-
- 958 boxylase in mastocytoma P-815 cells. *Biochim Biophys Acta* 1992,
- 959 1133:179–186
- 960 42. Tanabe K, Gamo K, Aoki S, Wada K, Kiyama H: Melanocortin re-
- 961 ceptor 4 is induced in nerve-injured motor and sensory neurons of
- 962 mouse. *J Neurochem* 2007, 101:1145–1152
- 963 43. Akiyama T, Carstens E: Neural processing of itch. *Neuroscience* 2013,
- 964 250:697–714
- 965
- 966
- 967
- 968
- 969
- 970
- 971
- 972
- 973
- 974
- 975
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993 **Supplemental Figure S1** Expression of MC1R, MC5R, and GAPDH mRNA in the Pam212 cells treated with siRNA. Typical examples of the products  
994 obtained from the reverse transcription and PCR (two examples each). The method is described in *Materials and Methods*. GAPDH, glyceraldehyde 3-phosphate  
995 dehydrogenase; MC1R, melanocortin 1 receptor; MC5R, melanocortin 5 receptor. 1002

996  
997 **Supplemental Figure S2** Concentration of intracellular cAMP in the Pam212 cells. SQ 22,536 (100 mmol/L), EGTA (1 mmol/L), or VH (0.1% dimethyl  
998 sulfoxide) was treated 30 minutes before 200 mmol/L  $\alpha$ -MSH application. The amount of intracellular cAMP 5 minutes after  $\alpha$ -MSH application was measured  
999 with the enzyme immunoassay kit (GE Healthcare Bio-Sciences Co., Piscataway, NJ).<sup>18</sup> Data are expressed as means  $\pm$  SEM.  $n = 6$  wells. \* $P < 0.05$  versus  
1000 Pam212 cells treated with VH; <sup>†</sup> $P < 0.05$  versus Pam212 cells treated with VH and  $\alpha$ -MSH (Holm–Šidák multiple comparisons).  $\alpha$ -MSH,  $\alpha$ -melanocyte-  
1001 stimulating hormone; VH, vehicle. 1003  
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UNCORRECTED PROOF

Supplemental Figure 1



# Supplemental Figure-2

