Transcriptome Profiling of Anhidrotic Eccrine Sweat Glands Reveals that Olfactory Receptors on Eccrine Sweat Glands Regulate Perspiration in a Ligand-Dependent Manner

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Sweat maintains systemic homeostasis in humans. Although sweating disorders may cause multifaceted health problems, therapeutic options for sweat disorders have not yet been established. To gain new insight into the mechanism underlying the regulation of perspiration, we compared eccrine sweat gland transcriptomes from hidrotic and anhidrotic lesions from patients with anhidrosis and found out that olfactory receptors were expressed differentially in anhidrotic and hidrotic eccrine sweat glands. We then confirmed OR51A7 and OR51E2 expression in human eccrine sweat glands by in situ hybridization and immunohistochemistry. An alkaline phosphatase–TGFα shedding assay revealed that β-ionone activates G-proteins through OR51A7 or OR51E2. The effect of topically applied β-ionone on sweating was examined with the quantitative sudomotor axon reflex test, which showed that responses to β-ionone differed between sexes. Topical β-ionone attenuated female sweating and augmented male sweating. Taken together, this study suggests that olfactory receptors expressed in eccrine sweat glands may regulate sweating in response to odorous ligands on the basis of sex. These unexpected results indicate that olfactory receptors may modulate sweating and that olfactory receptor modulators may contribute to the management of sweat disorders.

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INTRODUCTION

In humans, perspiration is essential to maintain skin homeostasis. Sweat contains many substances, including metabolites, antibiotic peptides, and electrolytes. Sweat regulates body temperature, immune defense, and moisture retention (Murota et al., 2015; Sato et al., 1989). Therefore, a reduction in sweating may affect wellness in various ways.

Acquired idiopathic generalized anhidrosis (AIGA) is extensive anhidrosis of unknown etiology characterized by fatal heatstroke and skin dryness. Although AIGA symptoms impair patient QOL, there are few promising treatment strategies for the disease (Munetsugu et al., 2017). AIGA is diagnosed in patients presenting with diffuse hypohidrosis or anhidrosis detected by the sweat test (e.g., starch-iodine technique, Minor's method). Patients with AIGA have no apparent abnormalities in the central, peripheral, and autonomic nervous systems (Munetsugu et al., 2017). Thus, AIGA is believed to be a result of dysfunctional eccrine sweat glands (Munetsugu et al., 2017). It has been hypothesized that increased blood carcinoembryonic antigen levels in some patients with AIGA may contribute to the destruction of eccrine sweat glands (Honma et al., 2015). Histopathological observation showed no specific patterns associated with eccrine sweat gland atrophy or massive lymphocyte infiltration around sweat glands (Iwama et al., 2015; Suma et al., 2014).

To investigate the etiology of AIGA, we analyzed gene expression profiles of eccrine sweat glands in hidrotic and anhidrotic skin specimens from patients with AIGA. Coincidently, our RNA sequencing–based transcriptome analysis found that several olfactory receptors (ORs) were expressed in eccrine sweat glands in hidrotic areas but downregulated in anhidrotic areas. ORs are generally expressed in the nasal mucosa and are involved in olfactory functions. ORs expressed in other organs are expected to have different functions (Maßberg and Hatt, 2018). For example, OR15, which is expressed in pancreatic β cells, promotes glucose-stimulated insulin secretion (Munakata et al., 2018). It was

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Abbreviations: AIGA, acquired idiopathic generalized anhidrosis; AP-TGF, alkaline phosphatase–TGFα; HEK, human embryonic kidney; KC, keratinocyte; OR, olfactory receptor; QSART, quantitative sudomotor axon reflex test

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also reported that an odorant factor induces wound healing in human keratinocytes (KCs) through OR2AT4. Several ORs have been found to be expressed in HaCaT cells (Kang et al., 2021). *Trans*-retinoic acid inhibits KC proliferation through decreased expression of OR7A17 (Kim et al., 2021). OR10G7 is strongly expressed in undifferentiated KCs in atopic dermatitis and is involved in skin-induced chemosensory responses (Tham et al., 2019). OR2AT4/7 and OR51B5 are expressed on suprabasal KCs, with OR2AT4/7 involved in IL-1 production and OR51B5 in KC migration and IL-6 production (Tsai et al., 2017). To the best of our knowledge, there have been no reports of OR expression in sweat glands. In this study, we showed that OR51A7 and OR51E2 are expressed in sweat glands and that they regulate sweating through β-ionone.

RESULTS

RNA sequencing–based transcriptome analysis of eccrine sweat glands from patients with anhidrosis

Existing stored tissue samples derived from patients with AIGA, a rare disease, were used in this study to complement the small number of patients. Eccrine sweat glands in hidrotic and anhidrotic skin areas were excised from paraffin-embedded skin specimens from four patients with AIGA, and gene expression was profiled by RNA sequencing. Because RNA could not be extracted from one patient owing to lack of sample material in the hidrotic lesion, we analyzed RNA derived from three hidrotic and four anhidrotic lesions. We identified 102 genes by filtering transcripts with significant differences ($P < 0.01$) and with >2-fold differences in gene expression. OR51A7, OR6C74, and OR4A15 were expressed highly in sweat glands in hidrotic lesions, whereas their expression levels were downregulated in anhidrotic sweat glands (Figure 1). Because ORs are members of the G-protein-coupled receptor superfamily, which transduces cellular signals, ORs may function in sweating in addition to odor sensory systems.

Expression of ORs in human eccrine sweat glands

To determine the expression and localization of OR mRNAs in anhidrotic and hidrotic specimens from a patient with AIGA, in situ hybridization was performed with digoxigenin-labeled synthetic oligo-DNAs. As shown in Figure 2a, *OR51A7* mRNA was detected in the cytoplasm of acinar cells in sweat glands. *OR51A7* mRNA was also detected in the myoepithelial cells surrounding the sweat glands (Figure 2b). *OR4A15* and *OR6C74* mRNAs were not detected in eccrine sweat glands from patients with AIGA and healthy donors under the conditions used in this study (data not shown).

To further evaluate OR expression, conventional immunohistochemistry with antibodies against OR51A7 was performed on anhidrotic and hidrotic skin specimens from patients with AIGA. OR51A7 protein was detected in eccrine sweat glands of patients with AIGA, and there was no apparent difference in the staining intensities of OR51A7 in the hidrotic and anhidrotic areas (Figure 3). OR51A7 expression was also detected in a specimen derived from a healthy donor. Because the ligands for OR51A7 have not yet been identified, we compared the expression pattern and ligand recognition of OR51A7 with those of OR51E2, a member of the OR51 receptor family whose ligand has been identified as β-ionone. We examined OR51E2 protein expression in eccrine sweat glands from patients with AIGA and a healthy donor by immunohistochemistry. OR51E2 protein was expressed in eccrine sweat glands from both hidrotic and anhidrotic skin lesions from patients with AIGA. OR51E2 protein was also expressed in eccrine sweat glands of the healthy donor. These data suggest that OR51A7 and OR51E2 may play roles in sweating from eccrine sweat glands.

Effect of β-ionone on OR51A7-mediated signal transduction

We examined the effect of β-ionone on OR51A7- or OR51E2-mediated signal transduction on the basis of the redundant recognition of odor ligands by ORs. We used an alkaline phosphatase–TGFα (AP-TGFα) shedding assay system with human embryonic kidney (HEK) 293T reporter cells (Figure 4a). In this assay system, when odorous ligands bind to ORs, a signal is transduced through G-proteins, such as Golf and Gαq/i1. Gαq/i1 is a chimera Gαq-protein with the six C-terminal Gαq amino acid residues replaced with the six C-terminal amino acid residues of Gαi resulting in ectodomain shedding of the AP-TGFα reporter by TNFα-converting enzyme (TACE, also known as ADAM17). Alkaline phosphatase activity is measured with the substrate *p*-nitrophenyl phosphate.

After HEK293T cells were transiently transformed with plasmids containing AP-TGFα, OR51A7 or OR51E2, Gαq/il, and/or Golf, the cells were challenged with β-ionone, and shedding of the extracellular domain of the AP-TGFα fusion protein was measured. As shown in Figure 4b, β-ionone activated G-proteins in a concentration-dependent manner in the presence of OR51A7 or OR51E2, Golf, and Gαq/i1. We increased the β-ionone dose up to 300 μM and found that the minimum dose for OR51A7 was 100 μM and the minimum dose for OR51E2 was 250–300 μM in the presence of both Golf and Gαq/il. These results indicated that OR51E2 could serve as the positive control as reported previously even though its sensitivity to β-ionone was weaker than that of OR51A7. Expression of both Golf and Gαq in human eccrine sweat glands was confirmed by immunohistochemical staining for GNAL (Golf subunit α) and GNAQ (Gαq) (Figure 5).

Effect of β-ionone on the sudomotor axon reflex and odor perception

On the basis of the above findings, topical β-ionone likely affects sweating in humans through OR51A7 or OR51E2. Thus, we examined the effect of topically applied β-ionone on human perspiration using the quantitative sudomotor axon reflex test (QSART) with glycerol as the control (Table 1). When β-ionone was applied to the skin of female donors, sweating decreased. By contrast, lower axon reflex–mediated sweating increased in male donors in response to the same odorant, showing a sex-based difference in OR signal transduction in response to β-ionone. This prompted us to examine the effect of β-ionone on odor perception in males and females. A β-ionone whiff test performed among the same subjects revealed that all the female subjects could smell the β-ionone, whereas most of the male

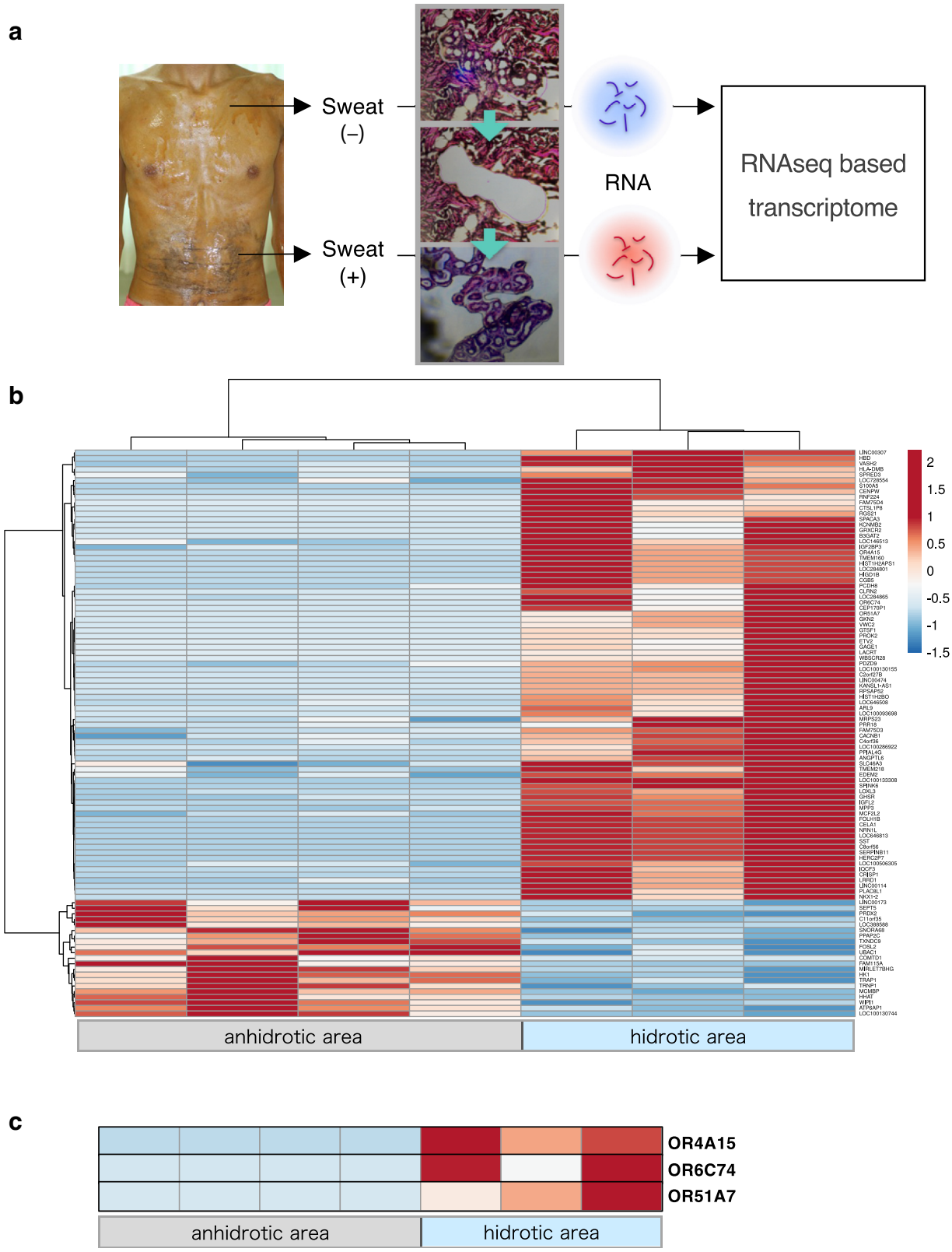

Figure 1. Transcriptome analyses of skin lesions from patients with AIGA. (**a**) Schematic representation of the strategy for identifying genes responsible for sweating. Hidrotic and anhidrotic lesions were identified by the conventional Minor's test. We obtained written informed consent from the patient for the publication of clinical images (left). Skin tissues were biopsied from the hidrotic and anhidrotic lesions, and sweat glands were excised from the tissues by standard laser microdissection (middle). RNAs were extracted from the samples and subjected to transcriptome analyses (right). (**b**) Classification of genes identified by transcriptome analyses. Heatmap of the differential expression levels of the mRNAs in anhidrotic eccrine sweat gland and hidrotic eccrine sweat gland harvested by laser microdissection. Left 4 columns represent anhidrotic eccrine sweat gland, and right 3 columns represent hidrotic eccrine sweat gland, and rows represent mRNAs. Dark red indicates high expression, and dark blue indicates low expression. A total of 102 genes were upregulated in the hidrotic

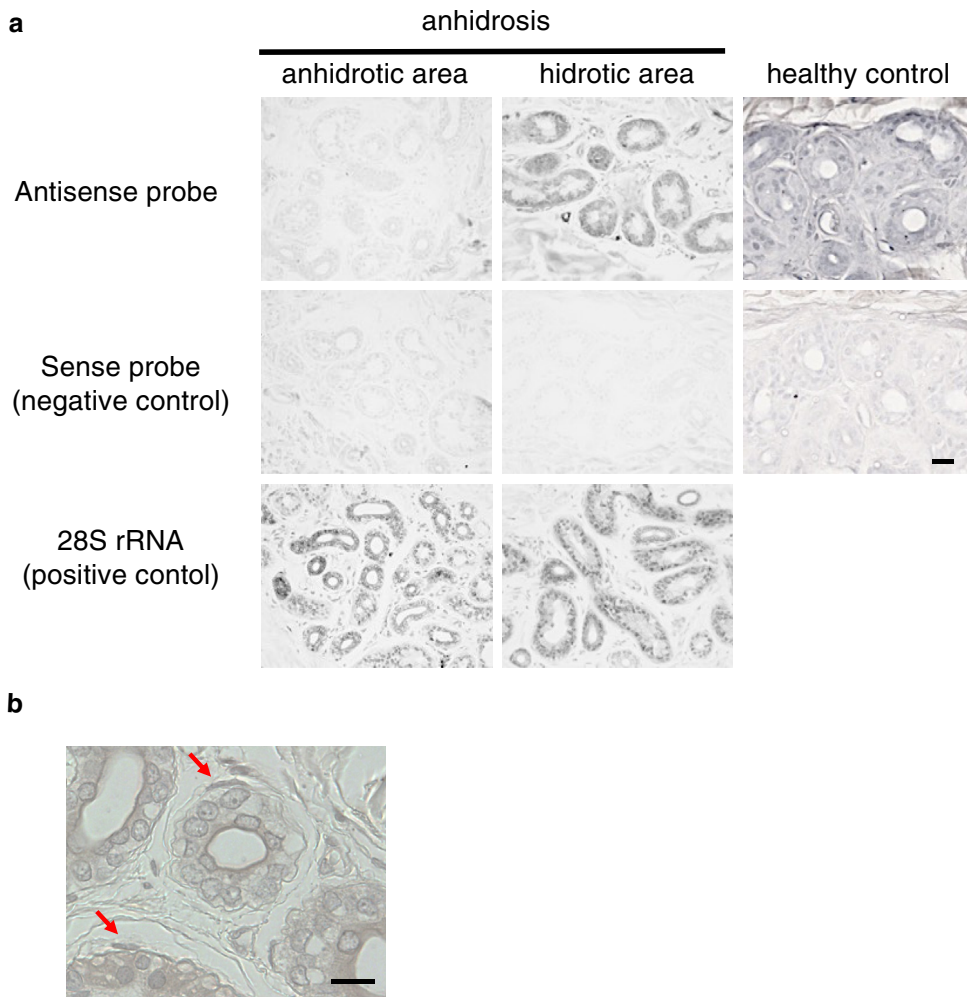

Figure 2. *OR51A7* mRNA levels in sweat glands of patients with AIGA and a healthy donor. (**a**) ISH analyses of ORs in sweat glands. The two columns on the left show the anhidrotic and hidrotic areas of an anhidrosis patient, and the single column on the right shows the ISH image of healthy control. Hybridization results with antisense probe, sense probe as negative control, and 28S rRNA as positive control are shown, respectively. Of the three ORs evaluated, only *OR51A7* mRNA was detected in sweat glands from sweating areas of patients with AIGA and the healthy donor. Bar = 20 μm. (**b**) Expression of OR51A7 in myoepithelial cells surrounding sweat glands. *OR51A7* mRNA was expressed in the acinar cells of sweat glands and in the myoepithelial cells surrounding sweat glands (red arrow). Bar = 20 μm. AIGA, acquired idiopathic generalized anhidrosis; ISH, in situ hybridization; OR, olfactory receptor; rRNA, ribosomal RNA.

subjects (five of seven) could not, suggesting a sex-based difference in the sensing of β-ionone through ORs.

DISCUSSION

In this study, we conducted transcriptome analysis to identify the genes responsible for AIGA, and we unexpectedly found OR expression in eccrine sweat glands. Histochemical and functional experiments confirmed OR expression in eccrine sweat glands and OR ligand-dependent activation of perspiration. This finding identifies an unprecedented mechanism underlying perspiration.

There are diverse causes of anhidrosis, including neurological trauma, angiopathy, sweat gland disorder, and decreased responses to acetylcholine (Murota et al., 2015). Moreover, hereditary diseases, systemic disorders, drug administration, and iatrogenic injuries can also cause abnormal sweating (Chia and Tey, 2013). AIGA is an intractable disease of unknown cause with acquired sudden onset (Munetsugu et al., 2017). Historically, tropical anhidrotic asthenia, which is characterized by extended lesions of anhidrosis accompanied by miliaria, has been thought to be caused by obstruction of sweat ducts due to miliaria (Sulzberger et al., 1946).

Histamine, mast cells, blood vessels, microneuropathies, sweat leakage from sweat glands, and a high state of anxiety have also been shown to be involved in AIGA pathogenesis (Fukunaga et al., 2009; Kijima et al., 2012; Kitaba et al., 2011; Matsui et al., 2014; Munetsugu et al., 2017; Suma et al., 2014; Takahashi et al., 2016; Yamaga et al., 2018). Thus, the etiological picture of AIGA varies by case. Decreased sweating function leads to difficulties in acclimation to heat and causes heat retention and heat stroke. Usually, patients with AIGA complain that their sweating stopped suddenly despite sweaty conditions. According to clinical guidelines, AIGA is diagnosed by the presence of an area of reduced sweating accompanied by the absence of neurological symptoms (Munetsugu et al., 2017). Thus, disorders in sweat glands or their microenvironments may contribute to the development of AIGA. Originally, this study aimed to delineate the molecular mechanism of anhidrosis by comparing gene expression profiles between hidrotic and anhidrotic eccrine sweat glands. During the gene expression

lesions and downregulated in the anhidrotic lesions ($P < 0.01$). (**c**) Identification of ORs as factors that contribute to sweating. Three ORs (OR51A7, OR6C74, and OR4A15) were downregulated in sweat glands from anhidrotic lesions. The columns and rows of the heat map follow the description in (**b**). AIGA, acquired idiopathic generalized anhidrosis; OR, olfactory receptor

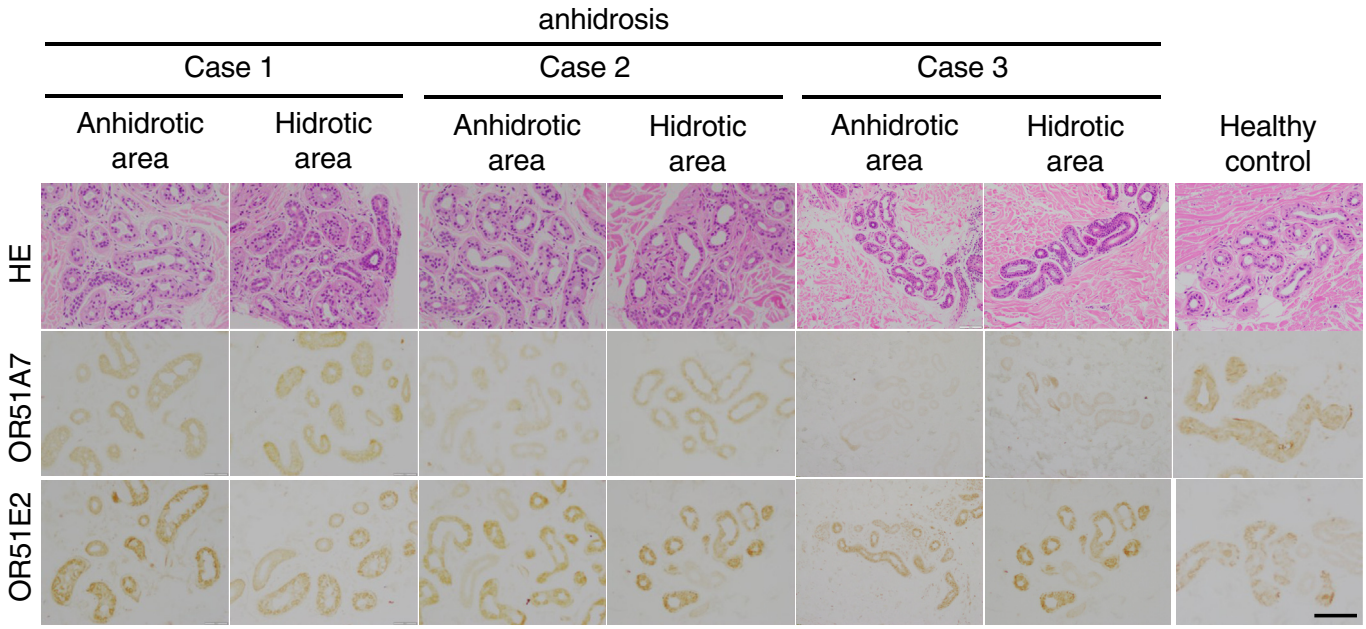

Figure 3. Immunohistochemical analyses of OR51A7 and OR51E2. Immunohistochemical staining was performed to confirm the expression and localization of the OR51A7 protein. OR51E2 protein expression was also examined because it has been shown to recognize the chemically defined odorous compound β-ionone. Both OR51E2 and OR51A7 proteins were expressed in the sweat glands of patients with AIGA and a healthy donor. OR51E2 and OR51A7 were expressed in both sweating and nonsweating areas of patients with AIGA. Bar = 100 μm. AIGA, acquired idiopathic generalized anhidrosis.

analysis, we found OR expression in eccrine sweat glands in patients with AIGA. This serendipitous finding expands the role of odorants on sweat regulation.

In the RNA sequencing–based transcriptome analysis, an individual patient was used to compare anhidrotic and hidrotic lesions to reduce nonspecific gene expression differences resulting from individual differences. The weak point of this procedure was that we could not ensure the persistency of the sweating ability in each collected sample. Nonetheless, skin samples were collected from regions in which sweating ability was confirmed just before the biopsy. Statistical analyses of RNA sequencing–based transcriptomes led us to focus on ORs that were expressed highly in hidrotic eccrine sweat glands when compared with those in anhidrotic sweat glands. ORs were cloned originally by Buck and Axel (1991) and were found to discriminate odors by interacting with odorous ligands. To the best of our knowledge, the expression of ORs in human eccrine sweat glands has not yet been reported. Although we cannot prove that both transcriptome analysis and in situ hybridization achieved the highest accuracy, we focused on the expression level of OR51A7, which showed consistency between those assays, whereas other ORs showed inconsistency. Because OR51A7 ligands had not been identified before this study, we set out to identify the odorous compounds recognized by this receptor. On the basis of receptor family analysis, we hypothesized that β-ionone is an odorous ligand of OR51A7. It was previously shown that OR51E2 is expressed in cancer cells and that β-ionone promotes cancer cell migration through OR51E2, indicating that β-ionone is an OR51E2 ligand (Gelis et al., 2017; Sanz et al., 2014). In this study, the HEK293T cell–based shedding assay system using AP-TGFα– and G-protein–containing plasmids confirmed the recognition of β-ionone by OR51E2 in the presence of Gαq/il and/or Golf. It is possible that the OR51A7 and OR51E2 signaling pathways may be the same. Notably, sweat glands express both Gαq and Golf at the transcriptional level, suggesting that β-ionone may transduce perspiration signals through OR51A7 and/or OR51E2.

Consistent with the hypothesis that ORs deliver sweat gland signals in response to β-ionone, QSART analyses showed that topically applied β-ionone induces skin perspiration in humans. Thus, β-ionone may affect axon reflex–mediated perspiration. Unexpectedly, QSART measurements revealed a sex-based difference in the induction of perspiration in response to β-ionone. Sweat volumes decreased in female subjects upon topical application of β-ionone to the skin, whereas sweat volumes increased in male subjects under the same conditions. In addition, all the female subjects could smell the aroma of β-ionone, but most of the male subjects could not. It has been reported that genetic and demographic phenotypes contribute to sex variance in human odorant perception (Trimmer et al., 2019). In a study of mice that were maintained separately on the basis of their sex, extensive differences in olfactory sensory receptor repertoires between the sexes were found (van der Linden et al., 2018). Sex differences in odorant perception have been explained partly by levels of sex steroid hormones, such as progesterone and 17-β-estradiol (Kanageswaran et al., 2016). These findings suggest that sex variance in odorous ligand-mediated sweating is associated with odor perception. In this study, the effect of odor perception of β-ionone through the nasal cavity on the QSART results cannot be excluded. Although a side-by-side comparison of β-ionone and glycerol was performed simultaneously, the effect of the topical glycerol control may have also been influenced by odor

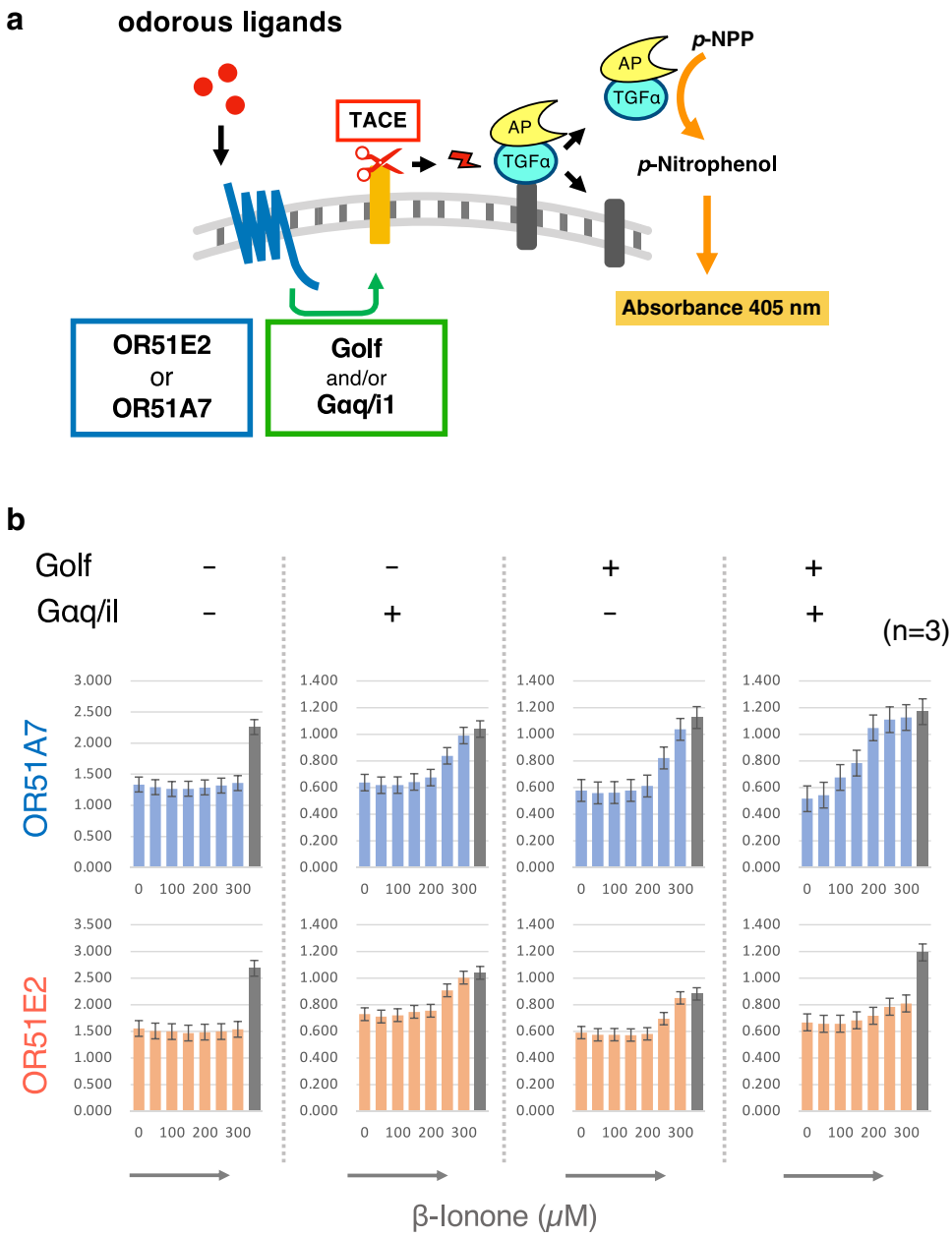

Figure 4. Recognition of β-ionone by OR51A7 in an AP-TGFα shedding assay. (**a**) Schematic of the AP-TGFα shedding assay. When an odorous ligand binds to an OR, such as OR51A7 or OR51E2, a signal is transduced through a G-protein, such as Golf or Gαq/i1, and TACE is activated, resulting in the liberation of AP-TGFα. AP activity was assessed using the AP substrate *p*-NPP. (**b**) Specific recognition of β-ionone by OR51A7 in a G-protein− and dose-dependent manner. After HEK293T cells were transiently transfected with AP-TGFα, ORs, Golf, and/or Gαq/i1, the transfectants were challenged with 0, 50, 100, 150, or 200 μM β-ionone, and the liberated AP-TGFα was measured at 405 nm using a spectrophotometer. PMA was used as a positive control. Experiments were performed in triplicate, and data are shown as mean ± SD (n = 3). AP, alkaline phosphatase; HEK, human embryonic kidney; OR, olfactory receptor; *p*-NPP, *p*-nitrophenyl phosphate; PMA, phorbol 12-myristate 13-acetate.

perception. Thus, topical β-ionone may affect sweating through percutaneous penetration.

This study has its limitations. The primary limitation was the small number of human samples because AIGA is a rare intractable disease. In addition, the in vitro reporter cell assay was established using HEK293T cells rather than sweat gland cells because we have not yet established a physiologically relevant sweat gland acinar cell line. Furthermore, β-ionone dose dependence was not investigated in the sweat test owing to ethical concerns regarding the burden on the study subjects. Moreover, our results cannot explain the sex-based difference in perspiration observed upon β-ionone application. Despite these limitations, our findings showed that perspiration can be regulated through an unknown pathway involving the activation of ORs by odorous substances. The etiology and nature of AIGA remain unknown, and information on regional differences and similarities of anhidrosis are lacking and should be elucidated in the future. The involvement of ORs in AIGA pathogenesis was not proven in this study and should be addressed in future studies.

MATERIALS AND METHODS

Preparation of skin samples for transcriptome analyses

Patients with AIGA and healthy donors were recruited after the study was approved by the Institutional Review Board of Osaka University Hospital (Osaka, Japan) (653-4) and Nagasaki University Hospital (Nagasaki, Japan) (identification 20042025). Participants provided written informed consent, and one of them also agreed to publish the image in Figure 1a. Biopsy samples were manipulated according to the Declaration of Helsinki protocols. Subjects with anhidrosis were diagnosed on the basis of the conventional Minor's test, also called

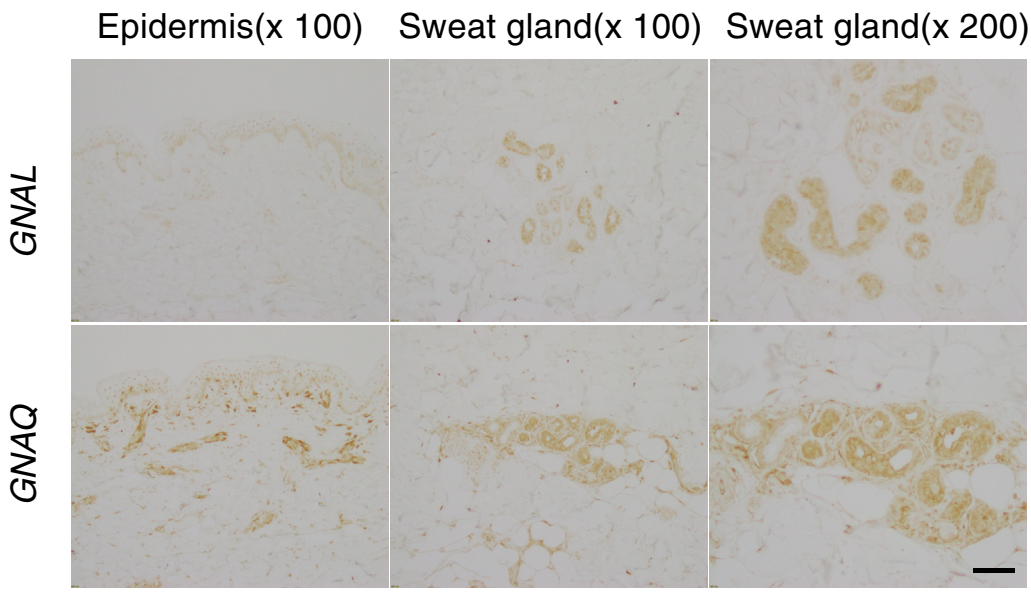

Figure 5. Expression of Gαq and Golf in sweat glands. Human skin samples were examined for protein expression of Gαq, which is encoded by *GNAL*, and Golf, which is encoded by *GNAQ*, by immunohistochemistry. Bar = 100 μm.

the starch-iodine sweat test, in which 2% iodine tincture was applied evenly to the skin followed by coating with a mixed suspension of starch-castor oil (50–100 and 100 g, respectively). Sweating was induced by sauna bathing at 60 °C for 10 minutes. After the Minor's test confirmed that subjects suffered from AIGA, skin punch biopsies (4–5 mm in diameter) were taken from anhidrotic (uncolored) and hidrotic (colored) areas. RNA for transcriptomic analysis was prepared from stored biopsy samples collected from four patients after the Minor's test as described earlier. Details of the cases are described in Table 2. During this process, sweat glands, isolated from those specimens using laser microdissection (LMD7000, Leica Microsystems, Wetzlar, Germany), were used as the source of RNA preparation.

RNA sequencing analysis

Total RNA was extracted from cells using a miRNeasy FFPE kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Next-generation sequencing library preparation was conducted using the SMARTer Stranded Total RNA Sample Prep Kit-Pico Input Mammalian (Clontech, Takara Bio, Kusatsu, Japan) kit according to the manufacturer's instructions. Sequencing was performed on an Illumina HiSeq 2500 platform in 75-base single-end mode. Illumina Casava1.8.2 software was used for base calling. Sequenced reads were mapped to the human reference genome sequence (hg19) using TopHat software, version 2.0.13, in combination with Bowtie2, version 2.2.3, and SAMtools software, version 0.1.19. Fragments per kilobase of exon per million mapped

Table 1. QSART Analyses of Forearm Skin Areas Treated with Topical β-Ionone

		QSART (mg/5 minutes)				
Age	Sex	β-Ionone	Control	Daily Exercise Habit	Smell	Impact of Topical β-Ionone on Sweating
50	F	0.039	0.305	+	+	Decrease
49	F	0.29	0.242	–	+	Not apparent
47	F	0.403	0.995	–	+	Decrease
46	F	0.077	0.27	–	+	Decrease
62	F	0.308	0.456	–	+	Decrease
41	F	0.516	0.807	+	+	Decrease
51	M	1.862	1.52	–	–	Increase
35	M	1.424	0.456	+	+	Increase
30	M	0.59	0.257	+	–	Increase
41	M	1.484	0.219	+	+	Increase
39	M	1.463	0.695	–	–	Increase
39	M	0.528	0.573	+	–	Not apparent
28	M	0.327	0.546	–	–	Decrease

Abbreviations: F, female; M, male; QSART, quantitative sudomotor axon reflex test.

The effect of β-ionone on sweating was examined using conventional QSART. The integrated volumes of sweating for 5 minutes after application of β-ionone and the control are shown. A difference <0.05 mg/5 min compared with the control was not considered significant. The subjects' exercise habits and their sensitivities to β-ionone as an odor are also shown.

Table 2. Demographic and Clinical Characteristics of the Four Patients with AIGA

case	Age/Sex	Duration of Disease	Treatment	Occupation	Underlying Comorbidities	No Sweat Area	Sweating Area
1	43/M	1 y	Antihistamine	Construction worker	None	Back	Forearm
2	32/M	1 y	Steroid pulse	Construction worker	None	Back	Chest
3	49/F	5 mo	—	Exercise Instructor	Cholinergic urticaria	Upper arm	Thigh
4	43/F	17 y	Herbal medicine	—	None	Right back	Left back

Abbreviations: AIGA, acquired idiopathic generalized anhidrosis; F, female; M, male.
Four patients with AIGA were recruited into this study after approval from the Institutional Review Board of Nagasaki University Hospital. Two of the patients were male, two were female, and all provided written informed consent.

fragments were calculated with Cuffnorm, version 2.2.1. Raw data were deposited in the National Center for Biotechnology Information Gene Expression Omnibus database (GSE 193125).

In situ hybridization

In situ hybridization was performed as described previously (Htun et al., 2021, Koji and Brenner, 1993). Briefly, sections were deparaffinized, rehydrated, treated with 0.2 N hydrogen chloride for 20 minutes, and then treated with 50 μg/ml proteinase K (Wako, Osaka, Japan) at 37 °C for 15 minutes. After fixation with 4% paraformaldehyde in 0.1 M PBS (pH 7.4) for 5 minutes, sections were immersed in 2 mg/ml glycine in PBS for 15 minutes and then maintained in 40% deionized formamide in 4x saline sodium citrate buffer (1x saline sodium citrate buffer: 0.15 M sodium chloride and 0.015 M sodium citrate, pH 7.0) until hybridization. Hybridization was carried out at 37 °C overnight with 2 μg/ml digoxigenin-labeled sense or antisense oligo-DNAs (Thermo Fisher Scientific, Waltham, MA) dissolved in a hybridization medium containing 10 mM Tris-hydrogen chloride (pH 7.4), 1 mM EDTA, 0.6 M sodium chloride, 1x Denhardt's solution, 250 mg/ml yeast transfer RNA, 125 mg/ml salmon sperm DNA, and 40% deionized formamide. After hybridization, sections were washed four times with 40% deionized formamide in 2x saline sodium citrate buffer for 1 hour at 37 °C, immersed in blocking solution for 1 hour, treated with horseradish peroxidase–conjugated goat anti-digoxigenin antibody overnight, and washed three times with 0.075% Brij 35 in PBS for 15 minutes. After rinsing with PBS, visualization was performed with 3,3′-diaminobenzidinetetrahydrochloride (Dojindo, Kumamoto, Japan), hydrogen peroxide, cobalt chloride, and nickel sulfate. Antisense oligo-DNA sequences complementary to portions of human OR51A7, OR6C74, and OR4A15 sense sequences were selected (Table 3). We also prepared oligo-DNA complementary to part of human 28S ribosomal RNA as a positive control probe (Yoshii et al., 1995).

Immunohistochemical staining

The skin samples used in the immunohistological study were obtained from a subject different from that for the samples used in the transcriptome assay. Skin samples were fixed in a 10% formalin-neutral buffer solution overnight. For staining, paraffin-embedded skin samples were sectioned (4 μm), deparaffinized, and dehydrated. Some sections were stained with H&E, and other sections underwent antigen retrieval in 10 mM sodium citrate buffer (pH 6.0) at 121 °C for 3 minutes. Samples were blocked with 5% normal goat serum/Tris-buffered saline with tween 20 for 60 minutes, incubated with primary antibodies at 4 °C overnight, and then washed three times with PBS. The following antibodies were used: OR51A7 (Invitrogen, Waltham, MA), OR51E2 (Invitrogen), GNAL (Abcam, Cambridge, England), and GNAQ (Abcam). Then, samples were incubated with secondary antibodies (Dako, LSAB2 system-horseradish peroxidase, Agilent, Santa Clara, CA) at room temperature for 30 minutes and washed three times with PBS. Signals were visualized using the 3,3′-diaminobenzidinetetrahydrochloride substrate kit (BD, Franklin Lakes, NJ). As a negative control, 5% BSA in PBS was used instead of the primary antibody (data not shown).

Construction of OR51A7 and OR51E2 expression vectors

Full-length canonical human OR51A7 and OR51E2 cDNAs were purchased from GenScript Biotech (Piscataway, NJ) and cloned into the pCAGGS vector (Riken, Tokyo, Japan) containing the

Table 3. List of RNA Probes Used for ISH

Object Gene	Arrangement	Gene Bank No.	Numbers of Bases
OR51A7		NM_001004749	
Sense probe	GCTGTGCTCACCTTCTATGTGCCCATCATCACCCTGGCTGCC		42
Antisense probe	GGCAGCCAGGGTGATGATGGGCACATAGAAGGTGAGCACAGC		
OR6C74		NM_001005490	
Sense probe	CTGGCTGCCATGTCCTATGAGCGCTATGTGGCC		33
Antisense probe	GGCCACATAGCGCTCATAGGACATGGCAGCCAG		
OR4A15		NM_001005275	
Sense probe	GCTGGTGCTGAAGTCATTCTTCTGGTGGTAATGGCC		36
Antisense probe	GGCCATTACCACCAGAAGAATGACTTCAGCACCAGC		
28S rRNA		NR_003279	
Antisense probe	TGCTACTACCACCAAGATCTGCACCTGCGGCGGC		34

Abbreviations: GC, guanine-cytosine; ISH, in situ hybridization; NCBI, National Center for Biotechnology Information; No, number; rRNA, ribosomal RNA.
Total RNA sequences were searched for using the NCBI database. ISH probes were designed on the basis of the number of bases and the GC ratio. The 28S rRNA was used as the positive control.

Table 4. OR1A7 and OR51E2 cDNA sequence data

OR51A7 cDNA (NM_001004749.1)

ATGTCTGTTCTCAATAACTCCGAAGTCAAGCTTTTCCTTCTGATTGGGATCCCAGGACTGGAACATGCCCACATTTGGTTCTCCATCCCCATTTGCCTCATGTA
CCTGCTTGCCATCATGGGCAACTGCACCATTCTCTTTATTATAAAGACAGAGCCCTCGCTTCATGAGCCCATGTATTATTTCCTTGCCATGTTGGCTGTCTCTG
ACATGGGCCTGTCCCTCTCCTCCCTTCCTACCATGTTGAGGGTCTTCTTGTTCAATGCCATGGGAATTTCACCTAATGCCTGCTTTGCTCAAGAATTCTTCATT
CATGGATTCACTGTCATGGAATCCTCAGTACTTCTAATTATGTCTTTGGACCGCTTTCTTGCCATTCACAATCCCTTAAGATACAGTTCTATCCTCACTAGCAACA
GGGTTGCTAAAATGGGACTTATTTTAGCCATTAGGAGCATTCTCTTAGTGATTCCATTTCCCTTCACCTTAAGGAGATTAAAATATTGTCAAAAGAATCTTCTTT
CTCACTCATACTGTCTTCATCAGGATACCATGAAGCTGGCCTGCTCTGACAACAAGACCAATGTCATCTATGGCTTCTTCATTGCTCTCTGTACTATGCTGGA
CTTGGCACTGATTGTTTTGTCTTATGTGCTGATCTTGAAGACTATACTCAGCATTGCATCTTTGGCAGAGAGGCTTAAGGCCCTAAATACCTGTGTCTCCCACA
TCTGTGCTGTGCTCACCTTCTATGTGCCCATCATCACCCTGGCTGCCATGCATCACTTTGCCAAGCACAAAAGCCCTCTTGTTGTGATCCTTATTGCAGATAT
GTTCTTGTTGGTGCCGCCCCTTATGAACCCCATTGTGTACTGTGTAAAGACTCGACAAATCTGGGAGAAGATCTTGGGGAAGTTGCTTAATGTATGTGGG
AGATAA

OR51E2 cDNA (NM_030774.3)

ATGAGTTCCTGCAACTTCACACATGCCACCTTTGTGCTTATTGGTATCCCAGGATTAGAGAAAGCCCATTTCTGGGTTGGCTTCCCCCTCCTTTCCATGTATGT
AGTGGCAATGTTTGGAAACTGCATCGTGGTCTTCATCGTAAGGACGGAACGCAGCCTGCACGCTCCGATGTACCTCTTTCTCTGCATGCTTGCAGCCATTG
ACCTGGCCTTATCCACATCCACCATGCCTAAGATCCTTGCCCTTTTCTGGTTTGATTCCCGAGAGATTAGCTTTGAGGCCTGTCTTACCCAGATGTTCTTTATT
CATGCCCTCTCAGCCATTGAATCCACCATCCTGCTGGCCATGGCCTTTGACCGTTATGTGGCCATCTGCCACCCACTGCGCCATGCTGCAGTGCTCAACAAT
ACAGTAACAGCCCAGATTGGCATCGTGGCTGTGGTCCGCGGATCCCTCTTTTTTTTCCCACTGCCTCTGCTGATCAAGCGGCTGGCCTTCTGCCACTCCAAT
GTCCTCTCGCACTCCTATTGTGTCCACCAGGATGTAATGAAGTTGGCCTATGCAGACACTTTGCCCAATGTGGTATATGGTCTTACTGCCATTCTGCTGGTCA
TGGGCGTGGACGTAATGTTCATCTCCTTGTCCTATTTTCTGATAATACGAACGGTTCTGCAACTGCCTTCCAAGTCAGAGCGGGCCAAGGCCTTTGGAACC
TGTGTGTCACACATTGGTGTGGTACTCGCCTTCTATGTGCCACTTATTGGCCTCTCAGTGGTACACCGCTTTGGAAACAGCCTTCATCCCATTGTGCGTGTT
GTCATGGGTGACATCTACCTGCTGCTGCCTCCTGTCATCAATCCCATCATCTATGGTGCCAAAACCAAACAGATCAGAACACGGGTGCTGGCTATGTTCAAG
ATCAGCTGTGACAAGGACTTGCAGGCTGTGGGAGGCAAGTGA

The cDNA sequences of OR51A7 and OR51E2 were extracted from the NCBI database.

cytomegalovirus immediate-early enhancer, chicken β-actin, and the rabbit β-globulin heterozygous promoter for expression in mammalian cells. Sequences are shown in Table 4.

AP-TGFα shedding reporter assay

The AP-TGF-α shedding assay was performed as described previously (Inoue et al., 2019) with minor modifications. HEK293T cells were cultured in RPMI1640 medium supplemented with 10% fetal calf serum, 100 IU/ml penicillin, and 100 μg/ml of streptomycin at 37 °C in a humidified atmosphere with 5% carbon dioxide. Cells (8 ′ 10^5 cells/4 ml) were transfected with the AP-TGFα–encoding plasmid (4 μg), the OR-encoding plasmid (3.2 μg), and/or G-protein–encoding plasmids (1.6 μg) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). After 12 hours, the transfected cells were harvested using trypsin/EDTA, pelleted by centrifugation at 600*g* for 5 minutes at 4 °C, washed once with DMEM, and resuspended in Hanks' balanced salt solution. Cell suspensions (180 μl each) were seeded into 96-well culture plates, 20 μl of β-ionone solution (10′, diluted in Hanks' balanced salt solution containing 0.01% [w/v] fatty acid–free and protease-free grade BSA; Sigma-Aldrich, St. Louis, MO) was added to each well, and the plates were incubated for 60 minutes at 37 °C. Then, the plates were centrifuged at 600*g* for 2 minutes at 4 °C, cell supernatants (80 μl) were transferred to fresh 96-well plates, and 80 μl of alkaline phosphatase reaction solution (10 mM *p*-nitrophenyl phosphate, 120 mM Tris-hydrogen chloride buffer, pH 9.5, 40 mM sodium chloride, and 10 mM magnesium chloride) was added to each well. Before and after a 2-hour incubation at 37 °C, absorbances at 405 nm were measured using a microplate reader.

Impact of topical β-ionone on sweating measured by the QSART

In this study, the volume of acetylcholine-induced sweating was measured quantitatively by QSART on the basis of the method established by Lee et al. (2009). Briefly, subjects were asked to remain quiet for 20 minutes before undergoing QSART in a hospital outpatient clinic at constant temperature (20 °C) and humidity (60%). The multicompartmental sweat capsule used in QSART consists of two independent compartments. Acetylcholine (100 mg/ml) applied iontophoretically to the skin from the outer compartment stimulates the underlying sweat glands directly; simultaneously, the central compartment of the capsule collects the sweat on the skin surface and measures sweat volume during the 5 minutes of iontophoresis. The integrated value of the sweat volume during the 5 minutes was regarded as the sweating ability (Kijima et al., 2012; Takahashi et al., 2016).

The effect of β-ionone topical application was evaluated in a side-by-side comparison with that of glycerol on both forearms of healthy subjects. This study was approved by the Institutional Ethical Committee of Nagasaki University (identification 20062602), and written informed consent was obtained from all study subjects. Briefly, 100 μl of β-ionone or glycerol (control) was applied on each forearm topically and separately. Immediately after topical application, ventilated sweat capsules that functioned in iontophoresis of acetylcholine (100 mg/ml) and that were attached to an additional outer compartment of sponge were placed onto the application areas. Integrated values of sweat volumes during the 5-minute acetylcholine iontophoresis after β-ionone and glycerol treatment were compared.

QSART activates postganglionic nerve fibers by both acetylcholine and electric stimulation, and once an axon is activated, the effect will be sustained for a certain period. Therefore, this test was performed once on the same subject to avoid erroneous results.

Data availability statement

Datasets related to this article can be found at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE193125], hosted at GSE 193125.

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CONFLICT OF INTEREST

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AUTHOR CONTRIBUTIONS

Conceptualization: HM, NM; Data Curation: DO; Formal Analysis: HM, NM; Funding Acquisition: HM, YT, NM; Investigation: YT, NM, TM; Methodology: YT, NM; Project Administration: HM, NM; Resources: YT, JA, AI; Software: YT, NM; Supervision: HM; Validation: YT, NM; Visualization: NM; Writing - Original Draft Preparation: NM; Writing - Review and Editing: NM, HM

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