

Article

Psychosocial Stress Induces Orofacial Mechanical Allodynia Due to the Enhancement of Transient Receptor Potential Ankyrin 1 Expression in Trigeminal Ganglion Neurons via the Increment of the Trace Amine-Associated Receptor 7f Expression

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Abstract: (1) Background: Chronic psychosocial stress can lead to oral dysesthesia with tongue pain. We examined whether psychosocial stress causes orofacial pain, and analyzed the comprehensive gene expression patterns of circulating cells and transient receptor potential ankyrin 1 (TRPA1) expression in trigeminal ganglion (TG) neurons in a mouse model of psychosocial stress. (2) Methods: Mice were divided into two groups: one group was kept in confrontational housing, and the other group was kept in single housing. Blood, adrenal gland, and tongue were collected. The head withdrawal threshold (HWT) of mechanical stimulation to the whisker pad skin was measured. TRPA1-positive TG neurons were immunohistochemically examined. DNA microarray analysis and quantitative reverse transcription polymerase chain reaction analysis were performed. (3) Results: The HWT was significantly lower in mice under the psychosocial stress condition compared to non-stressed mice. In stress-loaded mice, the number of TRPA1-positive TG neurons was significantly increased. Moreover, we showed that trace amine-associated receptor 7f expression was upregulated in circulating cells in blood and downregulated in the tongue. (4) Conclusions: Our results indicated that chronic psychosocial stress induced the orofacial mechanical allodynia through enhancement of TRPA1 expression in TG neurons with changes in the levels of trace amine-associated receptor 7f.

Keywords: psychosocial stress; transient receptor potential ankyrin 1; trace amine-associated receptor 7f; mechanical allodynia; trigeminal ganglion neuron

1. Introduction

Chronic psychosocial stress induces hypothalamic-pituitary-adrenal axis dysregulation, resulting in diseases, such as inflammatory disorders, metabolic syndrome, and major depressive disorder [1]. These disorders sometimes lead to chronic pain conditions [2]. In the oral and maxillofacial region, burning mouth syndrome (BMS), which presents as chronic oral dysesthesia with tongue pain (a burning sensation), is associated with stress,

anxiety, and depression [3]. Several studies have shown the relation between psychosocial stress and BMS [4,5]. The effectiveness of selective serotonin reuptake inhibitors for BMS has been reported by previous papers, suggesting that BMS is a somatoform disorder [6,7]. On the other hand, accumulating evidence has indicated that BMS is a trigeminal small-fiber sensory neuropathy [8,9].

Transient receptor potential ankyrin 1 (TRPA1), which belongs to the transient receptor potential channel, is expressed in nociceptive trigeminal neurons [10,11]. TRPA1 is involved not only in nociceptive pain, but also in peripheral neuropathic pain, including cold hyperalgesia, diabetic neuropathy, and chemotherapy-induced peripheral neuropathy [12–14]. Eliitt et al. have shown that TRPA1 expression in trigeminal sensory afferents is dramatically increased in mice with transgenic overexpression of artemin in tongue epithelium [15]. It is conceivable that these mice mimic the disease states of orofacial neuropathic pain.

Recently, Unno et al. developed a method to analyze the effect of psychosocial stress in an experimental rodent model [16]. This method is advantageous as it does not involve any attack between a dominant animal and a subordinate animal. Using the psychosocial stress mouse model of Unno et al., we examined the changes in the orofacial pain threshold and analyzed the comprehensive gene expression patterns of circulating cells.

2. Results

2.1. The Psychosocial Stress Decreased the Orofacial HWT

First, to examine whether psychosocial stress modulates corticosterone production, we assessed CYP11B1 expression in the adrenal gland, because CYP11B1 is essential for corticosterone synthesis. The level of CYP11B1 in the confrontational housing (CfH) group was significantly lower than that in the single housing (SiH) group (Figure 1; $n = 4$ each; Student's *t*-test). This result is consistent with a previous study that reported that the corticosterone level was decreased in the CfH group when compared to the SiH group [17]. Second, to assess orofacial mechanical allodynia, the HWT was measured for 5 weeks in both groups. The HWT in the CfH group was significantly lower than that of the SiH group from day 18 to day 27 (Figure 2; $n = 8$ each; two-way repeated measures analysis of variance, followed by Sidak's multiple comparisons test).

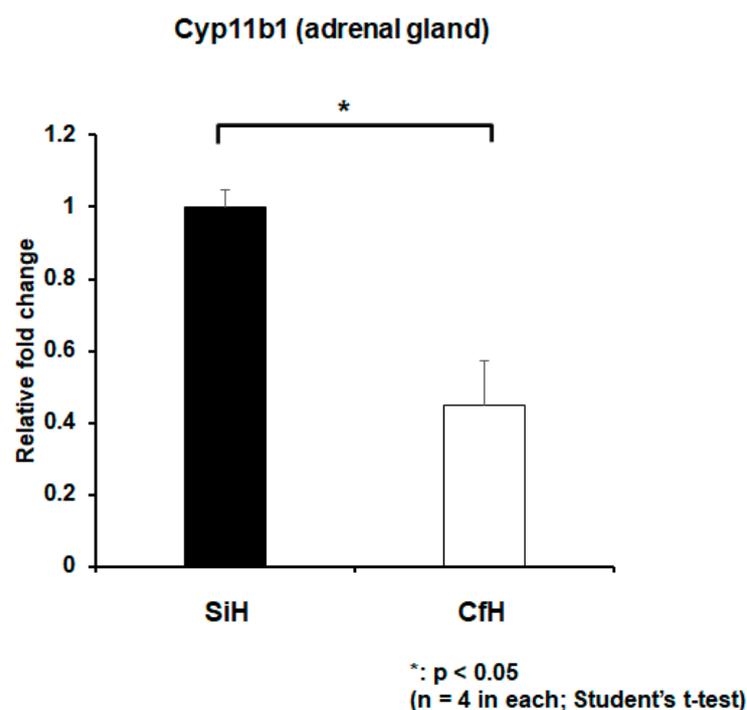
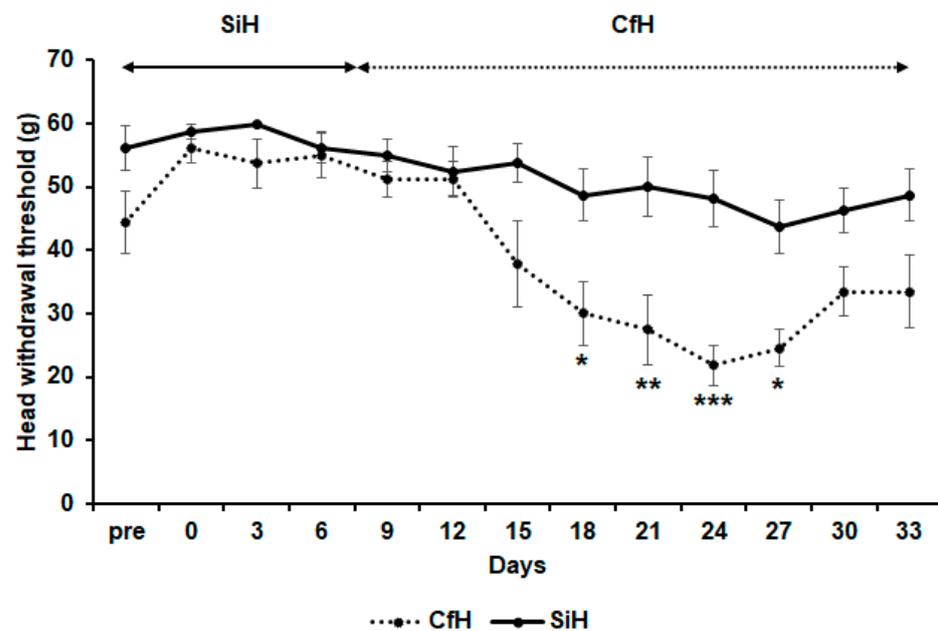


Figure 1. Gene expression of CYP11B1 in the adrenal gland. *: $p < 0.05$ vs. the SiH group (Student's *t*-test).



*: $p < 0.05$, **: $p < 0.01$, and ***: $p < 0.001$

($n = 8$ each; two-way repeated measures analysis of variance, followed by Sidak's multiple comparisons test)

Figure 2. Changes in the HWT in the whisker pad skin over 5 weeks in the SiH and CfH groups. *: $p < 0.05$, **: $p < 0.01$, and ***: $p < 0.001$ vs. the SiH group (two-way repeated measures analysis of variance, followed by Sidak's multiple comparisons test).

2.2. TRPA1-Positive Trigeminal Ganglion Neurons Increased in Mice under Psychosocial Stress Conditions

Next, we assessed whether TRPA1 in trigeminal ganglion neurons is involved in psychosocial stress. On day 24, the number of medium-sized (cell sizes: 401 to 500 μm^2 , 501 to 600 μm^2 , and 601 to 700 μm^2 in diameter) TRPA1-positive trigeminal ganglion neurons were significantly higher in the CfH group than in the SiH group (Figure 3A; $n = 4$ in each; Student's *t*-test). We also showed the immunostaining of TRPA1-positive trigeminal ganglion neurons in both groups (Figure 3B).

2.3. TAAR7f Expression Increased in Circulating Cells and Decreased in Tongue under Psychosocial Stress Conditions

To identify potential markers that are upregulated in blood by psychosocial stress, we collected circulating cells from blood, and conducted a DNA microarray analysis. In the DNA microarray analysis, we focused on TAAR7f, which is a G protein-coupled receptor that is a specific receptor for trace amines, because trace amine and trace amine-associated receptor (TAAR) signaling is implicated in several neuropsychiatric disorders [18]. The expression level of TAAR7f was 2.43-fold higher in the CfH group than in the SiH group (Table 1). We then examined TAAR7f expression in the tongue, because the tongue is affected by psychosocial stress in BMS. The level of TAAR7f was significantly lower in the CfH group than in the SiH group (Figure 4; $n = 4$ each; Student's *t*-test).

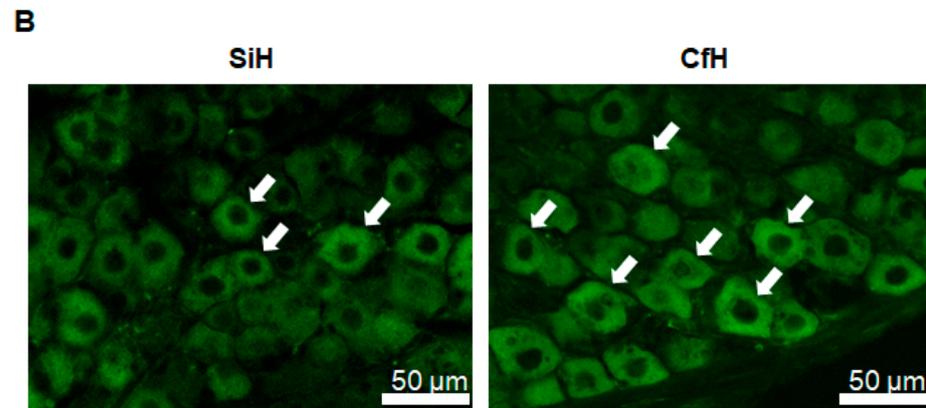
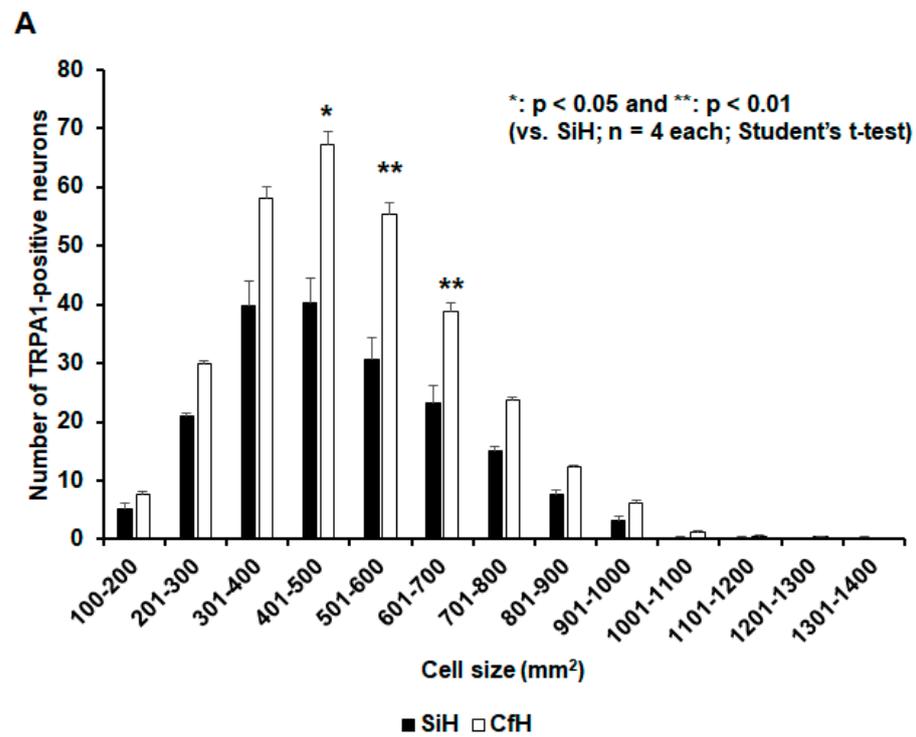


Figure 3. (A) The number of TRPA1-positive trigeminal ganglion neurons on day 24 in the SiH and CfH groups. (B) The immunostaining of TRPA1-positive trigeminal ganglion neurons (arrows) on day 24 in the SiH and CfH groups. Scale bars 50 μ m. *: $p < 0.05$ and **: $p < 0.01$ vs. the SiH group (Student's t -test).

Table 1. DNA microarray analysis in blood sample.

Gene Symbol	Description	Fold Change
Taar7f	Trace amine-associated receptor 7F	2.43

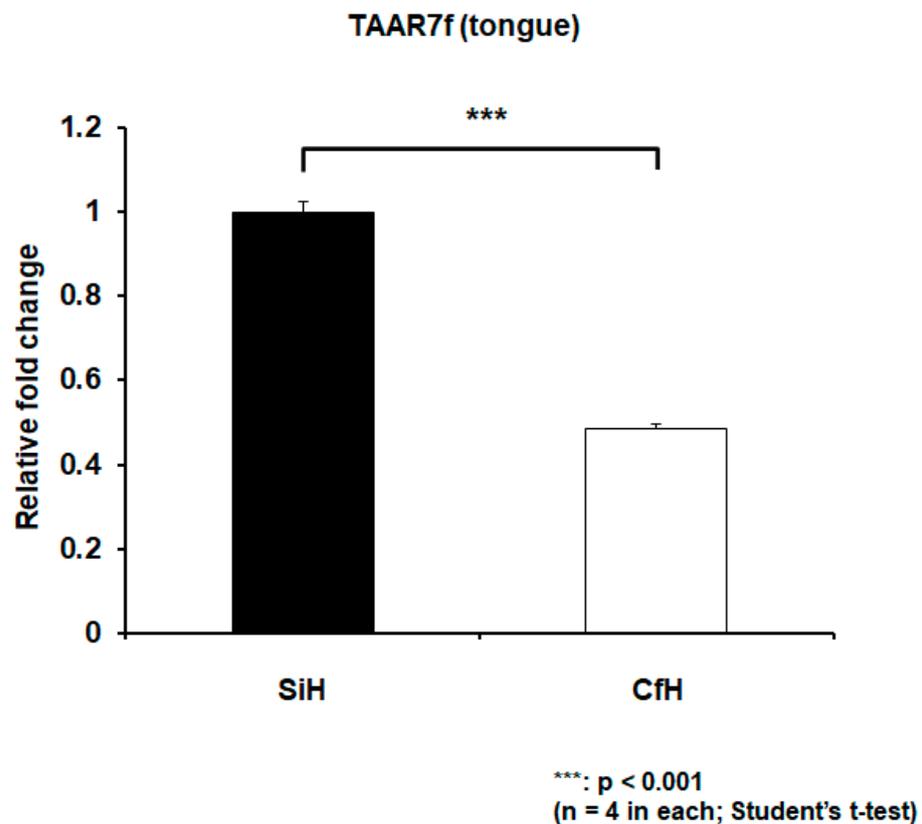


Figure 4. Gene expression of TAAR7f in tongue. ***: $p < 0.001$ vs. the SiH group (Student's *t*-test).

3. Discussion

Chronic psychosocial stress is an extraneous factor in BMS patients, and trigeminal small-fiber sensory neuropathy is associated with abnormal pain [8,9]. The TRPA1 channel is involved in nociceptive, neuropathic, cancer, and dysfunctional pain [19]. However, the involvement of TRPA1 in psychogenic pain has not yet been clarified. Our present study demonstrated for the first time that chronic psychosocial stress induced orofacial mechanical allodynia through increased TRPA1 expression in trigeminal ganglion neurons, and we found evidence of a connection between orofacial pain and psychosocial stress. TRPA1 signaling is involved in the development of social anxiety disorders due to reduced social behavior [20], and social anxiety disorders cause psychogenic pain [21]. We speculated that the induction of orofacial abnormal pain by chronic psychosocial stress is dependent on the plastic changes in TRPA1 expression in the trigeminal ganglion neurons.

Other types of TRP channels are associated with orofacial allodynia [22]. Transient receptor potential vanilloid 1 (TRPV1) depletion in genetic mice reveals remission of mechanical allodynia and the activation of TRPV1 induces mechanical allodynia [23]. Inhibition of transient receptor potential vanilloid 4 (TRPV4) attenuates mechanical allodynia in a rat model of intraoral wire-induced mucositis [24]. The inhibition of transient receptor potential melastatin 8 (TRPM8) with capsazepine significantly reduces cold pain, suggesting that TRPM8 may play a role in cold allodynia [25].

TAARs were initially discovered to have a high affinity for several psychotropic agents, and TAAR1 is expressed in the brain, pancreas, stomach, and gut [26–28]. Liberles and Buck showed that other members of TAARs (except for TAAR1) are involved in chemosensory function in the nasal olfactory epithelium [29]. Recently, other organs, such as the stomach, intestine, spleen, and testis, were shown to express several TAARs [30], and immune cells were also shown to express various TAARs [31,32]. Although the level of trace amines in mammal tissues is very low, trace amines have a functional role in physiological aspects. Given that a high expression level of TAAR1 is associated with an increased risk

of gastrointestinal illness and neuropsychiatric disorders, trace amines are assumed to be released into nerve endings and blood vessels. Although members of the TAAR7 subfamily are expressed by nasal olfactory epithelial cells and leukocytes [33], the functional role of the TAAR7 subfamily still remains unclear.

Since special sensory neurons, such as olfactory sensory neurons, express TAARs [29], and an agonist of human TAAR5 activates somatosensory regions in the brain [34], it is possible that TAARs are also expressed in trigeminal ganglia. We propose the following hypothesis by which trace amine induces orofacial pain. Trace amines in blood induced by psychosocial stress bind to the TAAR7f expressed by trigeminal ganglion neurons, resulting in increased phosphorylation of extracellular signal-regulated kinase (ERK) 1/2 since activation of TAARs is induced through ERK1/2 pathway [35]. Phosphorylation of ERK1/2 induces facial pain because it promotes the expression of TRPA1 protein [36].

We also found that circulating cells expressed TAAR7f. Leukocytes are the most abundant cells among circulating cells, and they express some TAARs [33], indicating that leukocytes may also express TAAR7f. We hypothesize that trace amines induced by psychosocial stress bind to the TAAR7f of leukocytes in the blood. Thus, the upregulation of TAAR7f in the blood may be a marker of psychosocial stress.

This study has some limitations. First, we did not measure the concentration of trace amines in the blood and tongue. Second, to obtain evidence of the involvement of TRPA1, we need to show that psychosocial stress does not affect the abnormal pain threshold in the absence of TRPA1. Finally, the relationship between TAAR7f and psychosocial stress still remains unclear in this study. To elucidate whether TAAR7f upregulation is specific to psychosocial stress, it is necessary to show that physical stress does not induce TAAR7f expression.

4. Materials and Methods

4.1. Animals

Male mice (ddY, 4-week-old) obtained from Japan SLC Co., Ltd., (Shizuoka, Japan) were maintained in an environmentally controlled clean room at Nihon University (approval number. AP16D007-4) and Saitama Medical University (approval number.2749), Japan. The experiments were conducted according to the institutional guidelines for ethical animal experiments.

4.2. Psychosocial Stress Experiment

Mice were housed in a group of ten in a cage for 5 days for acclimation, and they were subsequently divided into two groups: one group was kept in CfH, and the other group was kept in SiH. The psychosocial stress experiment was conducted according to a previous report [17]. Briefly, for SiH, two mice were housed in the same cage for 5 weeks. For CfH, a standard polycarbonate cage was divided into two identical subunits by a stainless-steel partition (floor size of each subunit: 140 × 170 mm). Two mice were housed separately in the cage for 7 days with one mouse in each subunit. The mice could not look at or be in contact with each other as a result of the partition (Figure 5A,B). Additionally, then the partition was removed to expose the mice to confrontational stress as shown in Figure 5C. The two mice were kept in cohabitation thereafter in the same cage for 4 weeks.

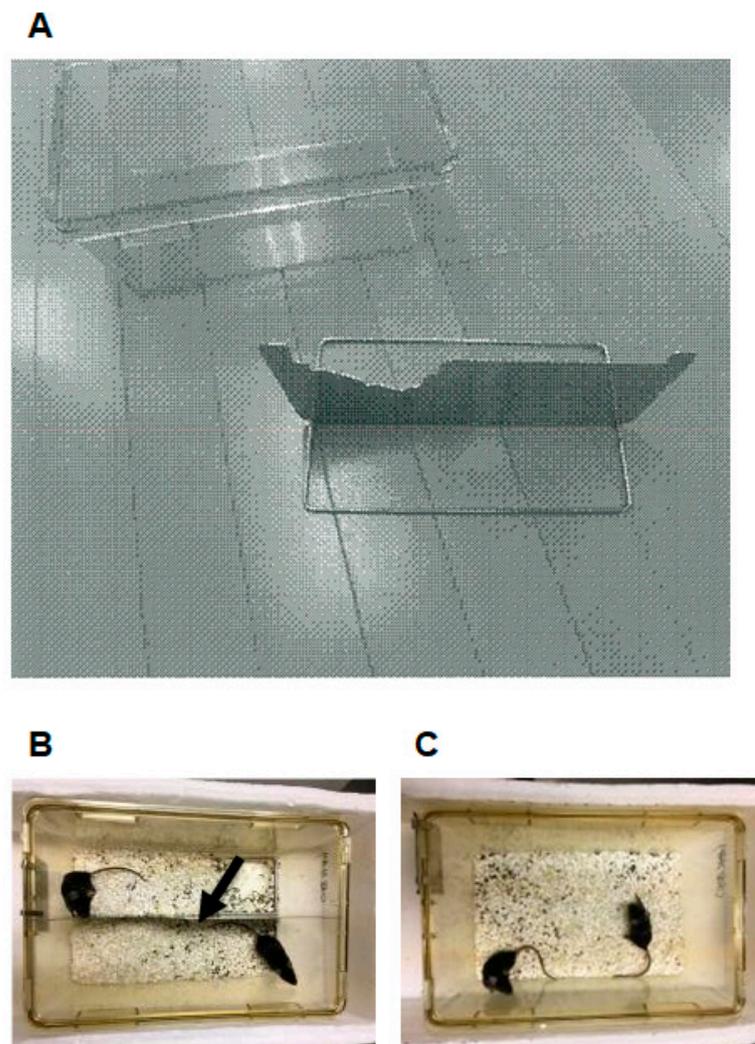


Figure 5. The photo of a cage, a partition and confrontational housing. (A) A partition and a cage. (B) Two mice were housed separately in the cage (Arrow indicates partition). (C) The partition was removed.

4.3. Blood, Adrenal Gland, and Tongue Sampling

Mice were sacrificed after 2 weeks. Collected blood was centrifuged at 3000 g for 10 min at 4 °C. The adrenal gland and tongue were homogenized using Cell Destroyer (Biomedical Science, Tokyo, Japan).

4.4. RNA Isolation

RNA was extracted using the miRNeasy Mini Kit (Qiagen, Hilden, Germany). The purity of the RNA was checked using Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). The RNA concentration was measured using the Qubit RNA Assay Kit (Thermo Fisher Scientific). The integrity of the RNA was assessed using the RNA Nano 6000 Assay Kit with the Agilent Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA).

4.5. Mechanical Pain Sensitivity in Whisker Pad Skin

Mice were laid in a dark, sound-insulated manufactured restraint device, which was equipped with a small fenestration, and their snout could protrude from the fenestration. Mechanical stimuli (intensity: 1, 2, 4, 6, 8, 10, 15, 26, 35, 50, or 60 g) to the whisker pad skin that protruded from the fenestration were applied by von Frey filaments (Touch-Test Sensory Evaluator, North Coast Medical, Morgan Hill, CA, USA) in order of increasing intensity. The mice were free to escape the mechanical stimuli of the whisker pad skin.

The HWT was defined as the minimum mechanical stimulus intensity that caused snout withdrawal three or more times out of five mechanical stimuli (duration: 1 s). The cutoff was 60 g of mechanical stimulus to prevent whisker pad skin injury. Under blind conditions, the HWT was measured every 2 days for 5 weeks under the same conditions.

4.6. Immunohistochemistry

On day 24, TRPA1-positive trigeminal ganglion neurons were immunohistochemically examined in both groups. Mice were deeply anesthetized by the intraperitoneal administration of butorphanol (2.5 mg/kg), medetomidine (0.375 mg/kg), and midazolam (2.0 mg/kg), followed by transcardial perfusion with 4% paraformaldehyde dissolved in 0.1 M phosphate buffer (pH 7.4). Trigeminal ganglion neurons were extracted from the cranial cavity and immersed in 4% paraformaldehyde for 4 h. After cryoprotection by immersion in 20% sucrose, the samples were embedded and sliced (thickness of 15 μ m) at -20°C . Then, the sections were rinsed in 0.01 M phosphate-buffered saline and incubated with anti-TRPA1 polyclonal rabbit antibody (1:1000, ab62053, Abcam, Cambridge, UK), 4% normal goat serum (Sigma-Aldrich Japan, Tokyo, Japan), and 0.3% TritonX-100 in 0.01 M phosphate-buffered saline at 4°C for 12 h. After rinsing, the sections were incubated with Alexa Fluor 568-conjugated goat anti-rabbit IgG (1:200, ab175471, Thermo Fisher Scientific) for 2 h. Subsequently, the sections were cover-slipped and air-dried in a dark room. TRPA1-immunoreactive neurons in the trigeminal ganglia were identified by fluorescence microscopy (BZ-9000; Keyence, Osaka, Japan). Samples with the absence of primary antibodies showed no specific immunoreactivity. Trigeminal ganglion neurons expressing a two-fold or greater intensity than the average background intensity were considered to be immunoreactive. The trigeminal ganglion neurons were divided into groups according to the cell sizes, i.e., 100–200, 201–300, 301–400, 401–500, 501–600, 601–700, 701–800, 801–900, 901–1000, 1001–1100, 1101–1200, 1201–1300, 1301–1400 (mm^2), and the mean number of TRPA1-immunoreactive trigeminal ganglion neurons was calculated in each group.

4.7. DNA Microarray Analysis

For the microarray analysis, total RNA was obtained using the GeneChip WT PLUS Reagent Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. Briefly, we performed reverse transcription using the total RNA, followed by second-strand cDNA synthesis, in vitro transcription, 2nd-Cycle cDNA synthesis, fragmentation, and biotin labeling. Biotin-labeled cDNA was then hybridized to the GeneChip Clariom D Mouse Array for 16 h, then washed and labeled with GeneChip Fluidics Station 450, and scanned with GeneChip Scanner 3000 7G (Thermo Fisher Scientific). The obtained expression data were analyzed by Transcriptome Analysis Console 4.0 software (Thermo Fisher Scientific).

4.8. Quantitative Reverse Transcription Polymerase Chain Reaction Analysis

To validate the changes in gene expression levels, quantitative reverse transcription polymerase chain reaction analysis was performed using the Step One Plus Real-Time PCR System (Thermo Fisher Scientific) according to the manufacturer's instructions. The reverse-transcription reaction was performed with a High-Capacity cDNA Reverse Transcription kit (Thermo Scientific). TaqMan Gene Expression Assays for cytochrome P450 family 11 subfamily B member 1 (CYP11B1) (Mm01204952_m1) and trace amine-associated receptor 7f (TAAR7f) (Mm03025211_gH; Thermo Scientific) were inventoried products. Mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Mm99999915_g1) was used as an endogenous control. Values were normalized to those of GAPDH using the $2^{-\Delta\Delta\text{Ct}}$ method.

4.9. Statistical Analysis

Comparisons between the two groups were analyzed using Student's t-tests. Differences between more than three groups were analyzed by two-way repeated measures analysis of variance, followed by Sidak's multiple comparisons test. All values are represented as the mean \pm S.E.M. *: $p < 0.05$, **: $p < 0.01$.

5. Conclusions

In conclusion, we found that chronic psychosocial stress induced orofacial pain due to the plastic changes in TRPA1 expression in TG neurons. We also showed that TAAR7f expression was upregulated in the circulating cells in the blood and downregulated in the tongue. However, because of the many limitations of this study, it is difficult to conclude whether chronic psychosocial stress induces an increase in TAAR7f expression in the blood via TRPA1. For example, it is necessary to show that chronic psychosocial stress does not upregulate blood TAAR7f expression in mice lacking TRPA1. Further examinations are needed to clarify whether chronic psychosocial stress is involved in chronic oral dysesthesia.

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