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Direct evidence that the brain reward system is involved in the control of scratching behaviors induced by acute and chronic itch

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ABSTRACT

In the present study, we demonstrated that there is a direct relationship between scratching behaviors induced by itch and functional changes in the brain reward system. Using a conditional place preference test, the rewarding effect was clearly evoked by scratching under both acute and chronic itch stimuli. The induction of ΔFosB, a member of the Fos family of transcription factors, was observed in dopamine transporter (DAT)-positive dopamine neurons in the ventral tegmental area (VTA) of mice suffering from a chronic itch sensation. Based on a cellular analysis of scratching-activated neurons, these neurons highly expressed *tyrosine hydroxylase (TH)* and *DAT* genes in the VTA. Furthermore, in an *in vivo* microdialysis study, the levels of extracellular dopamine in the nucleus accumbens (NAcc) were significantly increased by transient scratching behaviors. To specifically suppress the mesolimbic dopaminergic pathway using pharmacogenetics, we used the TH-cre/hM4Di mice. Pharmacogenetic suppression of mesolimbic dopaminergic neurons significantly decreased scratching behaviors. Under the itch condition with scratching behaviors restricted by an Elizabethan collar, the induction of ΔFosB was found mostly in corticotropin-releasing hormone (CRH)-containing neurons of the hypothalamic paraventricular nucleus (PVN). These findings suggest that repetitive abnormal scratching behaviors under acute and chronic itch stimuli may activate mesolimbic dopamine neurons along with pleasant emotions, while the restriction of such scratching behaviors may initially induce the activation of PVN-CRH neurons associated with stress.

1. Introduction

Chronic pruritus associated with illness is a serious problem that impairs sleep and daily life, leading to significantly reduced quality of life (QOL). Millions of people worldwide suffer from chronic pruritus [1], which is mainly observed in skin diseases, such as atopic dermatitis, but also occurs in visceral and mental diseases [2]. Therefore, it is a severe clinical problem that chronic itching caused by various diseases not only causes unpleasant emotions, but also causes severe skin damage due to scratching of the affected

Abbreviations: DAT, dopamine transporter; TH, tyrosine hydroxylase; VTA, ventral tegmental area; CRH, corticotropin-releasing hormone; PVN, paraventricular nucleus; M4Di, Gi-coupled human muscarinic M4 DREADD.

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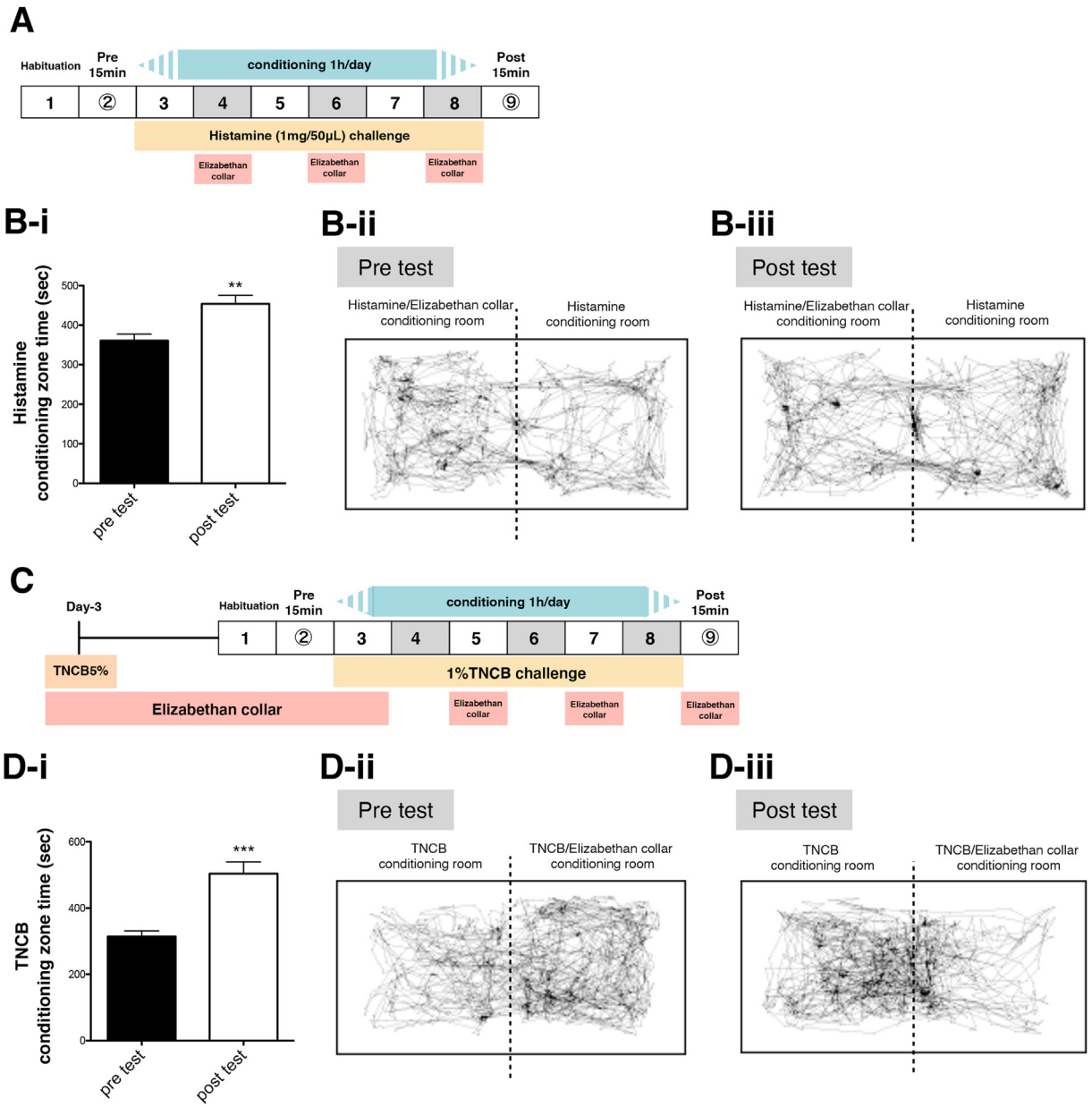

Fig. 1. Place preference induced by an itch sensation and scratching behavior resulting from the intra-dermal injection of histamine and the application of TNCB to skin. (A) Schedule for the conditioned place preference paradigm. (B) Effects of enabled scratching behavior accompanied by an itch sensation caused by the intradermal injection of histamine on the conditioned place preference (i). Ordinate: mean differences (sec) between times spent in the pre- and post-conditioning tests. Each column represents the mean conditioning score with S.E.M. of 8 animals. Typical traces of locomotion during the pre- (ii) and post-conditioning (iii) tests in histamine-treated mice ($n = 8$, $^{**}p < 0.01$ vs. Pre-test). (C) Schedule for the conditioned place preference paradigm. (D) Effects of enabled scratching behavior accompanied by an itch sensation caused by the application of TNCB to skin on the conditioned place preference (i). Ordinate: mean differences (sec) between times spent in the pre- and post-conditioning tests. Each column represents the mean conditioning score with S.E.M. of 8 animals. Typical traces of locomotion during the pre- (ii) and post-conditioning (iii) tests in TNCB-treated mice ($n = 8$, $^{***}p < 0.01$ vs. Pre-test).

area. In a skin condition such as atopic dermatitis, the skin is hypersensitive to itch, and itch is not suppressed by stimuli, such as scratching, which should normally suppress itch. These conditions lead to the prolongation and exacerbation of skin lesions. Thus, a vicious cycle of itching and scratching, an “itch-scratch cycle”, is generated, and may become intractable [3]. In addition, through the use of magnetic resonance tomography (fMRI) to examine changes in brain activity by inducing scratching behavior during itch stimulation, a strong response in the brain area associated with the reward system has been observed [4]. On the other hand, it has been documented that the application of itch stimulation to mice causes aversive behaviors [5]. The itch stimulus itself can induce unpleasant emotions. However, we would expect that scratching behavior activates the brain region related to pleasant emotions, and may lead to a craving for scratching behavior. This may lead to further destructive behavior and eventually to skin disorders.

It has been supposed that central sensitization may be caused by scratching behavior associated with itching, which may lead to chronic pruritus [6]. However, detailed studies have not been conducted on the brain circuit. In the present study using genetically modified animals and pharmacogenetic techniques, we investigated the direct relationship between scratching behaviors under itch stimuli and functional changes in the brain reward system.

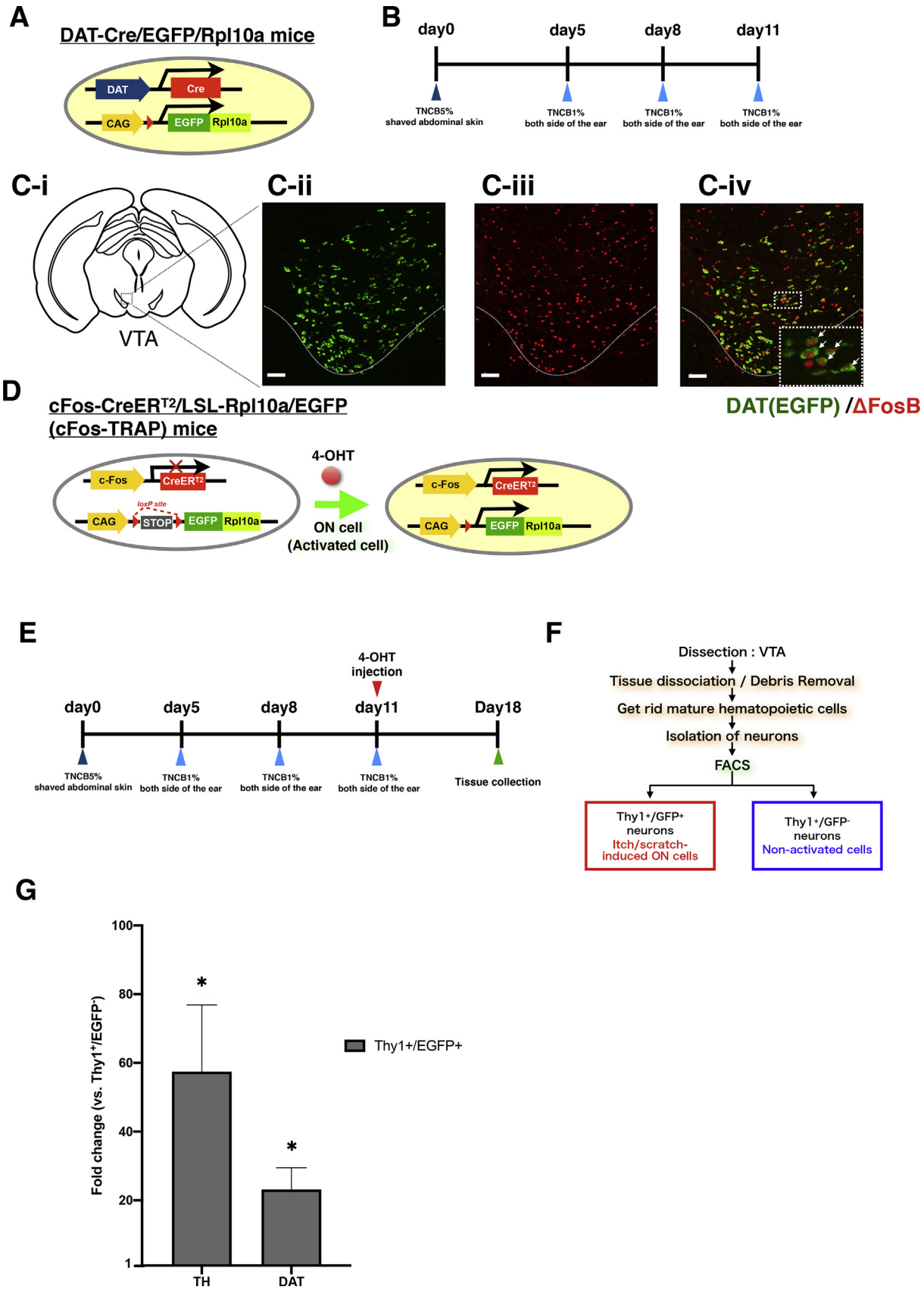

Fig. 2. Identification of activated-neurons induced by scratching behaviors following an itch sensation in the VTA. (A) DAT-EGFP/Rpl10a mice gene structure; these mice were produced by breeding DAT-cre mice and LSL-EGFP/Rpl10a mice. (B) Experimental timeline. (C) Schematic illustration of the staining site for the present study (i). Localizations of DAT (green, ii)-and ΔFosB (red, iii)-positive cells, and merged image of DAT and ΔFosB (iv) in a coronal section of the VTA of DAT-TRAP mice, which were repeatedly treated with TNCB and allowed to perform scratching behavior. (D) cFos-TRAP mice gene structure; these mice were produced by breeding cFos-creER^{T2} mice and LSL-EGFP/Rpl10a mice. (E)

2. Materials and methods

The present study was conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals, Hoshi University, as adopted by the Committee on Animal Research of Hoshi University.

2.1. Animals

The animals used in the present study were male C57BL/6J mice (7–13 weeks) (Jackson Laboratory, Bar Harbor, ME, USA), cFos-Targeted Recombination in Active Populations (TRAP) mice made by crossing B6.129 (Cg)-Fos < tm1.1 (cre/ERT2) Luo>/J mice (cFos-creERT2, The Jackson Laboratory) and B6.Cg-Gt (ROSA) 26Sor < tm9 (EGFP/Rpl10a) Amc>/J mice (The Jackson Laboratory), dopamine transporter (DAT)-TRAP mice made by crossing B6.SJL-Slc6a3<tm1.1(cre)Bkmn>/J mice (DAT-cre; The Jackson Laboratory) and B6;129S4-Gt(ROSA)26Sor < tm9 (EGFP/Rpl10a)Amc>/J mice, and corticotropin-releasing hormone (CRH)-TRAP mice made by crossing B6(Cg)-Crh < tm1(cre)Zjh>/J mice (CRH-cre; The Jackson Laboratory) and B6;129S4-Gt(ROSA)26Sor < tm9(EGFP/Rpl10a) Amc>/J mice. Tyrosine hydroxylase (TH)-hM4Di mice were generated by microinjection of AAV-FLEX-hM4Di-mCherry into the ventral tegmental area (VTA) of TH-cre mice to produce Gi-coupled human muscarinic M4 DREADD (hM4Di) based on the FLEX switch system. All mice were housed up to six mice per cage and kept in a temperature- and humidity-controlled room (24 ± 1 °C, 55 ± 5% relative humidity) under a 12-h light–dark cycle (light on at 8 a.m.). Food and water were available *ad libitum*. All procedures for the animal study were performed at the laboratory of Hoshi University following their Guiding Principles during the light cycle. All experimental procedures minimized the number and suffering of the animals.

2.2. Drugs

Drugs used in the present study were 2,4,6-trinitrochlorobenzene (TNCB; Tokyo Chemical Industry Co., Ltd. Tokyo), histamine (Sigma Aldrich. Inc., MO, USA), 4-hydroxytamoxifen (4-OHT; Sigma Aldrich. Inc.), and clozapine *N*-oxide (CNO; Abcam plc., Cambridge, UK). TNCB was dissolved in acetone (Fujifilm Wako Pure Chemical Co., Ltd., Osaka) at 5% or 1%. Histamine and CNO were dissolved in physiological saline, and 4-OHT is dissolved in ethanol (Fujifilm Wako Pure Chemical Co., Ltd.) and corn oil (Fujifilm Wako Pure Chemical Co., Ltd.).

2.3. Chemical-induced itch in mice

Histamine (1 mg/50 μL) was injected intradermally into the rostral back of mice. Mice were initially sensitized by painting 100 μL 5% TNCB on their shaved abdominal skin at day 0, and this was boosted by the application of 20 μL of 1% TNCB to ears at 5, 8 and 11 days after initial sensitization based on a previous paper [7].

2.4. Place conditioning

Place conditioning studies were conducted using a shuttle box (15 × 30 × 25 cm: w × l × h; Ohara & Co., Ltd., Tokyo, Japan) composed of an acrylic resin board divided into two equal-sized compartments (black or white). In brief, the place-conditioning schedule consisted of three phases (pre-conditioning test, conditioning, and post-conditioning test) as previously described [8,9]. In the pre-conditioning test, the position (whether black or white box) of mice was monitored automatically using an infrared beam sensor (TimeLD4; Ohara & Co., Ltd., Tokyo, Japan). Conditioning sessions (3 with an Elizabethan collar: 3 without an Elizabethan collar) were conducted once a day after the pre-conditioning test. Immediately after histamine injection (1 mg/50 μL, i.d.), the animals were placed in the compartment opposite that in which they had spent the most time in the preconditioning test for 1 h without an Elizabethan collar. On alternate days, immediately after histamine injection (1 mg/50 μL, i.d.), the animals were placed in the other compartment for 1 h while wearing an Elizabethan collar. A post-conditioning test, which was identical to the pre-conditioning test, was performed 1 day after the final conditioning session without an Elizabethan collar.

In another set of experiments, mice with an Elizabethan collar were sensitized by treatment with 5% TNCB. In the conditioning session, immediately after treatment with 1% TNCB, animals were placed in the compartment in which they had spent the most time in the pre-conditioning test for 1 h with an Elizabethan collar on conditioning days 1, 3 and 5. On the other days, these animals without an Elizabethan collar were placed in the other compartment for 1 h on days 2, 4 and 6. A post-conditioning test that was identical to the pre-conditioning test was performed 1 day after the final conditioning session with an Elizabethan collar.

2.5. Immunohistochemistry

Immunohistochemistry was conducted based on our previous paper [10]. Under isoflurane (3%, inhalation) anesthesia, the brain was fixed transcardially using 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), and then post-fixed and cryoprotected in 20–30 (w/v)% sucrose. Brain frozen sections were cut on a cryostat (CM1860; Leica Microsystems, Heidelberg, Germany) after being embedded in OTC compound (Tissue Tek; Sakura Fine Technical, Tokyo, Japan). After blocking, the brain sections were incubated with a primary antibody for ΔFosB 1:500) (goat polyclonal; Santa Cruz Biotechnology, Inc., TX, USA) in 7% NHS. After washing, they were incubated with an appropriate secondary antibody conjugated with Alexa 546. Fluorescence was detected using a light microscope (BX53; Olympus, Tokyo, Japan) and scanned with a digital camera (CoolSNAP HQ; Photometrics. Inc., AZ, USA).

2.6. Labeling and isolation of scratching-activated neurons

Labeling of scratching-activated neurons was performed using cFos-TRAP mice [11], which express an EGFP/Rpl10a by a tamoxifen-induced Cre/loxP system in the presence of 4-OHT (50 mg/kg, i.p.) 30 min after the final application of 1% TNCB. Samples were isolated 7 days after 4-OHT injection.

To obtain activated cells, which were labeled with enhanced green fluorescent protein (EGFP), an adult brain dissociation kit (Miltenyi Biotec., Bergisch Gladbach, Germany) was used to isolate the cells, and these lineage cells (hemocyte cells) were then segregated by a lineage cell depletion kit (Miltenyi Biotec) and magnetically activated cell sorting (MACS). Thereafter, glial cells were removed from the lineage cell-negative fraction using a neuron isolation kit (Miltenyi Biotec). Thy1.2 (a marker for a nerve cell surface antigen, 1:200, BioLegend Inc., CA, USA)-positive/EGFP-

Experimental timeline. (F) Experimental flowchart for the isolation of activated neurons by itch-associated scratching behavior from the VTA of cFos-TRAP mice by MACS and FACS. (G) The mRNA expression levels of dopamine transporter (DAT) and tyrosine hydroxylase (TH) in activated and non-activated neurons in the VTA of cFos-TRAP mice, which were repeatedly treated with TNCB and allowed to perform itch-associated scratching behavior. Each column represents the mean ± S.E.M. of 3 samples (*p < 0.05 vs. $Thy1^{+}/GFP^{-}$ cells). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

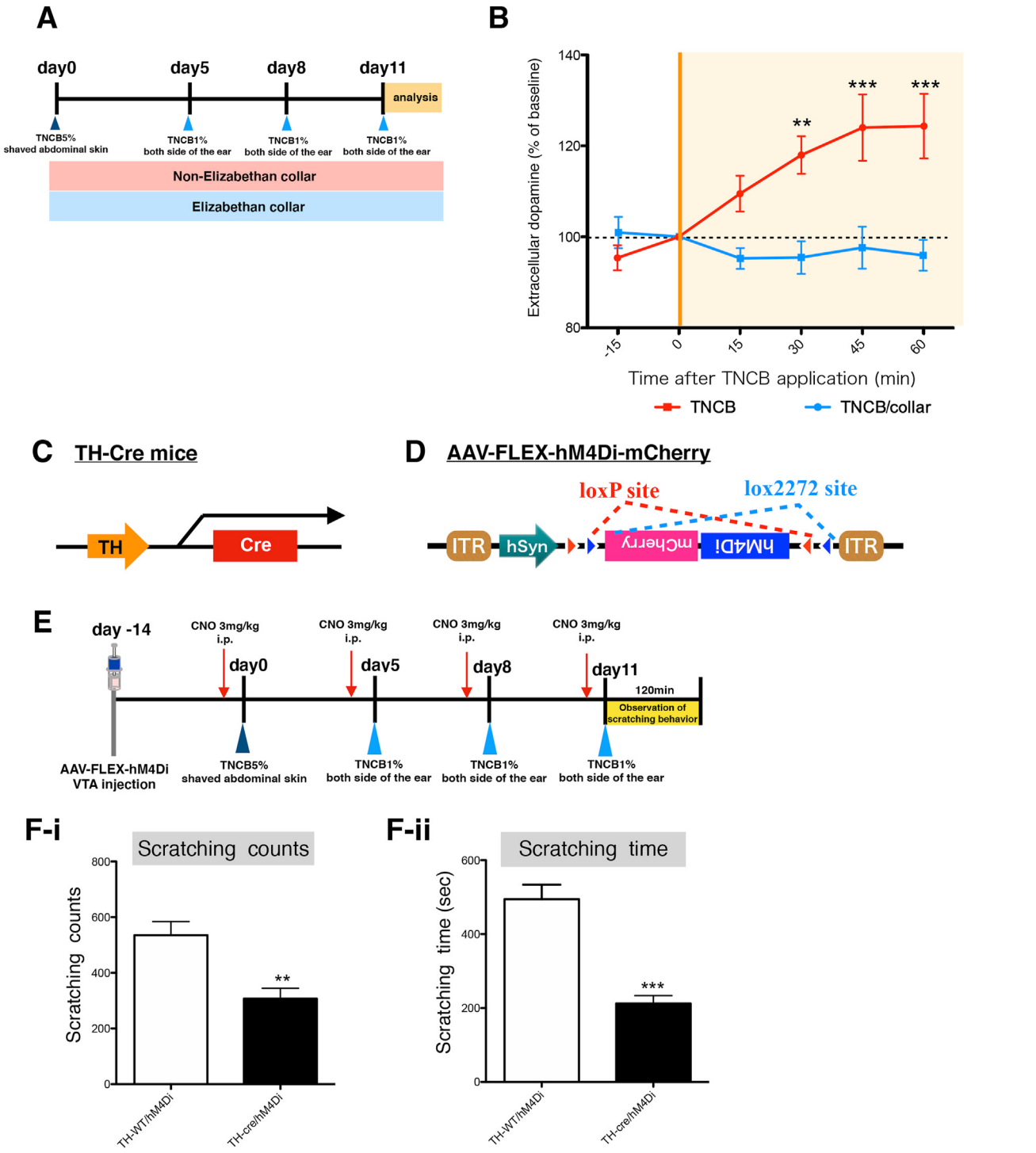

Fig. 3. Changes in the release of dopamine in the NAcc after TNCB application and effects of the suppression of mesolimbic dopaminergic neurons on itch-associated scratching behaviors induced by TNCB. (A) Experimental timeline. (B) Extracellular dopamine levels in the NAcc of TNCB sensitized mice and with or without an Elizabethan collar after the final application of TNCB to the ears (n = 6, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. TNCB/collar). (C) TH-cre mouse gene structure; The Cre gene is located downstream of the TH promotor. (D) Schematic construct of Cre-dependent AAV (AAV-hM4Di-mCherry); The gene is doubly flanked by two incompatible sets of *lox* sites. In the presence of Cre recombinase, hM4Di-mCherry is inverted to enable transcription from the hSyn promoter. (E) Experimental timeline. (F) Effects of the suppression for the mesolimbic dopaminergic neuronal activity by a DREADD system on the number of scratching behaviors (i) and the duration of scratching (ii) in mice that were repeatedly treated with TNCB. Each column represents the mean ± S.E.M. of 6 animals ($^{**}p < 0.01$, $^{***}p < 0.001$ vs. WT).

positive fraction was defined as scratching-activated cells, and the Thy1.2-positive/EGFP-negative fraction, was defined as scratching-activated cells, were purified by using a BD FACSAria™ III cell sorter (BD Bioscience, Inc., NJ, USA). After pre-amplifying specific transcripts for 18 cycles using PreAmp Master Mix (Fluidigm Co., CA, USA), real-time RT-PCR was then performed based on our previous paper [12].

TH; forward: 5′- TTCGAGGAGAGGGATGGAAA-3′

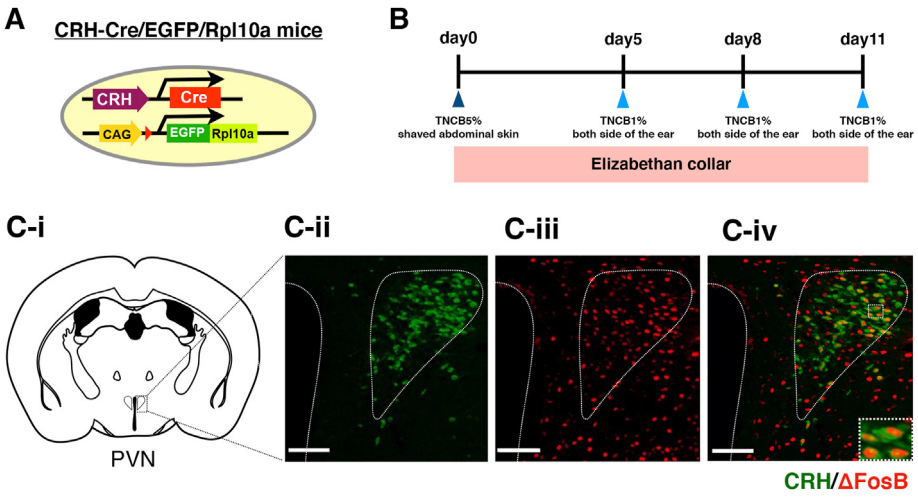

Fig. 4. Localization of itch-associated "ON" cells in the PVN of mice in which scratching behaviors were restricted. (A) CRH-EGFP/Rpl10a mouse gene structure; these mice were produced by breeding CRH-cre mice and LSL-EGFP/Rpl10a mice. (B) Experimental timeline. (C) Schematic illustration of the staining site for the present study (i). Localization of CRH (green, ii) and ΔFosB (red, iii), and merged image of CRH and ΔFosB (iv) in a coronal section of the PVN of CRH-TRAP mice in which scratching behavior was restricted. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

reverse: 5′- GGTGGATTTTGGCTTCAAATG-3′
DAT; forward: 5′- GCTGCTGGTGTCTGGAAGATC-3′
reverse: 5′- GTAGTGCAGTGCCCATGCAA-3′

2.7. In vivo microdialysis and high-performance liquid chromatography

In vivo microdialysis to measure dopamine release from the nucleus accumbens (NAcc) (AP, +1.4 mm; ML, +1.5 mm; DV, −3.6 mm; angle, 10°) was conducted as described previously [10]. The probe was continuously perfused with artificial cerebrospinal fluid (0.9 mM $MgCl_2$, 147.0 mM NaCl, 4.0 mM KCl, and 1.2 mM $CaCl_2$), and outflow fractions were collected every 15 min. After more than two baseline fractions were collected, TNCB was applied to both ears of the mice. Dialysis samples were analyzed by high-performance liquid chromatography with electrochemical detection (HTEC-500; Eicom). The amount of dopamine was quantified by calculations using the peak area, and data are expressed as a percentage of the corresponding baseline peak area.

2.8. Designer receptors exclusively activated by designer drugs (DREADD)

To selectively manipulate the mesolimbic dopaminergic neurons by DREADD, TH-hM4Di mice, which expressed hM4Di in the VTA, were used in this experiment. Suppression of neuronal activity through the DREADD system was achieved by the administration of CNO (3 mg/kg, i.p.) to each mouse.

2.9. Measurement of scratching behavior

The measurement of scratching behavior due to the itch sensation as an itch model in mice was performed after the final application of 1% TNCB in TNCB-sensitized mice. 1% TNCB was applied to both ears of mice under anesthesia by isoflurane (2%, inhalation). The number and duration of scratching behaviors were observed for 120 min.

2.10. Statistics

The data are presented as the mean S.E.M. The sample size was selected based on similar publications in the field. The statistical significance of differences between the groups was assessed by two-way analysis of variance followed by the Bonferroni post-hoc test or unpaired *t*-test. All statistical analyses were performed with GraphPad Prism 5.0 (GraphPad Software).

3. Results

3.1. Place preference induced by an itch sensation and scratching behavior resulting from the intra-dermal injection of histamine and the application of TNCB to the skin

We first confirmed that intradermal administration of histamine produced a robust scratching behavior just after the administration (data not shown). In the conditioned place preference paradigm (Fig. 1A), scratching behaviors in response to histamine-induced itch sensation caused a significant place preference (Fig. 1B–i $^{**}p < 0.01$ vs. Pre-test).

We examined the effects of temporary scratching behaviors after a sustained itch sensation caused by the application of TNCB to the skin in TNCB-sensitized mice on a conditioned place preference (Fig. 1C). As shown in Fig. 1D, temporary scratching behavior produced a significant place preference in mice that had experienced a sustained itch sensation (Fig. 1D–i $^{***}p < 0.001$ vs. Pre-test).

3.2. Identification of neurons activated by scratching behaviors in response to an itch sensation in the VTA

We identified scratching behavior-induced activated cells, so-called activated "On cells", in the VTA during an itch sensation using DAT-EGFP/Rpl10a mice (Fig. 2A). Immunohistochemically, ΔFosB expression after the application of TNCB merged with the EGFP reaction inside cells of the VTA of TNCB-sensitized mice (Fig. 2C).

We identified and measured the mRNA levels of dopamine-related genes in scratching behavior-induced "On" cells under sustained itch sensation caused by the application of TNCB to the skin in cFos-TRAP mice (Fig. 2D–G). The mRNA levels of dopamine-related makers, such as TH and DAT, in "ON" cells were extremely high in the VTA of cFos-TRAP mice (Fig. 2G $^{*}p < 0.05$ vs. $Thy1^{+}/GFP^{-}$).

3.3. Changes in the release of dopamine in the NAcc after the application of TNCB and effects of the suppression of mesolimbic dopaminergic neurons on itch-associated scratching behaviors induced by TNCB

The scratching behavior caused by the itch sensation after the application of TNCB to the ears of TNCB-sensitized mice significantly increased the release of extracellular dopamine in the NAcc, whereas the dopamine level was not changed in scratching behavior-restricted mice that wore an Elizabethan collar, despite the presence of an itch sensation (Fig. 3B).

By using selective manipulation of the mesolimbic dopaminergic system by the DREADD technique, we found that the suppression of mesolimbic dopaminergic neurons by CNO (3 mg/kg) significantly reduced the number and duration of scratching behaviors over 120 min after the application of TNCB (Fig. 3F–i,ii **$p < 0.01$ vs. WT, ***$p < 0.001$ vs. WT) in TNCB-sensitized TH-hM4Di mice, which were TH-Cre mice that were microinjected with hM4Di-mCherry (Fig. 3D) into the VTA (Fig. 3C).

3.4. Localization of itch-associated "ON" cells in the PVN of mice with restricted scratching behaviors

We determined the localization of itch-associated "ON" cells in the hypothalamic paraventricular nuclei (PVN) of CRH-EGFP/Rpl10a mice in which scratching behaviors were restricted (Fig. 4). The restriction of scratching behaviors in the presence of an itch sensation produced by the application of TNCB induced ΔFosB expression ("ON" cells) in the PVN area. Such expression of ΔFosB by the restriction of scratching behaviors in the PVN was merged with the CRH-positive EGFP reaction (Fig. 4C).

4. Discussion

At first, using the CPP test, we found that significant increase in place preference was clearly observed by scratching behaviors under a histamine-induced itch stimulus, which is widely recognized as an acute mouse model. Similar to the results in the acute mouse model, we also found a significant increase in the time spent in the conditioned area in a chronic mouse model with contact dermatitis induced by the application of TNCB. These results indicate that scratching under acute and chronic itch stimuli could be regarded as reward stimuli.

It has been established that, among the brain reward systems, the mesolimbic dopaminergic system plays an important role in the scratching behavior during pruritus as well as drug dependence [4,13]. In this study, we generated mice expressing EGFP/Rpl10a specifically for DAT-positive cells to identify scratching-activated dopamine neurons induced by TNCB injection. We confirmed that scratching-activated cells in the VTA labeled by the neuronal activation marker ΔFosB were mostly DAT-positive dopamine-containing neurons. As well as the present immunohistological staining, we identified that, according to a FACS system using cFos-TRAP mice that expressed EGFP/Rpl10a when cFos was expressed by scratching, the scratching-activated cells in the VTA were rich in TH, the rate-limiting enzyme for dopamine, and DAT, which plays a role in dopamine reuptake. Taken together, these results suggest that scratching behaviors may be associated with the activation of VTA-dopaminergic neurons.

Next, we investigated whether the suppression of mesolimbic dopamine neurons could directly reduce the induction of scratching behaviors according to pharmacogenetic techniques. For this purpose, we generated TH-Cre mice expressing the recombinant Cre enzyme in TH-positive cells, with the injection of AAV-FLEX-hM4Di-mCherry into the VTA. Using the transgenic mice, a reduction in scratching behaviors caused by an allergic reaction was clearly observed by specifically suppressing dopaminergic neurons in the VTA. These data provide direct evidence that the induction of repeated scratching behaviors is related to fluctuations in the activity of mesolimbic dopaminergic neurons.

In addition to the VTA, the NAcc is also known to play a role in itch cognition and pleasure. The NAcc is a major target of VTA-dopamine neurons. It has been demonstrated that the dopamine fibers in the NAcc are strongly activated during the onset of itch-evoked scratching behavior [14]. In the present study, we investigated the possible change in dopamine release due to temporary scratching in the NAcc region of mice that had been treated with TNCB using an *in vivo* microdialysis assay. In the presence of itch stimulation by the application of TNCB, a significant increase in dopamine release was observed in the group that was capable of scratching behaviors compared to the group in which scratching behaviors were restricted. These findings suggest that, in addition to the VTA, the NAcc also plays a crucial role in the itch-scratch sensation.

Clinically, stress is one of the main causes of worsening itch in patients with atopic dermatitis [15]. In an atopic dermatitis mouse model, it has been documented that CRH is involved in the exacerbation of dermatitis due to stress [16] . It has been recognized that activation of CRH neurons in the hypothalamic PVN is involved in the stress response, resulting in the induction of aversive behaviors [17]. These findings provide the hypothesis that the restriction of scratching behaviors in the presence of itch stimuli may elicit a stress response, and the activation of CRH in the PVN is partly involved in the itch-induced craving for scratching. In the present study, using a genetically modified mouse in which GFP was specifically expressed in PVN-CRH-positive cells, we demonstrated that the ΔFosB-positive PVN cells activated by itching when scratching behaviors were restricted by an Elizabeth collar were mostly rich in CRH. These results suggest that changes in the activities of both VTA dopamine neurons and PVN-CRH neurons may be implicated in scratching behaviors under chronic itch condition. Especially, the abnormal and repetitive scratching behaviors found in numerous skin disorders may be triggered by the activation of mesolimbic dopamine neurons, and could induce cravings for scratching. Thus, we expect that a strong desire for bruising behaviors followed by a strong itch may spur further scratching behaviors.

In conclusion, using genetically modified animals and pharmacogenetic techniques, we provide here direct evidence that activation of the mesolimbic dopaminergic system is involved in repetitive abnormal scratching behaviors induced by acute and chronic itch stimuli. Furthermore, the restriction of such scratching behaviors could initially activate PVN-CRH neurons, which is related to stress, and could eventually trigger a desire and craving for scratching behaviors. The present data support the idea that a further approach is needed to establish a new treatment that focuses on the control of the neural circuits in the brain for chronic pruritus, instead of treating only the affected peripheral area.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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