Intense chronic itch significantly reduces quality of life for atopic dermatitis patients, impairing daily activity. Although abnormal itch sensation can be induced by innocuous stimuli, known as alloknesis, the mechanisms driving this process remain obscure. Psychological and environmental stimuli are known to aggravate atopic dermatitis symptoms. Recently, the enzyme 11β-hydroxysteroid dehydrogenase-1 (HSD11β1), which is expressed in keratinocytes, has been implicated in maintaining homeostasis against environmental stimuli by activating endogenous glucocorticoids. To investigate the role of HSD11β1 in keratinocytes, we generated keratinocyte-specific Hsd11b1-knockout (Hsd11b1<sup>KC−/−</sup>) mice and analyzed skin phenotype. Hsd11b1<sup>KC−/−</sup> mice exhibited abnormal cutaneous innervation and skin sensitivity, including light mechanical stimulus-evoked itch (i.e., alloknesis). Attenuated endogenous glucocorticoid activation induced by aberrant artemin production in keratinocytes was involved in alloknesis in Hsd11b1<sup>KC−/−</sup> mice. Finally, we observed a significant negative correlation between expression of HSD11β1 and artemin in human skin with and without AD. These results suggest that endogenous glucocorticoids that maintain skin homeostasis in the epidermis affect both skin innervation and cutaneous sensation. Modulation of HSD11β1 activation could be a therapeutic target for sensitive or itchy skin.
Therefore, we hypothesized that an endogenous GC deficiency might negatively affect itch symptoms induced by environmental stimulus.

In this study, we found excessive cutaneous innervation and touch-evoked itch in keratinocyte-specific Hsd11b1 knockout (Hsd11b1KC/−/−) mice. We show that a nerve elongation factor produced by keratinocytes is suppressed by homeostatic local cortisol activation in healthy and atopic human skin. These results suggest that endogenous GCs that maintain skin homeostasis in epidermis affect both skin innervation and cutaneous sensation.

RESULTS

Hsd11b1KC/−/− mice exhibit abnormal cutaneous innervation in the absence of skin lesions

We first investigated the cutaneous phenotype of Hsd11b1KC/−/− mice, including skin inflammation, barrier function, and innervation, which are pathologic features of AD. Hsd11b1KC/−/− mice did not present with significant skin lesions, such as hyperkeratosis, acanthosis, or immune cell infiltration (Figure 1a). However, we frequently observed epidermal nerve sprouting and nerve fibers just under the epidermis in the footpad, tail, and dorsal skin from Hsd11b1KC/−/− mice (Figure 1b). We calculated the percentage of protein gene product 9.5-positive area in the epidermis of the footpad, tail, and dorsal skin. Compared with wild-type (WT) mice, the fold change in protein gene product 9.5-positive area in the epidermis Hsd11b1KC/−/− mice (Figure 1c).

Then, we performed three-dimensional imaging of protein gene product 9.5-positive nerve fiber in dorsal skin to visualize the cutaneous innervation pattern. Thickened nerve fibers that extended toward the epidermis were observed in Hsd11b1KC/−/− mice (Figure 1d, and see Supplementary Movies S1 and S2 online).

Hsd11b1KC/−/− mice exhibit dysesthesia and susceptibility to light mechanical stimulus-evoked itch

To profile the skin sensitivity of Hsd11b1KC/−/− mice, including skin inflammation, barrier function, and innervation, which are pathologic features of AD. Hsd11b1KC/−/− mice did not present with significant skin lesions, such as hyperkeratosis, acanthosis, or immune cell infiltration (Figure 1a). However, we frequently observed epidermal nerve sprouting and nerve fibers just under the epidermis in the footpad, tail, and dorsal skin from Hsd11b1KC/−/− mice (Figure 1b). We calculated the percentage of protein gene product 9.5-positive area in the epidermis of the footpad, tail, and dorsal skin. Compared with wild-type (WT) mice, the fold change in protein gene product 9.5-positive area was significantly increased in the footpad (P < 0.05), tail (P < 0.05), and dorsal skin (P < 0.01) versus Hsd11b1KC/−/− mice (Figure 1c).

To investigate the cause of the alloknesis in Hsd11b1KC/−/− mice, we explored epidermal factors known to cause alloknesis. First, we assessed the gene expression of neurotrophins, including NGF, BDNF, NT-3, NT-4/5, and members of the glia cell-derived neurotrophic factor family, such as GDNF, ARTN, NRTN, and PSPN, in the epidermis of Hsd11b1KC/−/− mice. Quantitative real-time PCR (RT-qPCR) analysis showed that gene expression of ARTN was significantly up-regulated in the epidermis of Hsd11b1KC/−/− mice relative to that of WT mice (P < 0.01) (Figure 3a). We also assessed the expression levels of keratinocyte-derived cytokines, which are known to be associated with allergic inflammation (e.g., TSLP, IL-33, and IL-25) (Divekar and Kita, 2015). TSLP expression was significantly increased in the epidermis of Hsd11b1KC/−/− mice compared with that of WT mice (P < 0.01) (Figure 3a).

The increased epidermal expression of the ARTN protein in Hsd11b1KC/−/− mice was confirmed using immunohistochemistry (Figure 3b and c). TSLP protein level was significantly increased in the supernatant of epidermal sheet homogenate from Hsd11b1KC/−/− mice compared with that from WT mice (P < 0.05) (see Supplementary Figure S2b).

Pruritogen-evoked alloknesis in Hsd11b1KC/−/− mice

Next, we investigated the degree of the alloknesis induced 30 minutes after administration of histaminergic or non-histaminergic pruritogens in WT and Hsd11b1KC/−/− mice. First, we counted the number of scratch bouts during the 30 minutes immediately following the administration of histamine (histaminergic) or chloroquine (non-histaminergic) to compare the baseline susceptibility to the pruritogen. Hsd11b1KC/−/− mice showed significantly higher susceptibility to chloroquine (P < 0.01) but a comparable response to histamine compared with WT mice (see Supplementary Figure 1b and c). Next, we evaluated the degree of alloknesis induced by histamine or chloroquine using a von Frey filament with 0.008 g of force, which was below the mechanical stimulus threshold for both WT and Hsd11b1KC/−/− mice. Stimuli were applied five times every 5 minutes. The mean alloknes score in Hsd11b1KC/−/− mice was higher than that in WT mice at all time points from 35 to 60 minutes after injection of histamine or chloroquine (see Supplementary Figure S1d and e), and the total alloknes score elicited by these pruritogens was significantly increased in Hsd11b1KC/−/− mice compared with WT mice (P < 0.01) (Figure 2e and f).

Artemin and TSLP productions are up-regulated in the epidermis of Hsd11b1KC/−/− mice

To investigate the cause of the alloknesis in Hsd11b1KC/−/− mice, we explored epidermal factors known to cause alloknesis. First, we assessed the gene expression of neurotrophins, including NGF, BDNF, NT-3, NT-4/5, and members of the glia cell-derived neurotrophic factor family, such as GDNF, ARTN, NRTN, and PSPN, in the epidermis of Hsd11b1KC/−/− mice. Quantitative real-time PCR (RT-qPCR) analysis showed that gene expression of ARTN was significantly up-regulated in the epidermis of Hsd11b1KC/−/− mice relative to that of WT mice (P < 0.01) (Figure 3a). We also assessed the expression levels of keratinocyte-derived cytokines, which are known to be associated with allergic inflammation (e.g., TSLP, IL-33, and IL-25) (Divekar and Kita, 2015). TSLP expression was significantly increased in the epidermis of Hsd11b1KC/−/− mice compared with that of WT mice (P < 0.01) (see Supplementary Figure S2a online). The increased epidermal expression of the ARTN protein in Hsd11b1KC/−/− mice was confirmed using immunohistochemistry (Figure 3b and c). TSLP protein level was significantly increased in the supernatant of epidermal sheet homogenate from Hsd11b1KC/−/− mice compared with that from WT mice (P < 0.05) (see Supplementary Figure S2b).

Although TSLP is known as a pruritogen, Hsd11b1KC/−/− mice do not develop spontaneous scratch behavior. The expression levels of related receptors on Hsd11b1KC/−/− dorsal root ganglion (DRG) (e.g., TSLP receptor, GFRα2 [a potent receptor for ARTN], transient receptor potential channels, CGRP, and VGLUT2) were not significantly different from those of WT dorsal root ganglion (Figure 3d). Furthermore, the expression levels of genes associated with Merkel cells (e.g., Krt8, Atoh1, Piezo1, and Piezo2) in the epidermis of
Hsd11b1KC/e mice were not significantly different from those of WT mice (Maksimovic et al., 2014; Maricich et al., 2009; Ranade et al., 2014; Woo et al., 2014). Therefore, we investigated whether increased epidermal ARTN might be involved in the alloknesis mechanism in Hsd11b1KC/e mice.

ARTN production is down-regulated by endogenous GC activation in keratinocytes
To examine if ARTN expression was regulated by endogenous GCs in keratinocytes, we evaluated the effect of corticosterone on ARTN gene and protein expression in keratinocytes in vitro. When primary Hsd11b1KC/e keratinocytes were co-cultured with corticosterone at physiological concentrations, ARTN expression up-regulation was decreased in a dose-dependent manner to a level comparable to that of primary WT keratinocytes (Figure 4a–c). The same results were seen with TSLP expression (see Supplementary Figure S3a and b online).

To determine if endogenous GC activation affects ARTN expression in human keratinocytes, we evaluated expression levels of ARTN and TSLP in normal human epidermal keratinocytes (NEHks) with HSD11b1 knocked down using small interfering RNA. ARTN and TSLP expression were up-regulated in HSD11b1-knockdown NEHks compared with NEHks transfected with scrambled small interfering RNA (control) (Figure 4d). In HSD11b1-knockdown NEHks co-cultured with cortisol at physiological concentrations, ARTN and TSLP expression up-regulations were also decreased in a dose-dependent manner to levels comparable to those of control

Figure 1. Cutaneous nerve fiber outgrowth toward the epidermis in Hsd11b1KC/e mice. (a) Hematoxylin and eosin-stained dorsal skin from WT and Hsd11b1KC/e mice. Scale bar = 20 μm. (b) Frozen sections of footpad, tail, and dorsal skin from WT and Hsd11b1KC/e mice stained with anti-PGP9.5. Scale bar = 50 μm. (c) Quantification of the percentage of PGP9.5-positive area in the footpad (n = 6–7), tail (n = 6), and dorsal epidermis (n = 4). Data are represented as the relative fold change normalized to WT mice and expressed as mean ± standard deviation. Each symbol represents an individual mouse. Statistically significant differences between groups are indicated by *P < 0.05 and **P < 0.01 (unpaired t test). (d) Three-dimensional images of cutaneous nerve fiber in WT and Hsd11b1KC/e mice. Scale bar = 50 μm. PGP9.5, protein gene product 9.5; WT, wild type.
alloknesis, we evaluated the effect of ARTN-neutralizing antibody (αARTN) on touch- and pruritogen-evoked alloknesis in Hsd11b1KC−/− mice. Figure 5a shows the experiment schedule. The total number of scratch responses to light touch was higher in Hsd11b1KC−/− mice treated with isotype-matched control IgG compared with WT mice treated with saline for 2 weeks after administration (Figure 5b). By contrast, the total number of scratch responses to light touch in αARTN-treated Hsd11b1KC−/− mice gradually decreased, and the differences with control IgG-treated Hsd11b1KC−/− mice were statistically significant starting at day 8 after administration and continuing thereafter (P < 0.05 and P < 0.01) (Figure 5b). ARTN neutralization also significantly attenuated the histamine- and chloroquine-evoked alloknesis score (P < 0.05) (Figure 5c and d). ARTN neutralization showed no significant effect on the number of histamine- and chloroquine-evoked scratch bouts in the 30 minutes immediately after administration or on epidermal nerve innervation (see Supplementary Figure S4a–c online). To confirm the effect of ARTN neutralization on skin, we assessed the gene expression of GFRα1–4, ARTN, and TSLP at the injection site of Hsd11b1KC−/− mice at 1 day after administration. GFRα1, GFRα3, and ARTN expression were significantly decreased at the injection site of αARTN-treated Hsd11b1KC−/− mice compared with control IgG-treated Hsd11b1KC−/− mice (P < 0.05) (see Supplementary Figure S4d).

Homeostatic cortisol activation negatively regulates ARTN production in human epidermis

To investigate the involvement of endogenous GC activation in the pathological consequences of itchy skin, we performed immunohistochemistry for epidermal HSD11B1, GR, and ARTN expression in lesional skin from AD patients. HSD11B1 staining intensity and GR-positive area were significantly decreased in the epidermis of AD patients compared with that of healthy control individuals (HCs) (P < 0.01) (Figure 6a). Similar to previous studies (Hidaka et al., 2017; Murota et al., 2012), ARTN stained intensely in the epidermis and papillary layer, and the ARTN-positive area on the epidermis of AD patients was significantly higher than that of HCs (P < 0.01) (Figure 6a and b). We next examined the Pearson correlation between HSD11B1, GR, and ARTN expression levels. The epidermis of HCs had high HSD11B1 and GR expression levels, whereas AD patients had low HSD11B1 and GR levels. We observed a significant positive correlation between the expressions of HSD11B1 and GR (r = 0.4759, P < 0.01) (Figure 6f). The epidermis of HCs had high HSD11B1 and low ARTN expression levels, whereas that of AD patients had low HSD11B1 and high ARTN levels. We observed a significant negative correlation between expression of HSD11B1 and ARTN (r = −0.4220, P < 0.05) (Figure 6g).

**DISCUSSION**

This study showed that decreased expression of HSD11B1 in keratinocytes increased susceptibility to pruritogen- or touch-evoked itch by up-regulating ARTN expression. These immunopathological findings in AD lesional skin potentially could explain the alloknesis observed in Hsd11b1KC−/− mice. Hsd11b1KC−/− skin had abnormal skin innervation characterized by an increased number of intraepidermal nerve
fibers and thickened dermal nerve fibers, which led to Hsd11b1KC−/− mice exhibiting various sensory disorders. Hsd11b1KC−/− mice exhibited thermal hyperalgesia, which could be caused by hypersensitization of TRPV1/VGLUTs-positive or CGRP-positive sensory nerves (McCoy et al., 2013; Murota and Katayama, 2016; Rogoz et al., 2014). Therefore, we assessed TRPV1 and CGRP mRNA levels in dorsal root ganglion using RT-qPCR. We found that the expressions of those genes in Hsd11b1KC−/− mice were comparable to those in WT mice, indicating that the abnormal sensitivity to thermal nociception in Hsd11b1KC−/− mice was not caused by expression changes of those thermal nociceptors/pruriceptors on sensory nerves. Exploratory gene expression analysis in epidermis of Hsd11b1KC−/− mice showed up-regulation of ARTN, which is known to cause hypersensitivity to warmth (Murota et al., 2012; Murota and Katayama, 2016). Thus, ARTN could be involved in the thermal hyperalgesia mechanism in Hsd11b1KC−/− mice. By
In contrast, Hsd11b1<sup>KC−/−</sup> mice exhibited reduced nociceptive mechanosensation compared with WT mice. At present, this abnormality has not been explained to our knowledge, and further studies are needed to clarify the role of epidermis-derived GCs on deep somatic sensation.

Unexpectedly, Hsd11b1<sup>KC−/−</sup> mice exhibited scratch behavior after touch stimulation with a von Frey filament at higher forces than WT mice. To explain this observation, we focused our investigation on Merkel cells. However, quantitative PCR analysis of Krt8 and Atoh1 in the epidermis of Hsd11b1<sup>KC−/−</sup> mice showed comparable expression to that in WT mice. We showed that neutralizing ARTN appeared to attenuate touch-evoked alloknesis and down-regulate the expression of ARTN and its receptors in Hsd11b1<sup>KC−/−</sup> mice without affecting epidermal innervation (see Supplementary Figure 5Ac). These findings indicate that nerve C-fiber could be directly sensitized by ARTN, and this sensitization could modify the neurotransmission associated with touch sensitivity. Our in vitro study showed that expression of a nerve elongation factor was up-regulated in keratinocytes of Hsd11b1<sup>KC−/−</sup> mice (Figure 4a). It is possible that some factors other than ARTN might contribute to abnormal skin innervation or that the period of administration could be too short to improve abnormal innervation in this model. TSLP, a prurito-genic chemokine, was also up-regulated in Hsd11b1<sup>KC−/−</sup> epidermis. Although increased or exogenously administered TSLP is known to induce scratch behavior (Wilson et al., 2013), Hsd11b1<sup>KC−/−</sup> mice did not exhibit abnormal scratch behaviors unprovoked. Because TSLP protein levels in Hsd11b1<sup>KC−/−</sup> epidermis were much lower than those injected in the previous study (Wilson et al., 2013), we hypothesized that epidermal TSLP levels in Hsd11b1<sup>KC−/−</sup> mice were insufficient to induce scratching and inflammation.

In our study, ARTN and TSLP expression were up-regulated in the epidermis of epidermis-specific GR-knockout mice (Sevilla et al., 2013), and TSLP levels were transcriptionally repressed by ligand-activated GR in keratinocytes (Surjit et al., 2011). These findings are consistent with our data indicating that homeostatic activation of Hsd11b1 helps regulate TSLP production in keratinocytes (Stante et al., 2005). A previous report suggested that the environmental stimulus-induced...
shown to exacerbate pruritus by inhibiting prostaglandin D2 production by mast cells in a murine dermatitis model (Yamaura et al., 2012). Based on these data, future itch therapies could target the local stress regulatory system in keratinocytes specifically or inhibit the ARTN signal pathway. Furthermore, agents that regulate HSD11B1 expression, such as topical exogenous cholesterol, could be used to treat sensitive skin (Murota et al., 2014).

AD patients suffer from itch that is exacerbated by commonplace factors, such as temperature and scratchy clothes (Murota and Katayama, 2017; Wahlgren, 1991). In human skin, either with or without AD, there was negative correlation between the staining intensity of HSD11B1 and ARTN. The mechanism of skin sensitivity in AD could be explained partly by decreased HSD11B1 expression and subsequent ARTN overexpression. However, the role of skin inflammation on HSD11B1-mediated alloknesis has not been clarified. In this study, we evaluated cutaneous sense in the absence of dermatitis and allergic inflammation in Hsd11b1Kc−/− mice. In Hsd11b1Kc−/− mice, we showed that endogenous corticosteroids secreted from epidermis to maintain skin homeostasis affect both skin innervation and cutaneous sensation. This knowledge could contribute to the development of a therapeutic intervention for sensitive/itchy skin. The observations in this study could contribute considerably to resolving outstanding issues in treating pruritus.

MATERIALS AND METHODS

Mice

Keratinocyte-specific Hsd11b1Kc−/− mice were generated as described previously (Terao et al., 2016). Briefly, Krt5-Cre+/− transgenic and Hsd11b1flox/flox mice were maintained on a C57BL6/J background and interbred to generate mice that carried Krt5-Cre transgene and a floxed Hsd11b1 allele (Krt5-Cre+/− Hsd11b1flox/flox). These mice were then inbred. Offspring that carried Krt5-Cre+/− Hsd11b1flox/flox were used for further analysis as conditional knockouts (HSD11b1Kc−/−). Krt5-Cre+/− Hsd11b1flox/flox litters and C57BL6/J mice purchased from JapanCLEA (Osaka, Japan) were used as WT in two experiments, respectively, and reproducibility of data was confirmed. Approximately 7- to 12-week-old mice were used to conduct this study. Animals received care in strict accordance with the Guiding Principles for the Care and Use of Laboratory Animals. All animal experiments were performed with the approval of the Animal Experiments Committee of Osaka University (Osaka, Japan).

Cell culture

Mouse primary keratinocytes were isolated from the tail and ear epidermal layer as described previously (Terao et al., 2011). NHEKs were purchased from DS Pharma Biomedical (Osaka, Japan). Mouse primary keratinocytes and NHEKs were cultured on type 1 collagen-coated plates (Asahi Techno Glass, Funabashi, Japan) in EpiLife medium (Life Technologies, Carlsbad, CA). Detailed information can be found in the Supplementary Materials and Methods online.

Human specimens

Human skin biopsy samples were obtained from AD patients and HCs. All participants provided written informed consent. All studies were approved by the ethical committee of Osaka University.
Detailed patient demographic characteristics (age, sex, and biopsy site) are described Supplementary Table S1 online.

**Pruritogen scratch behavior and alloknesis test**
Pruritogen scratch behavior and alloknesis were assessed using a previously reported method with minor modifications (Akiyama et al., 2012). Detailed information can be found in the Supplementary Materials and Methods.

**Touch alloknesis test**
Touch-evoked scratching behavior was evaluated using a previously reported protocol with minor modifications (Bourane et al., 2015). Detailed information can be found in the Supplementary Materials and Methods.

**Nociceptive behavior test**
Mechanical nociception was evaluated using the dynamic Planter Test (37450-Planter test instrument, Ugo Basile, Comerio VA, Italy). Thermal nociception was evaluated using the hot plate test (MK-350HC, Muromachi Kikai, Tokyo, Japan) and tail flick test (37360-Tail flick unit, Ugo Basile, Comerio, VA, Italy). Nociceptive behavior tests were performed according to manufacturers’ protocols. Detailed information can be found in the Supplementary Materials and Methods.

**GC agonist and antagonist treatment and small interfering RNA transfection**
Detailed information can be found in the Supplementary Materials and Methods.
Histopathological, RT-qPCR, and Western blot analyses
Detailed information can be found in the Supplementary Materials and Methods. Primer lists are described Supplementary Table S2 and S3 online.

Three-dimensional imaging of cutaneous nerve fibers
Whole-tissue immunostaining and clearing were performed according to the manufacturer's instructions for the RapiClear 1.52 (Sunjin Lab, Hsinchu, Taiwan). Z-stack images were collected using confocal microscopy (FV3000, Olympus, Tokyo, Japan). Detailed information can be found in the Supplementary Materials and Methods.

Administration of ARTN-neutralizing antibody
Mice were injected subcutaneously with 10 mg/kg ARTN-neutralizing antibody (MAB10851; R&D Systems, Minneapolis, MN) or rat IgG2A isotype control (MAB006, R&D Systems) once a week. Previous studies showed that 10 mg/kg of ARTN-neutralizing antibody was able to block ARTN-GFPRz signaling (DeBerry et al., 2015; Hidaka et al., 2017; Thornton et al., 2013). Detailed information can be found in the Supplementary Materials and Methods.

Statistical analysis
Statistical analyses were performed using GraphPad Prism (La Jolla, CA). Data were analyzed using a one-way analysis of variance with Dunnett multiple comparisons test or two-tailed unpaired t test. Error bars are mean ± standard deviation. P values less than 0.05 were considered statistically significant.

CONFLICT OF INTEREST
The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL
Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2018.02.010.

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Divekar R, Kita H. Recent advances in epithelium-derived cytokines (IL-33, IL-25, and thymic stromal lymphopoietin) and allergic inflammation. Curr Opin Allergy Clin Immunol 2015;15:98–103.

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