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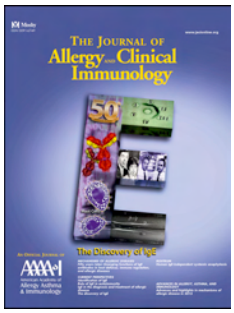

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New mechanism underlying IL-31-induced atopic dermatitis

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ABSTRACT

BACKGROUND: T helper type 2 cell-released interleukin 31 (IL-31) is a critical mediator in atopic dermatitis (AD), a prevalent and debilitating chronic skin disorder. Brain-derived natriuretic peptide (BNP) has been described as a central itch mediator. The importance of BNP in peripheral (skin-derived) itch and its functional link to IL-31 within the neuro-immune axis of the skin is unknown.

OBJECTIVE: To investigate the function of BNP in the peripheral sensory system and skin in IL-31-induced itch and neuro-epidermal communication in AD.

METHODS: Ca^{2+}-imaging, immunohistochemistry, quantitative real-time PCR, RNA-Seq, knockdown, cytokine/phosphor-kinase arrays, enzyme immune assay and pharmacological inhibition were subjected to examine the cellular basis of the IL-31-stimulated, BNP-related itch signaling in human DRG neurons (hDRG) and skin cells, transgenic AD-like mouse models, and human skin of AD and healthy subjects.

RESULTS: In hDRG, we confirmed expression and co-occurrence of OSMRβ and IL-31 receptor A in a small subset of neuronal population. Furthermore, IL-31 activated ~50% of endothelin-1-responsive neurons, and half of the latter also responded to histamine. In murine DRGs IL-31 upregulated *Nppb* and induced SNARE-dependent BNP release. In the Grhl3PAR2$^{/+}$mice, house dust mite-induced severe AD-like dermatitis was associated with *Nppb* upregulation. Lesional IL-31Tg mice also exhibited increased *Nppb* transcripts in DRGs and skin; accordingly, skin BNP receptor was elevated. Importantly, expression of BNP and its receptor were increased in AD patient skin. In human skin cells, BNP stimulated a pro-inflammatory, itch-promoting phenotype.

CONCLUSION: Our findings show, for the first time, that BNP is implicated in AD and that IL-31 regulates BNP in both DRGs and skin. IL-31 enhances BNP release and synthesis, and orchestrates cytokine and chemokine release from skin cells, thereby coordinating the signaling pathways involved in itch. Inhibiting peripheral BNP function may be a novel therapeutic strategy for AD and pruritic conditions.

KEY MESSAGES

- IL-31 induces SNARE-dependent BNP release and its synthesis in sensory nerves.
- BNP increases AD-related cytokine release from keratinocytes and dendritic cells through GSK3-dependent and C-Jun activation pathways, respectively.
- AD is associated with upregulation of BNP and its receptor.

CAPSULE SUMMARY

Our study provides a novel functional link between IL-31 and BNP within the neuro-immune axis of AD. Blockade of BNP release by inactivating SNAREs offers new strategy for management of AD and pruritic conditions.

KEY WORDS

Atopic dermatitis, pruritogens; brain-derived natriuretic peptide; pruritus; skin; dorsal root ganglion; keratinocytes; dendritic cells; SNAREs

ABBREVIATIONS USED

AD	atopic dermatitis
BNP	brain-derived natriuretic peptide
Calca	calcitonin gene-related peptide (CGRP) gene
CCL20	C-C Motif Chemokine Ligand 20
CXCL	chemokine (C-X-C motif) ligand
DAPI	4',6-diamidino-2-phenylindole
DRGs	dorsal root ganglionic neurons
ET-1	endothelin-1
FPKM	fragments per kilobase of transcript per million mapped reads
GSK3	glycogen synthase kinase 3
hDCs	human dendritic cells
hKCs	human primary keratinocytes
HDM	house dust mite
IL-31	interleukin-31
IL-31RA	interleukin-31 receptor alpha
IL-31Tg mice	IL-31-transgenic mice
MMP9	plasma matrix metalloproteinase-9
NeuN antibody	NEUronal Nuclei antibody
Nppb	BNP gene
NPR1	natriuretic peptide receptor A
NPR2	natriuretic peptide receptor B
OSMRβ	oncostatin M receptor beta subunit
PGP9.5	protein gene product 9.5
RT-PCR	real-time PCR
SNAP-25	synaptosomal associated protein 25k
SNARE	soluble N-ethylmaleimide-sensitive factor activating protein receptor
STX1	syntaxin 1
Tac-1	substance p (SP) gene
TG	trigeminal ganglia
Th2	T helper type 2
TSLP	thymic stromal lymphopoietin
VAMP	vesicle-associated membrane protein

133	V1	vesicle-associated membrane protein isoform 1
134	V7	vesicle-associated membrane protein isoform 7
135	WT	wild-type

INTRODUCTION

Atopic dermatitis (AD) is one of the most prevalent chronic inflammatory skin diseases world-wide. It is characterized by dysregulation of immunity, skin barrier as well as nerve function, resulting clinically in eczema and itch (1-4). The mediators of histamine-independent itch in AD are poorly understood (5). Candidates include endothelin-1 (ET-1), thymic stromal lymphopoietin (TSLP) and the cytokines interleukin (IL)-4, IL-13 and IL-31 (1). IL-31 is a critical cytokine in the pathophysiology of AD (1, 6, 7) playing a role in eczema (7), itch (8), and nerve growth (9). Elevated serum levels of IL-31 were found to correlate with disease severity in patients (6, 10). IL-31 serves as a critical neuron-immune link between T helper type 2 (Th2) cells and sensory nerves in the generation of T cell–mediated itch (8). Cutaneous and intrathecal injections of IL-31 evoked robust itch behavior in mice (8). IL-31 injection in mouse cheek induces itch, but not pain (8). IL-31-transgenic (Tg) mice that overexpress IL-31 develop severe pruritus and skin lesions similar to AD (7). In mice, IL-31 receptor A (IL-31RA) associates with oncostatin M receptor β (OSMRβ) to form the IL-31RA heterodimeric complex that binds to IL-31 (8). Targeting IL-31 pathway therapeutically has been proved to be effective in AD (11, 12). For examples, IL-31 neutralization in AD-models was effective in the treatment of IL-31-induced itch and dermatitis (13-17). Anti-IL-31RA antibody nemolizumab was evaluated in phase II with satisfied efficacy and safety for the treatment of patients with moderate-to severe AD disease (11). Despite this, it is not known if IL-31 stimulates neuropeptide release from central and/or peripheral primary afferent neurons to modulate itch transmission in skin and/or spinal cord. The importance of IL-31 in the regulation and release of neuropeptides from peripheral sensory nerves in the skin and the resulting neurogenic inflammation in AD, also remains elusive.

B-type natriuretic peptide (BNP) is a 32-amino-acid cyclic peptide expressed in a subset of primary afferent neurons (18-20). It binds to the natriuretic peptide receptor A (NPR1) and to a lesser extent NPR2 (21). BNP is the *Nppb* gene product and has been identified as an important neuropeptide for itch transmission from the sensory to the spinal cord (19). Intrathecal injection of BNP in mice induced a robust itch phenotype whereas *Nppb* $^{-/-}$ mice exhibited lack of scratching responses to many puritogens (19). In contrast to substance P (SP) and calcitonin gene-related peptide (CGRP) which facilitate pain processing and painful neurogenic vasodilation in mice, BNP has been characterized as a negative regulator of nociceptive transmission (20, 22). *Nppb* has been detected in the murine

primary puritogenic sensory dorsal root ganglionic neurons (DRGs), which overlapped with IL-31RA^{+} neurons (23). Despite of its importance in itch, it is unknown whether BNP can be released from peripheral sensory neurons in response to puritogens (i.e. IL-31 or histamine). The cellular and molecular basis for its pruritic action on skin and immune cells also remains unknown. Understanding the downstream signaling pathways of IL-31 and its potential interacting mediators like BNP, CGRP or SP is significant because it will not only expand our current knowledge and theoretical repertoire but may also aid the development of a more efficacious treatment for chronic skin inflammation and pruritus. Herein, we investigated the possible mechanistic relationship between IL-31 and BNP in itch. The aim of the current study was to: (i) examine the expression and function of IL-31 receptor in human DRGs (hDRGs); (ii) investigate the role of BNP in AD using mice models and human subjects; (iii) investigate IL-31-induced release of BNP and other neuropeptides in relation to itch; (iv) dissect the molecular components of the exocytotic machinery in IL-31-induced BNP release; (v) study the alteration of BNP receptor in AD skin; (vi) screen distinct intracellular kinases activated and cytokines induced by BNP in human skin cells. Overall, our results reveal a novel IL-31-mediated downstream target in itch induction and highlight the importance of BNP as a potential target for the treatment of AD and other itch disorders.

MATERIALS and METHODS

Materials

Human skin samples from 3 donors of AD and 3 donors of healthy control subjects were bought from Tissue Solutions Ltd., Glasgow, Scotland. Human DRG paraffin sections were purchased from Amsbio (Local distributor of Zyagen Laboratories). Antibodies, cells and reagents for tissue culture and others are detailed in the Supplementary Material and Methods.

Human and animal rights

Human DRGs were isolated as previously described (24) with full legal consent by Anabios Corporation (San Diego, CA, USA). DRGs were isolated and cultured from 1 donor (25 year old Hispanic female). Housing/handling of mice (C57BL6), IL-31Tg mice and experimental procedures had been approved by the University College Dublin Ethics Committee and the Irish Authorities. Housing/handling of Grhl3PAR2$^{/+}$ transgenic mice

model and experimental procedures following federal guidelines had been approved by the University of California, San Francisco Ethics Committee and by local authorities.

HDM application on Grhl3PAR2$^{/+}$ mouse model

The Grhl3PAR2$^{/+}$ mice were maintained in C57BL6/J-129X1/SvJ mixed strain and used for the experiments as an AD model. For details of HDM treatment, see Supplementary Material and Methods.

RNA-Seq

For gene expression experiment, trigeminal ganglia (TG) were harvested from wild-type (WT) and HDM-treated Grhl3PAR2$^{/+}$ mice and processed for RNA-Seq. For details, see Supplementary Material and Methods.

Quantitative real time PCR (RT-PCR)

RNA was isolated from cultured mouse DRG neurons (mDRGs) using the RNeasy kit (Qiagen, Hilden, Germany). TRIzol Reagent was used to isolate RNA from DRGs and skins of WT and IL-31Tg mice. The quantitation of mRNA levels was performed by real time fluorescence detection using SYBR Green ROX mix (ABI). For details, see Supplementary Material and Methods.

BNP, CGRP and SP release assay

For detailed preparation of mDRGs culture and their release of neuropeptides, see Supplementary Material and Methods.

Measurement of intracellular Ca^{2+} concentration

Human DRGs (hDRGs) and mDRGs in culture were loaded with calcium indicator before measurement of Ca^{2+} mobilization in response to various pruritogens. For details, see Supplementary Material and Methods.

Immunofluorescence staining

Cultured mDRGs, paraffin sections of human DRG and skin (15µm) were stained and imaged by a Zeiss LSM710 confocal microscope (Carl Zeiss MicroImaging). For details, see Supplementary Material and Methods.

Lentivirus-mediated knockdown of soluble N-ethylmaleimide-sensitive factor activating protein receptors (SNAREs)

Cultured mDRGs in 7d *in vitro* were treated with non-targeted control lentiviral particles or shRNA lentivirus specifically targeting to SNAREs for 7-10 days before measuring BNP release and protein expression. For details, see Supplementary Material and Methods.

Culture of human primary epidermal keratinocytes (hKCs) and human monocyte-derived dendritic cells (hDCs), cytokine antibody array and phosphor-kinase array

hKCs were cultured in the KBM-Gold™ medium with KBM-Gold SingleQuot KCs supplement (Lonza). hDCs were cultured by *in vitro* differentiation of $CD14^+$ monocytes using LGM-3™ Lymphocyte Growth Medium plus 50 ng/ml GM-CSF and 50 ng/ml IL-4 (Lonza). Cells were maintained in the above medium for 3 d before use. For detailed cytokine antibody and phosphor-kinase arrays, see Supplementary Material and Methods.

Statistical Data Analysis

Data expressed as mean ± S.E.M. n ≥ 3 independent experiments, probability values are determined with the use of Student's 2-tailed t test; P values <0.05 are considered significant; non-significant N.S. P>0.05; *P < 0.05; **P < 0.01; *** P<0.001. Data analysis was performed with Prism software.

RESULTS

IL-31RA and OSMRβ are co-expressed in a small subset of human DRG neurons

In human AD, IL-31 levels correlate with disease severity (6, 10). However, subpopulations responding to IL-31 and distribution of its receptors (IL-31RA and OSMRβ) in hDRGs have not been fully characterized. Our study herein will help to define the functional contribution of neuronal subpopulations to the skin inflammation of AD in humans. Using double-immunohistochemistry, we observed IL-31RA and OSMRβ expression in hDRG sections (Fig 1A-D). IL-31RA was detected in ~3% of total neurons visualized with a neuronal marker NEUronal Nuclei (NeuN) antibody, which stained strongly neuronal nuclei and distal cytoplasm (Fig 1A). IL-31RA also occurred in a small proportion of cells immunoreactive to protein gene product 9.5 antibody (PGP9.5) (Fig 1B), another marker for sensory nerves. Similarly, OSMRβ was found in a small subset of

PGP9.5$^+$ neurons (Fig 1C). Co-staining of IL-31RA and OSMRβ confirmed that in hDRG all of IL-31RA$^+$ neurons were OSMRβ$^+$ (Fig 1D, E). However, ~5% OSMRβ$^+$ neurons did not appear to be strongly labeled by anti-IL-31RA (Fig 1D arrowed neuron).

IL-31 induces intracellular calcium mobilization in a distinct subset of hDRGs, which also responds to ET-1

Functional identities of neuronal subsets were further assayed in hDRG using intracellular Ca^{2+} imaging upon application of individual or combined puritogens to cultured hDRGs. We found that ~4% hDRG neurons were directly activated by ET-1 (n=100 neurons) (Fig 2A, B top panels). The response consisted of a transient increase in intracellular calcium. In comparison, glia cells consistently exhibited large responses with slow kinetic. In several cases after ET-1 application, glial cells generated oscillatory calcium signals that persisted even after the washout of ET-1 (Fig 2A, B top panels). In our observation, about ~50% of neurons pre-exposed to ET-1 also responded to histamine (Fig 2A middle panel and C). Following application of ET-1 and histamine some neurons exhibited spontaneous calcium transients. Co-application of ET-1 and histamine did not potentiate the response. On the contrary, de-sensitization was apparent in some neurons (Fig 2A bottom panel). For the IL-31-induced response, 1 of 50 neurons pre-exposed to ET-1 responded with a rapid calcium transient to IL-31 (Fig 2B top and middle panels). Likewise, co-application of ET-1 and IL-31 did not potentiate the response, except in one neuron with a delayed response (Fig 2B bottom panel). In summary, ~50% neurons responded to histamine, and ~4% of total neurons responded to ET-1. Half of the ET-1-responsive neurons also responded to histamine (Fig 2C). Overall, in hDRGs 2% of neurons were responding to IL-31, and these cells also responded to ET-1 (Fig 2C). Due to the importance of IL-31 and ET-1 in pathogenesis of AD, it is tempting to postulate that this neuronal subset mediates itch transmission in humans.

We further investigated whether murine DRGs (mDRGs) exhibit similar phenomenon in response to IL-31 and ET-1. Cultured mDRGs uploaded with Fluo-4AM were treated with IL-31 and ET-1, sequentially. In a total of 335 neurons recorded, IL-31 activated 9 neurons (~2.7%) and ET-1 activated 14 neurons (~4.2 %). 5 IL-31-responsive neurons did not respond to ET-1 (Fig 2D.). Thus, similarities as well as differences were observed between human and murine DRG with respect to IL-31 and ET-1 stimulation.

PAR2 transgenic mice treated with house dust mite (HDM) and IL-31Tg mice are associated with *Nppb* upregulation in sensory nerves

We investigated the possible involvement of BNP in AD using Grhl3PAR2$^{/+}$ mice (25). These mice develop atopic-like inflammation, scaly dry skin, epidermal hyperplasia, and itch behaviors, all characteristics of human AD (25). We applied HDM on the right cheek to induce severe dermatitis, as HDM have abundant proteases that can activate PAR2 constantly and stably. Ipsilateral (ipsi) and contralateral (contra) trigeminal ganglia (TG) from each mouse were analyzed by RNA-Seq (Fig 3A). Clinical scores for pathologic diagnosis (skin lesion severity) were evaluated by hematoxylin and eosin (HE) staining on cheek skin biopsy from each of the 8 HDM-applied mice and vehicle-treated WT mice (representative images shown in Fig 3B). Notably, in HDM-treated mice, the correlation coefficient between clinical score and fold-change of fragments per kilobase of transcript per million mapped reads (FPKM) for *Nppb* transcripts (ipsi/contra) is about 0.8 (Fig 3A). Average of fold change of *Nppb* for these 8 mice is ~2.3. In contrast, HDM did not induce significant change in TGs for NPR1, NPR2, IL-31, IL-31RA, and OSMR in Grhl3PAR2$^{/+}$ mice (Fig 3A). After comparing FPKM from TGs of ipsilateral (right cheek) *vs* contralateral (left cheek) in 3 high clinical score Grhl3PAR2$^{/+}$ mice (score was 8, 7, 7), we found that *Nppb* transcripts were upregulated by 3.5 times by application of HDM and ranked the third position among the highly upregulated genes (Fig 3C). Notably, the *Nppb* FPKM values in the contralateral (left) cheek of Grhl3PAR2$^{/+}$ mice were not significantly different from that of WT mice with or without vehicle (Vaseline) treatment (Supplementary Fig E1).

As IL-31Tg mice show skin phenotype closely resembling those of human AD (7), we used RT-PCR to compare *Nppb* transcripts in whole DRG tissue isolated from the IL-31Tg (lesional and non-lesional) and from age matched WT non-transgenic mice. Higher levels of *Nppb* mRNA were detected in the DRGs isolated from both lesional and non-lesional IL-31Tg mice (Fig 3D).

Collectively, our data indicate that BNP is implicated in AD and may be associated with severity of AD-like skin conditions in mice.

IL-31 augments release and upregulates synthesis of BNP but not CGRP or SP from cultured mDRGs

IL-31 directly activates peripheral sensory neurons to induce pruritus (8); however, its effect on BNP has not yet been reported. To explore this possibility, we cultured mDRGs

and incubated neurons with 300 nM IL-31 for 30 min before starting the release assay. The IL-31-inducd release was compared with the response to histamine, high (HK, 60 mM) and low (LK, 3.5 mM) potassium chloride buffers (Fig 4A). Notably, IL-31 elicited ~2.7 fold increase of BNP release over the basal level (LK). Histamine (His) and potassium depolarization (HK) also elicited BNP release (Fig 4A). Moreover, IL-31-induced BNP release was concentration- (Fig 4B) and time- (Fig 4C) dependent. No significant difference of BNP release (Fig 4B) and mRNA synthesis over 30 min (Supplementary Fig E2 A) was detected between 300 nM and 1 µM concentrations of IL-31. RT-PCR results revealed that 300 nM IL-31 induced *Nppb* mRNA synthesis peaked at 4h and declined at 8h (Supplementary Fig E2 B). In contrast, neither CGRP nor SP release was affected by IL-31 or His, although both were elicited by HK (Fig 4D). This finding demonstrates for the first time that IL-31 directly activates sensory neurons to release BNP rather than CGRP or SP. Consistently, incubation of mDRGs with IL-31 for 6h did not induce significant changes of mRNA levels of *Calca* (CGRP gene) or *Tac-1* (SP gene) (Fig 4E). In contrast, IL-31 increased *Nppb* mRNA levels by ~2 fold. Altogether, these findings confirm that IL-31 directly induces BNP release and upregulates its synthesis, indicating BNP might contribute to IL-31-mediated itch signaling.

Subsequently, we performed immunofluorescence studies using antibodies against BNP or CGRP with their specificities verified (Supplementary Fig E3) to investigate their expression and distribution pattern in mDRGs. Notably, BNP was expressed in both $CGRP^+$- and $CGRP^-$ - neurons (Fig 4F). This distinct, but overlapping distribution, suggests that BNP and CGRP may have discrete functional roles in the transduction of itch signaling.

IL-31-induces BNP release from cultured mDRGs via a SNARE-mediated vesicle fusion

BNP release from sensory neurons in response to itch stimuli might be regulated by selective exocytotic machinery. In terms of IL-31-induced BNP release, its mechanism is of particular importance because of its relevance in AD. SNAREs are critical membrane fusion proteins and serve as therapeutic targets for many neurological diseases (26). To explore their role in BNP release, SNAP-25 (synaptosomal-associated protein 25k) and two other vesicular SNARE proteins, vesicle-associated membrane protein (VAMP) isoform 1 (V1) and 7 (V7), were selectively knocked down using lentiviral shRNA particles. Notably, expression levels of each protein were substantially reduced compared to the non-

targeted controls (Fig 5A). Knockdown of SNAP-25 and V1, but not V7, resulted in complete blockade of IL-31-elicited BNP release (Fig 5B). These findings, for the first time, demonstrated that selective SNARE proteins control IL-31-induced BNP release.

Expression of BNP and its receptors is increased in the skin of patients with atopic dermatitis

To understand BNP-induced signaling and identify the functional consequence of BNP in human skin level, we examined the expression of BNP and its receptor NPR1 and NPR2 in cultured human keratinocytes (hKCs). Using immunostaining, we readily detected expression of NPR1 and NPR2 in cultured hKCs (Supplementary Fig E4 A). Both receptors showed punctate distribution. In a great contrast, BNP immune-signal was below the detection limit in these cultured cells (Supplementary Fig E4 A).

Next, similar experiments were performed on human skin sections from AD subjects and healthy controls. We found that in the epidermis, no staining of BNP was discernible in normal skin, similar to the observation in cultured KCs, but it was readily detected in AD skin (Fig 6A), indicating a disease-related upregulation of BNP expression. We also detected immune-signals of NPR1 and NPR2 in the epidermal KCs of healthy skin (Fig 6A), and both signals were enhanced in AD skin (Fig 6A). We further analyzed the detailed distribution of these receptors by taking high resolution confocal images in epidermal KCs. Distinct localization of NPR1 and NPR2 were revealed in epidermal KCs of AD skin sections. NPR1 predominantly resided along the plasma membrane of KCs, whereas NPR2 was detected both on plasmalemma and cytoplasm (Fig 6A). The enhanced labelling of NPR1 and NPR2 might not be simply due to the acanthosis, because we did observe increased immunoreactivity at single KC level by immunofluorescence staining.

We further investigated BNP, NPR1 and NPR2 expression in the dermis of human skin. BNP expression was detected in the dermis of healthy skin, and its level appeared increased in dermal structures including secretory portion of sweat glands and blood vessel of AD skin (Fig 6B). Interestingly, NPR2 (Fig 6C, left panel) and NPR1 (not shown) did not seem to colocalize with PGP9.5 in these structures. NPR1 and NPR2 co-occurred on the dermal structures including secretory portion of sweat glands and blood vessel (Fig 6C middle and right panels). There was no obvious incremental labelling of NPR1 or NPR2 in the dermal structures (Fig 6C) and immune-reactive cells in AD skin compared to healthy skin (Supplementary Fig E5).

Consistently, using RT-PCR we also found *Nppb* transcripts were upregulated in lesional and non-lesional skin, compared with that in WT skin (Supplementary Fig E6 A). Moreover, BNP receptor NPR1 mRNA level was also increased in the lesional skin from IL-31Tg mice (Supplementary Fig E6 B).

Taken together, these collective data suggest that BNP might be involved in promoting the dermatitis response through activated/upregulated receptor in AD.

BNP induces release of IL-17A, chemokine (C-X-C motif) ligand 10 (CXCL10) and matrix metallopeptidase 9 (MMP9) from cultured human keratinocytes through activation of GSK3>JNK>ERK1/2≥P38

KCs of AD patients exhibit a propensity to produce exaggerated cytokines, chemokines and proteases, which play major role in promoting and maintaining inflammation (27, 28). However, nothing is known about the influence of BNP on hKCs. We firstly investigated the possible BNP effect on hKCs. Cells in culture were exposed to BNP for 24 h before the supernatant was collected and used for proteome profiler human XL cytokine array, which allowed detecting 102 different cytokines simultaneously. An increase in IL-17A (~2.5 folds), CXCL10 (~2 fold), and to a lesser degree, MMP9 was detected in the culture supernatant (Fig 7A). In contrast, significantly lower levels of cytokine release were triggered by the same dose of SP. In comparison, incubation of hKCs with 1 µM human CGRP peptide for 24 h only induced minimal CXCL5 release (Fig 7A). IL-17A is a critical cytokine involved in the pathogenesis of AD (29, 30). Its expression in KCs was reported (31-33) and detected in our primary culture (Supplementary Fig E4 B). CXCL10 mediates cell adhesion, migration and inflammatory infiltrates in AD (34). Elevated levels of IL-17A and CXCL10 are implicated in patients with AD (28, 29, 35). Thus, peripheral BNP might promote the release of itch-related cytokines by acting on the KCs to propagate itch signals in AD.

Subsequently, we investigated the signaling pathways involved in the activation of the KCs using phospho-kinase arrays on the cell lysate of hKCs harvested after exposure to 1 µM human BNP for 8 min. Glycogen synthase kinase 3 (GSK3), c-Jun N-terminal kinases (JNK) pan (JNK kinase family includes three proteins named JNK1, 2, 3), ERK1/2 and P38 were significantly phosphorylated/activated by application of BNP. In contrast, ERK1/2, JNK and to a lesser extent GSK3 were activated by SP (Fig 7B). IL-17A is considered to be a potent stimulator of further inflammatory mediator production, amplifying the inflammatory response. Therefore, using selective phosphor-kinase inhibitors, we

investigated which intracellular kinase was responsible for the BNP-mediated IL-17A release from hKCs. 1 μM P38α inhibitor AL8697, GSK3 inhibitor SB216763 (inhibiting the activity of α and β isozymes of GSK-3), or JNK inhibitor JNK-IN-8, were incubated with cultured hKCs, for 1 h prior to and during 24 h incubation with BNP before measuring IL-17A release using IL-17A ELISA kit. Inhibition of GSK3 by SB216763 abolished the IL-17A release, whereas AL8697 only gave minor blockade (Fig. 7C). Because IL-17A is regarded as a key cytokine involved in the pathogenesis of AD (36, 37), targeting GSK3 may prove effective for the inhibition of itch transmission.

BNP stimulates release of CCL20 through c-Jun activation in human monocyte-derived dendritic cells (hDCs)

We then assess a possible functional consequence of BNP on skin immune cells, hDCs, known to express BNP receptors (38). To do this, monocyte-derived hDCs were maintained in the presence of IL-4 and GM-CSF to allow their differentiation before incubating with 1 μM human BNP for 24 h. We found CCL20 release was increased significantly by BNP, and this was not observed when incubated with 1 μM SP (Fig 7D). In the phospho-kinase array, BNP also activated c-Jun through phosphorylation, unlike SP which did not show any effect on c-Jun activation (Fig 7E). CCL20 level is strongly increased in lesional skin tissues with AD and it stimulates the migration of various immune cells (39-41). Thus, BNP induction of CCL20 release may constitute a novel neuron-immuno-modulatory mechanism that results in the activation of immature dendritic cells, which could contribute to the persistent itch.

DISCUSSION

AD patients experience a ‘vicious cycle of itching and scratching’ (42). The molecular basis for this phenomenon, however, is still unknown. Although it has recently been revealed that cytokines like IL-31 and IL4 or IL-13 play a role in immunity and itch transmission (8, 43, 44), it is currently unknown whether cytokines like IL-31 also regulate neuropeptide release from peripheral nerve endings thereby modulating neurogenic inflammation in AD (Fig 8). Understanding the importance of neuropeptide- and cytokine-mediated intercellular communication would further explain not only the interplay between inflammation and itch, but also how this debilitating symptomatology of inflammation and itch may be therapeutically interrupted. Here, we show for the first time that peripherally

released BNP is implicated in AD pathophysiology through IL-31 stimulation and subsequent regulation of cytokine, chemokine and MMP9 release. Moreover, we show BNP also activates several major cell types in skin which are pivotal in AD itch transmission such as keratinocytes and dendritic cells (Fig 8). Thus, we have revealed another mechanism how TH2 cells simultaneously impact itch and neuro-inflammation.

BNP was originally identified as an important contributor of *central* itch being released from central primary afferents to the dorsal horn of the spinal cord (19). Here we show for the first time that BNP is released from peripheral nerve endings upon stimulation by puritogens like IL-31 and histamine. These findings provide a new link between Th2 cells, mast cells and peripheral sensory nerves. Notably, this is unique to BNP as to other neuropeptides, such as CGRP and SP, because neither of them are synthesized nor released under the same conditions, despite both being linked to itch (45, 46). In addition, BNP together with its receptor, NPR1, are expressed in $CGRP^+$ small sensory neurons (47). In fact, though traditionally viewed primarily as a pain mediator (48), SP has also been implicated as an itch mediator in AD, with increased numbers of SP^+-nerve fibers found concomitantly with a decrease in cutaneous SP levels in lesional skin of AD (49, 50), and responsiveness to the NK1R antagonist aprepitant (45, 51). In particular, BNP-expressing sensory neurons are found not to be involved in acute, inflammatory or neuropathic pain (19, 52), unlike SP and CGRP. Therefore, our findings further differentiate BNP from CGRP and SP and highlight its importance in the neuropeptide-mediated pathogenesis of AD-associated itch.

Here, we show for the first time that IL-31RA and OSMRβ highly overlap in hDRGs, similar to the results observed previously in mDRGs (53). Moreover, in hDRGs IL-31 elicited Ca^{2+}-transient in ~50% ET-1-responsive neurons and ~2% of total neurons. Half of ET-1-responsive neurons also responded to histamine. To our knowledge, this is the first detailed characterization of human sensory sub-populations according their response to sequential and combined pruritogens. In contrast, IL-31 and ET-1 excited partly overlapping small subset of mDRG neurons. In response to IL-31, *Nppb* transcripts in cultured mDRGs were specifically upregulated, but this was not found for *Tac-1* or *Calca*. IL-31 was capable of eliciting ~2.7-fold increase of BNP release over the basal level. The elicited release was completely dependent on particular SNARE proteins, SNAP-25 and

VAMP1, which are also important for several other inflammatory diseases (54). In contrast to the minimal BNP release at resting condition, the IL-31-induced augmentation of peripheral BNP release may link to excessive itch in AD induced by a positive feed-back loop of amplifying itch mediators. This may also explain the difficulty to treat AD-associated itch.

Using deep sequence, we detected upregulation of *Nppb* transcripts in isolated TGs from the HDMs-treated PAR2 transgenic mice, which showed AD-like skin condition. Furthermore, our data confirm that the *Nppb* gene is implicated in severe AD conditions because mice with high clinical scores exhibit high levels of *Nppb* transcripts. Our findings provided the first evidence showing a functional association of BNP with severity of AD. Consistently, we established that *Nppb* transcripts in DRGs and skin of lesional IL-31Tg mice were also greatly upregulated. NPR1 transcripts were increased in lesional IL-31Tg skin. In fact, upregulation of BNP in murine AD-like skin has also been reported by a recent finding using global transcriptome, which identified a significant *Nppb* upregulation (>3 fold) in oxazolone-challenged AD-like dermatitis mice skin (55).

In a translational approach, we also provide evidence for the importance of BNP in AD using skin from AD patient and healthy control subjects. We detected increased protein levels of NPR1 and NPR2 in epidermis, particularly in KCs. Moreover, enhanced expression of BNP in both epidermis and dermis of AD patient skin was revealed. In fact, immunostaining signal of BNP, NPR1 and NPR2 protein seemed to be increased in single-cell level of KCs; however, we could not exclude that the possibility of the acanthosis might also contribute to the increment. The upregulated expression levels of BNP and its receptors in AD patients suggest a role for the IL-31/BNP/BNPR-axis in peripheral itch amplification in AD patients.

Although IL-31 rapidly induces itch in mice (7, 8) and is regarded as one of the major ‘drivers’ of itch in AD, it failed to induce immediate itch responses in humans as shown in a recent study including both AD patients and healthy subjects monitored by skin prick testing (56). Though there is a possibility of insufficient amount of IL-31 being administered which failed to induce immediate itch response, the late onset of IL-31-induced mild itch sensations might also be attributed to this cytokine exerting its pruritic effect indirectly via KCs and secondary mediators, rather than by a sole action on its receptors on cutaneous sensory nerves (56, 57). In addition, similar to IL-4 (44) IL-31 may

be a sensitizer in some patients and not a direct itch inducer. However, the variable expression levels of IL-31 and IL-31RA in AD patients may explain that some patients will respond more to IL-31 stimulation. Consequently, some patients may profit better from Anti-IL-31 therapy than others (11, 12). It will be interesting to learn as to whether IL-4 and IL-13 are also linked to BNP release.

We demonstrate that BNP acts as the downstream skin-derived effector of IL-31. In addition to its contribution to spinal processing of itch (19), excessive release of BNP from peripheral sensory nerves stimulates NPR1 receptor to activate multiple intracellular signaling pathways in skin, thereby contributing to skin inflammation in AD. We found BNP activates GSK3 pathway and leads to the secretion of several important inflammatory or itch modulators, such as IL-17A, CXCL10 or MMP9 from skin KCs. BNP activates C-Jun to release CCL20 from DCs. All of these cytokines are known to be increased in AD and represent an important potential component of the pathology of AD (58, 59). Upregulation of BNP receptor(s) in turn, could further augment secretion of the above important itch modulators from these cells. Thus, our results support a new pathway in which BNP is a peripheral contributor of IL-31-mediated neurogenic inflammation in AD, and establish the missing link between TH2-nerve mediated inflammation as well as pruritus.

In conclusion, we describe a novel functional link between TH2 cells and sensory nerves through IL-31 and BNP, and give first evidence between a functional association of peripheral BNP in AD using human and rodent DRGs, animal models and patient skin in a translational fashion. Indeed, Th2 cells, sensory neurons, keratinocytes and dendritic cells release a complex network of cytokines and chemokines establishing a local milieu and environment that favors AD skin inflammation. In this context, BNP seems to act as an important “relay center” for peripheral and central itch circuits to facilitate itch, but also neuroinflammation, in the pathogenesis of AD. We demonstrate that BNP acts through multiple mechanisms: 1) IL-31 modulates pruriceptive neurons to rapidly induce SNARE-dependent release of BNP, and 2) IL-31 increases the synthesis of *Nppb*; 3) BNP receptors are upregulated in AD; 4) BNP signals through its upregulated receptors to directly activate multiple intracellular kinases in skin cells to elicit the release of itch-related pro-inflammatory cytokines (Fig 8). Our findings provide new insights about BNP as an important regulator of neuro-inflammation as well as itch in the skin of AD, and close a missing link why the itch inflammatory skin disease AD has been also defined as

'neurodermatitis'. In addition, peripheral BNP signaling provides a new basis for the development of more effective therapies for AD and probably other skin diseases.

FIGURE LEGENDS

Fig 1. IL-31RA and OSMRβ co-occurred in a small population of hDRGs. (A, B) Representative immunofluorescence confocal images showing IL-31RA protein immunoreactivity only occurred in a subpopulation of neuronal cells in hDRG sections. Neuronal cells were stained by antibody against NeuN (**A**) or PGP9.5 (**B**). **C**, Co-staining of OSMRβ with PGP9.5 in hDRG section. D, Immunofluorescence staining of hDRG section showing IL-31RA and OSMRβ largely co-localised in a subset of neurons. Specimens were counterstained with DAPI to highlight all cell nuclei. Arrow pointed a neuron expressing OSMRβ but not stained strongly by IL-31RA antibody. **E**, Venn diagram depicts the relationship of IL-31RA$^+$, OSMRβ$^+$ and NeuN$^+$ populations in hDRG section. Total neuron numbers were counted based on the dual labelling of both NeuN and DAPI. A total of 573 cells were imaged.

Fig 2. Puritogens–induced calcium mobilization and characterization of IL-31–responsive cells in hDRGs and mDRGs. A, Representative traces for calcium measurement after sequential pruritogen mediators application. Traces show individual cell responding to ET-1 only (top), histamine only (middle), ET-1 plus histamine (bottom). Notably, there are some ET-1–responsive neurons that also responded to histamine. **B,** Neurons responding to ET-1 only (top), IL-31 only (middle), ET-1 plus IL-31 (bottom). **C**, Venn diagrams for percentage of neurons that responded to puritogenic compounds. Total number of hDRGs recorded=100. **D**. Four representative traces of mDRG cells responsive to IL-31 and/or ET-1. Note that cell 1 responded to both IL-31 and ET-1; cell 2 only responded to ET-1, whereas cell 3 failed to respond to either IL-31 or ET-1; cell 4 is an IL-31-responsive cell which did not respond to ET-1. Venn diagram showing relative proportions of mDRG neurons responsive to IL-31 and/or ET-1. A total of 335 mDRGs were recorded.

Fig 3. *Nppb* is upregulated in sensory ganglia from HDM-treated Grhl3PAR2$^{/+}$ mice and IL-31Tg mice. A, The FPKM for *Nppb,* NPR1, NPR2, IL-31, IL-31RA and OSMR gene from TGs of ipsilateral vs contralateral (ipsi vs contra) were analyzed by RNA-seq

and compared. Eight mice were treated with HDM on the right cheek and nothing on the left cheek, and the cheek biopsies were clinical scored using HE staining. Correlation coefficient between clinical score and fold change of *Nppb* is about 0.8, whereas for other genes analyzed the values are not significant. For these 8 mice the average of fold change of *Nppb* is 2.3. **B**, Representative HE staining images showing the severity of skin lesion from HDM-treated mice, compared with vehicle-treated WT control. Images were taken by 10x magnification objective. **C**, FPKM analyzed by RNA-seq of the top 3 high clinical score mice (Score is 8, 7 and 7) show the *Nppb* gene was upregulated by 3.5 times (ipsi vs contra). Average of fold change for genes upregulated and reached significance were plotted. A two-fold change in FPKM was deemed significant. **D**, RT-PCR analysis of *Nppb* mRNA levels in DRGs isolated from lesional, non-lesional IL-31Tg or WT mice. Values were normalized to the housekeeping gene GADPH. The results (mean ± S.E.M.) are pooled data from multiple animals. Significant difference is indicated; * $P<0.05$; ** $P<0.01$.

Fig 4. IL-31 induces the release and mRNA synthesis of BNP (but not CGRP or SP) from cultured mDRGs. Release of BNP **(A)**, CGRP and SP (**D**) over 30 min was measured by their specific ELISA or EIA, and the increment of release after each treatment was plotted relative to the basal level. The amount in pg/ml in basal (LK) release is: 15.3 ± 1.2 for BNP; 3.9 ± 0.9 for SP; 49.3 ± 11.8 for CGRP. LK, low potassium basal buffer; HK, high potassium stimulation buffer; HIS, histamine. **B**, BNP release induced by various dose of IL-31 over 30 min. **C**, Time-dependence of BNP release induced by 300 nM IL-31. **E,** RT-PCR for mRNA of *Nppb*, *Calca* and *Tac-1* in cultured mDRGs after treatment with 100ng/ml IL-31 for 6h. Data in **A** –**E** are presented as mean ± S.E.M. $n \geq 3$ independent experiments. Significant difference is indicated; N.S. $P>0.05$; *$P<0.05$; ** $P<0.01$; *** $P<0.001$. **F,** Immunofluorescence study for cellular localization of CGRP (red) and BNP (green) after dual labelling of DRGs with each specific antibody and counterstained with DAPI (blue). Scale bars are indicated.

Fig 5. IL-31-induced BNP release from cultured mDRGs requires SNARE proteins. A, Representative immunoblots show knock-down of SNAP-25 (S25), VAMP1 (V1) and VAMP7 (V7) protein expression in cultured DRGs using lentiviral shRNA particles. Syntaxin 1 (STX1) serves as an internal control in each blot. Non-targeted virus-treated samples were loaded to the gels for the comparison. **B,** Quantified plot demonstrates that

300 nM IL-31-elicited BNP release over 30min is inhibited after knockdown of S25 and V1 but not V7. Data are presented as mean ± S.E.M. n ≥ 3 independent experiments. Significant difference between specific shRNA-treated cells and non-targeted controls is indicated; N.S. P>0.05; *** P<0.001.

Fig 6. Immunohistochemical staining of BNP, NPR1 and NPR2 in the skin of human healthy control and AD patient. A. Representative fluorescent images show expression patterns of BNP, NPR1 and NPR2 in AD and healthy control skin. Note that epidermal keratinocytes layer of AD skin showed enhanced staining of BNP, NPR1 and NPR2 compared to healthy control skin. Boxed areas in the middle panels are shown at higher magnification in right panels. **B.** Images show increased BNP staining in the dermal structures including secretory portion of sweat glands and blood vessel in AD skin compared with the healthy skin. **C.** NPR2 does not co-localize with nerve marker PGP9.5 in the dermal structures, but highly co-localizes with NPR1. There is no obvious change of NPR1 and NPR2 expression in dermis between AD patient skin and control healthy skin. Scale bars are indicated. Paraffin-embedded human skin sections (n=10) from each donor of AD and healthy control subjects were stained for each condition. The white dotted lines denote the epidermal–dermal junctions.

Fig 7. Effect of BNP on cytokine/chemokine release and activation of intracellular phosphor-kinase. A, Release profile of cytokines from cultured hKCs detected by antibody array. Data from BNP-, SP- or CGRP-induced release were calculated relative to non-treated control (basal). **B,** Neuropeptide-induced activation of intracellular phosphor-kinases. Data plotted are the ratio of phosphorylated signal obtained from neuropeptide-treated cells relative to non-treated control. **C,** Percentages of inhibition by selective kinase inhibitors of BNP-induced IL-17A release from hKCs. **D,** CCL20 release from cultured hDCs induced by BNP or SP. **E**, Phosphorylation of intracellular c-Jun from hDCs after incubation with BNP or SP. Data are presented as mean ± S.E.M. n ≥ 3 independent experiments. Significant difference between stimulated and basal is indicated; * P<0.05; ** P<0.01; *** P<0.001.

Fig 8. Schematic diagram illustrates an important communication link between IL-31 and BNP, both of which are key players in the signaling pathways implicated in pruritus. Itch inducers such as IL-31 and histamine elicit BNP release via SNARE-

controlled mechanism. Augmented release and synthesis by IL-31 of BNP might contribute to central and peripheral itch signaling. In contrast, CGRP and SP are not elicited by these puritogens. Released BNP subsequently increases IL-17A, CXCL10 and MMP9 release from keratinocytes; however, SP and CGRP only elicit minimum release of CXCL10 and CXCL5, respectively. BNP also mediates CCL20 from dendritic cells. Taken together, it is postulated that these released cytokines/chemokines modulate itch transmission and pathogenesis of AD.

DECLARATION OF INTEREST

We declare no conflict of interest.

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Fig. 1

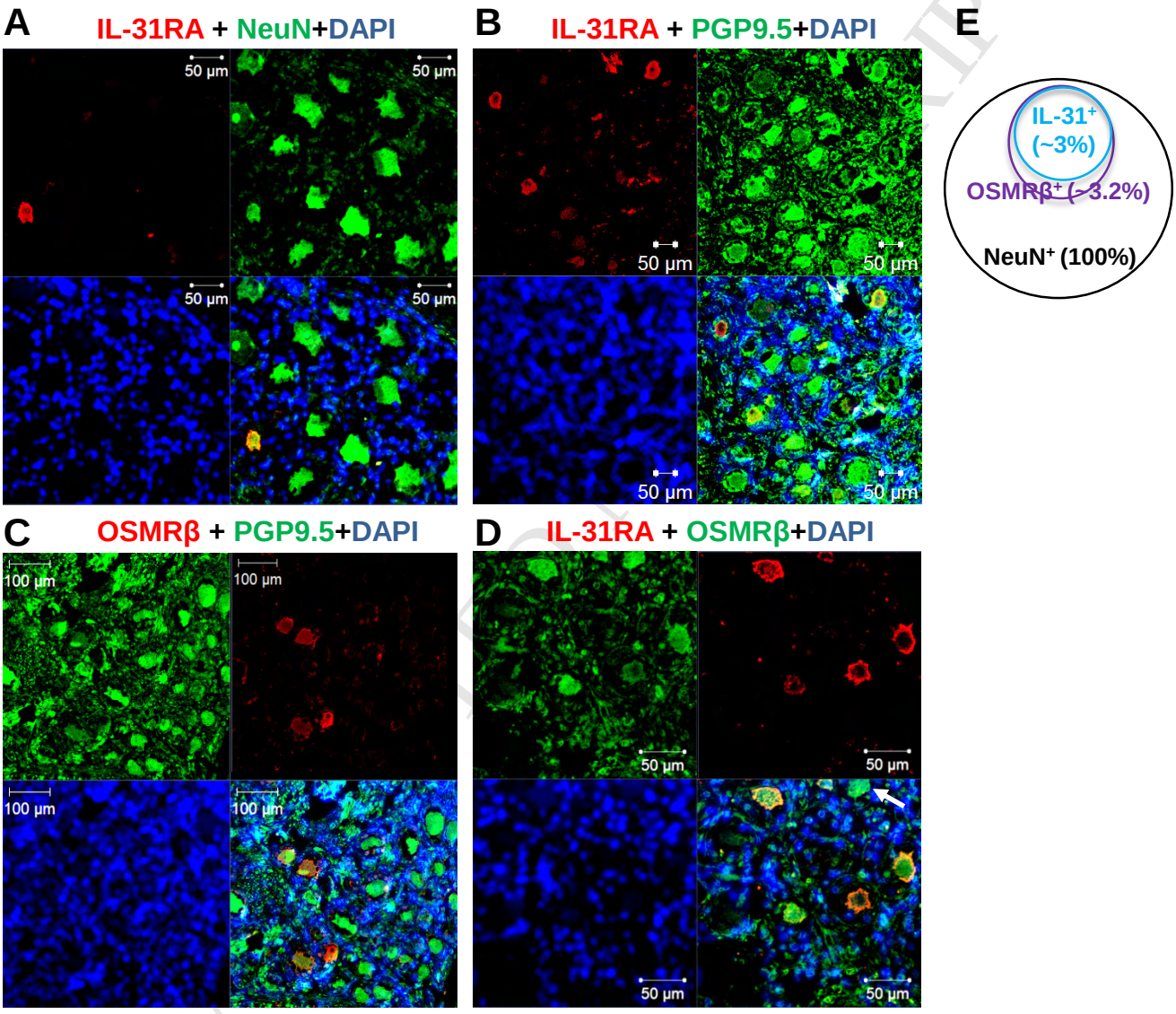

A
IL-31RA + NeuN+DAPI
B
IL-31RA + PGP9.5+DAPI
E
IL-31⁺ (~3%)
OSMRβ⁺ (~3.2%)
NeuN⁺ (100%)
C
OSMRβ + PGP9.5+DAPI
D
IL-31RA + OSMRβ+DAPI
50 μm
100 μm

Fig. 2

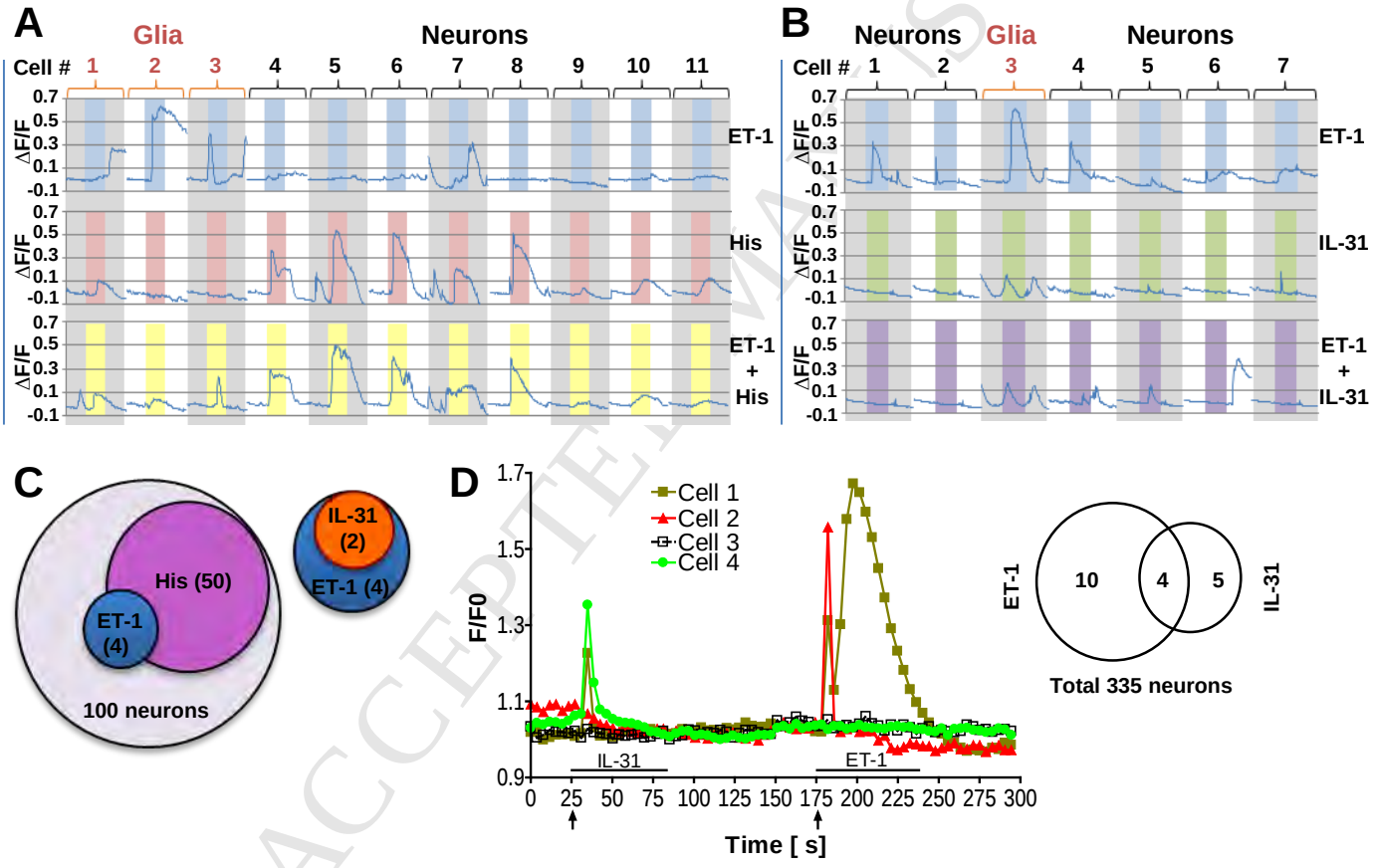


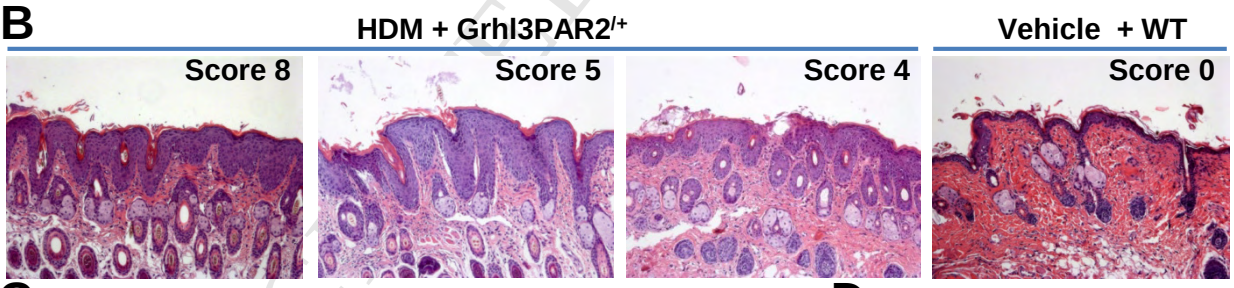
Fig. 3

A

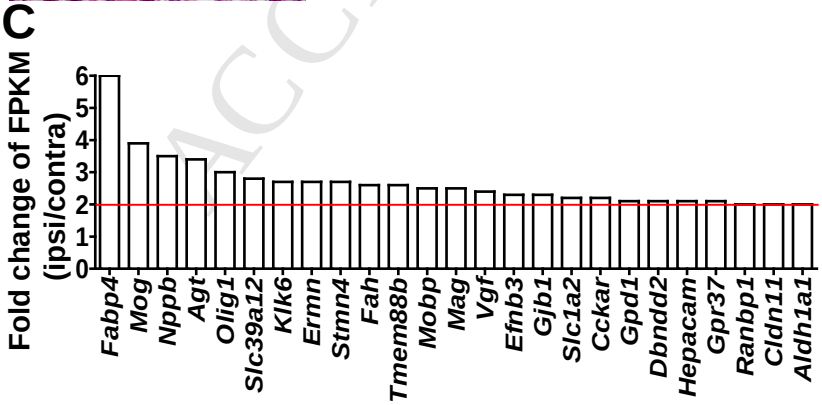
Nppb	FPKM		ipsi/contra	NPR1	FPKM		ipsi/contra	NPR2	FPKM		ipsi/contra
Clinial Score	ipsi	contra	Fold Change	Clinial Score	ipsi	contra	Fold Change	Clinial Score	ipsi	contra	Fold Change
8	21.1	8.7	2.4	8	0.5	0.7	0.6	8	15.2	13.4	1.1
7	17.6	3.9	4.5	7	0.6	0.3	2.4	7	14.6	14.9	1.0
7	29.4	7.7	3.8	7	0.8	1.2	0.7	7	15.9	14.0	1.1
5	19.7	8.1	2.4	5	1.0	0.7	1.3	5	11.8	14.5	0.8
4	10.2	8.8	1.2	4	1.1	0.7	1.5	4	16.1	15.0	1.1
3	20.0	12.2	1.6	3	0.4	0.3	1.2	3	15.7	14.5	1.1
3	12.5	6.8	1.8	3	0.7	0.3	2.2	3	21.1	15.5	1.4
2	5.4	13.4	0.4	2	1.4	0.6	2.5	2	19.1	18.8	1.0
correlation coefficient			0.799	correlation coefficient			-0.556	correlation coefficient			-0.126

IL-31	FPKM		ipsi/contra	IL-31RA	FPKM		ipsi/contra	OSMR	FPKM		ipsi/contra
Clinial Score	ipsi	contra	Fold Change	Clinial Score	ipsi	contra	Fold Change	Clinial Score	ipsi	contra	Fold Change
8	0.0	0.0	-	8	3.6	4.4	0.8	8	17.5	20.0	0.9
7	0.0	0.0	-	7	1.6	2.2	0.7	7	15.0	10.1	1.5
7	0.0	0.0	-	7	3.5	4.4	0.8	7	16.3	13.3	1.2
5	0.0	0.0	-	5	2.5	2.2	1.1	5	13.8	14.3	1.0
4	0.0	0.0	-	4	2.6	2.0	1.3	4	10.3	11.0	0.9
3	0.0	0.0	-	3	2.9	1.4	2.0	3	15.0	11.4	1.3
3	0.0	0.0	-	3	6.2	2.0	3.2	3	17.5	10.0	1.7
2	0.0	0.0	-	2	2.1	3.3	0.6	2	10.1	14.9	0.7
correlation coefficient			-	correlation coefficient			-0.500	correlation coefficient			0.020

B



C



D

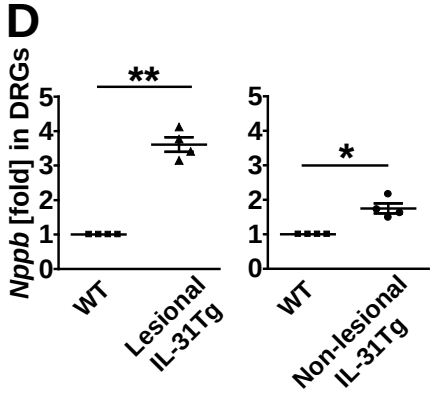


Fig. 4

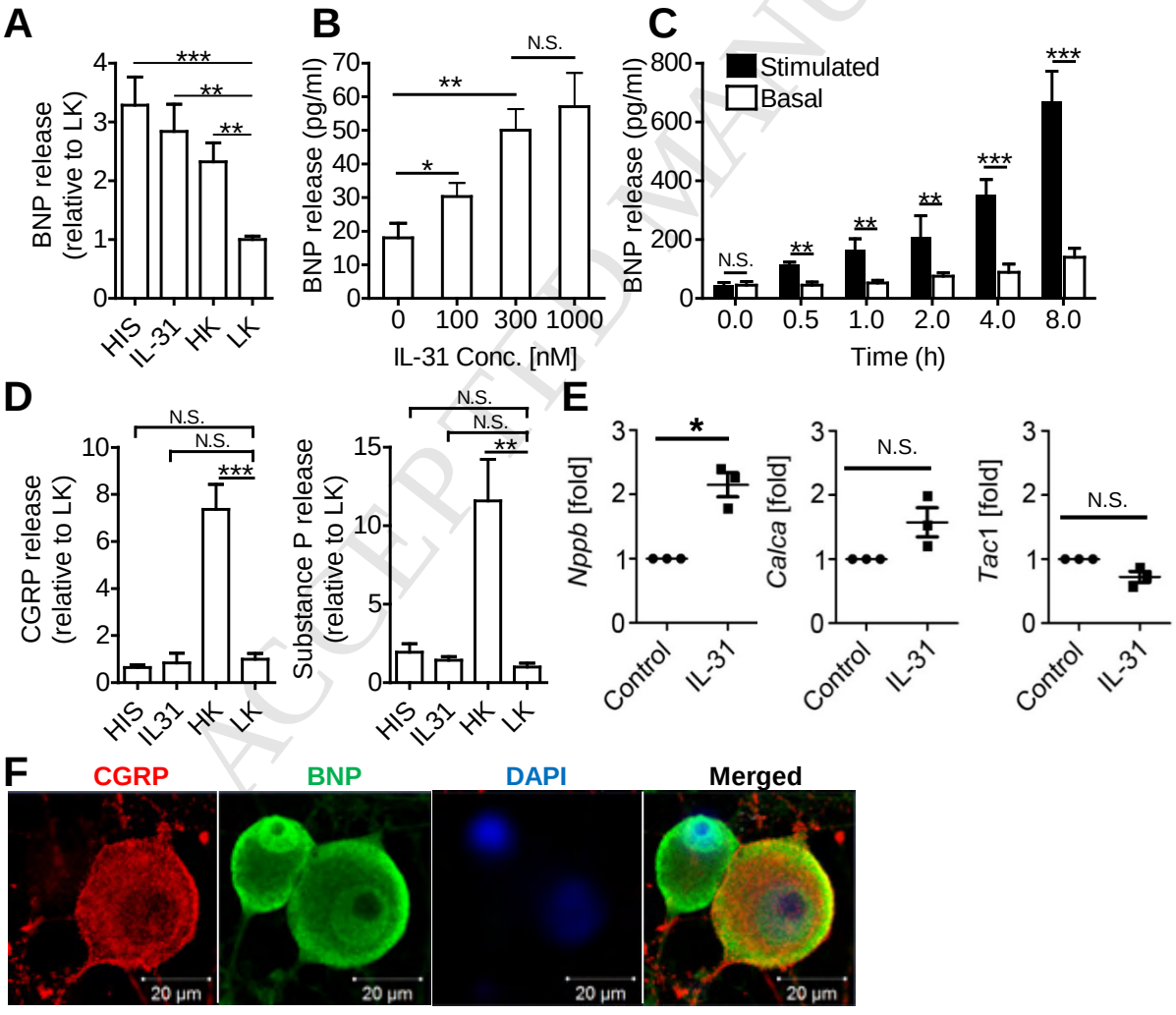


Fig. 5

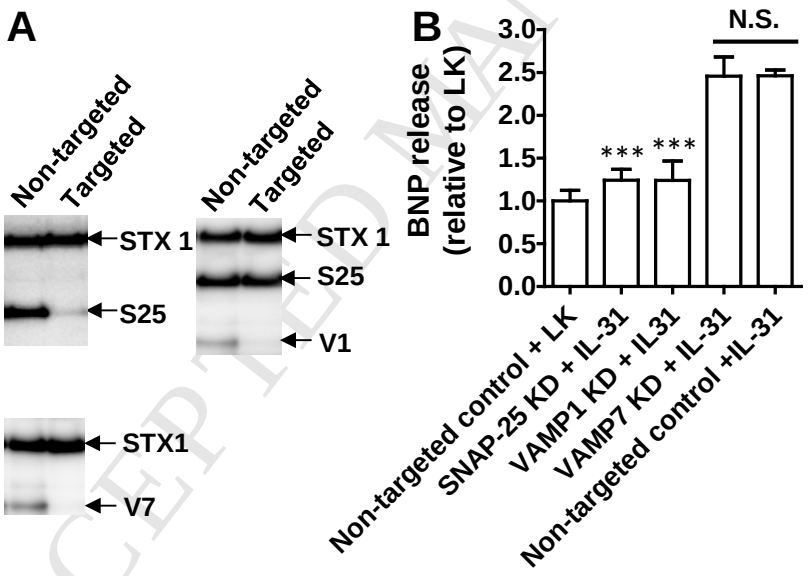


Fig. 6

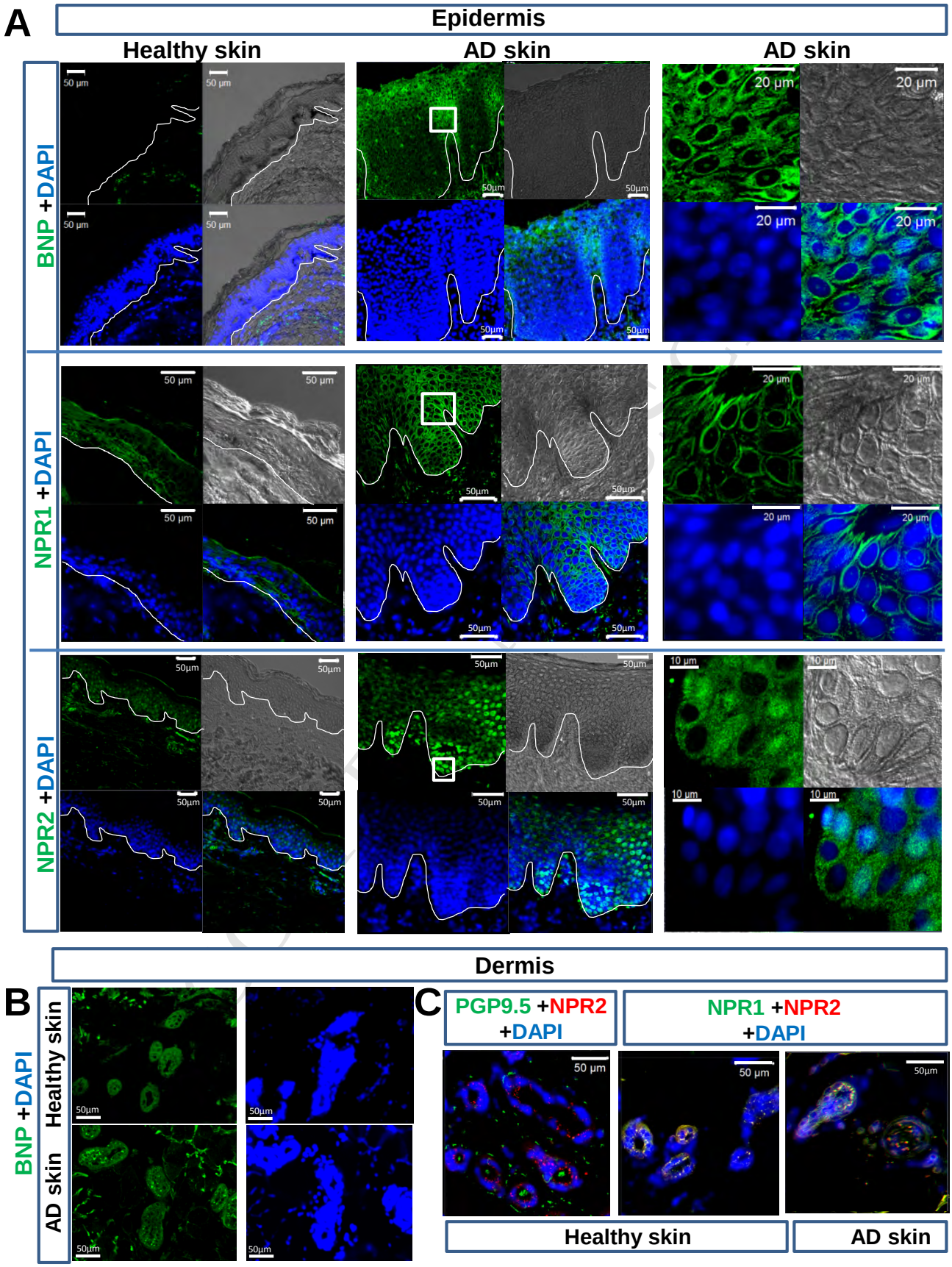


Fig. 7

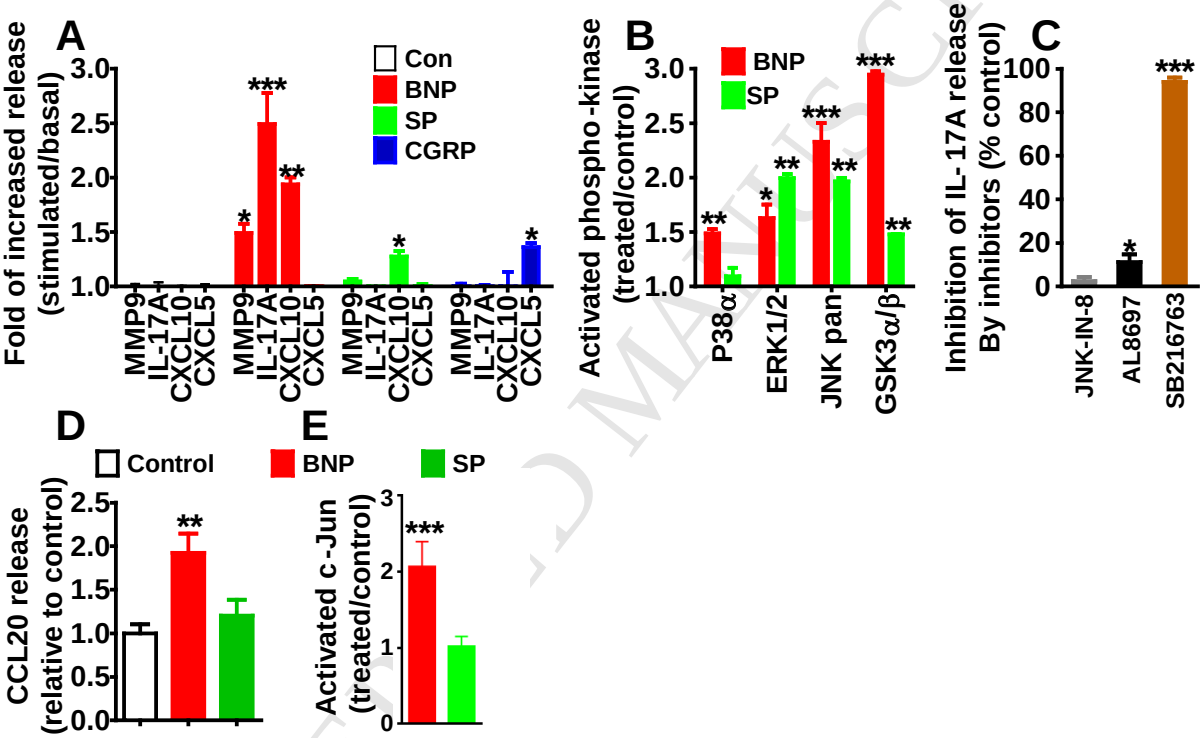
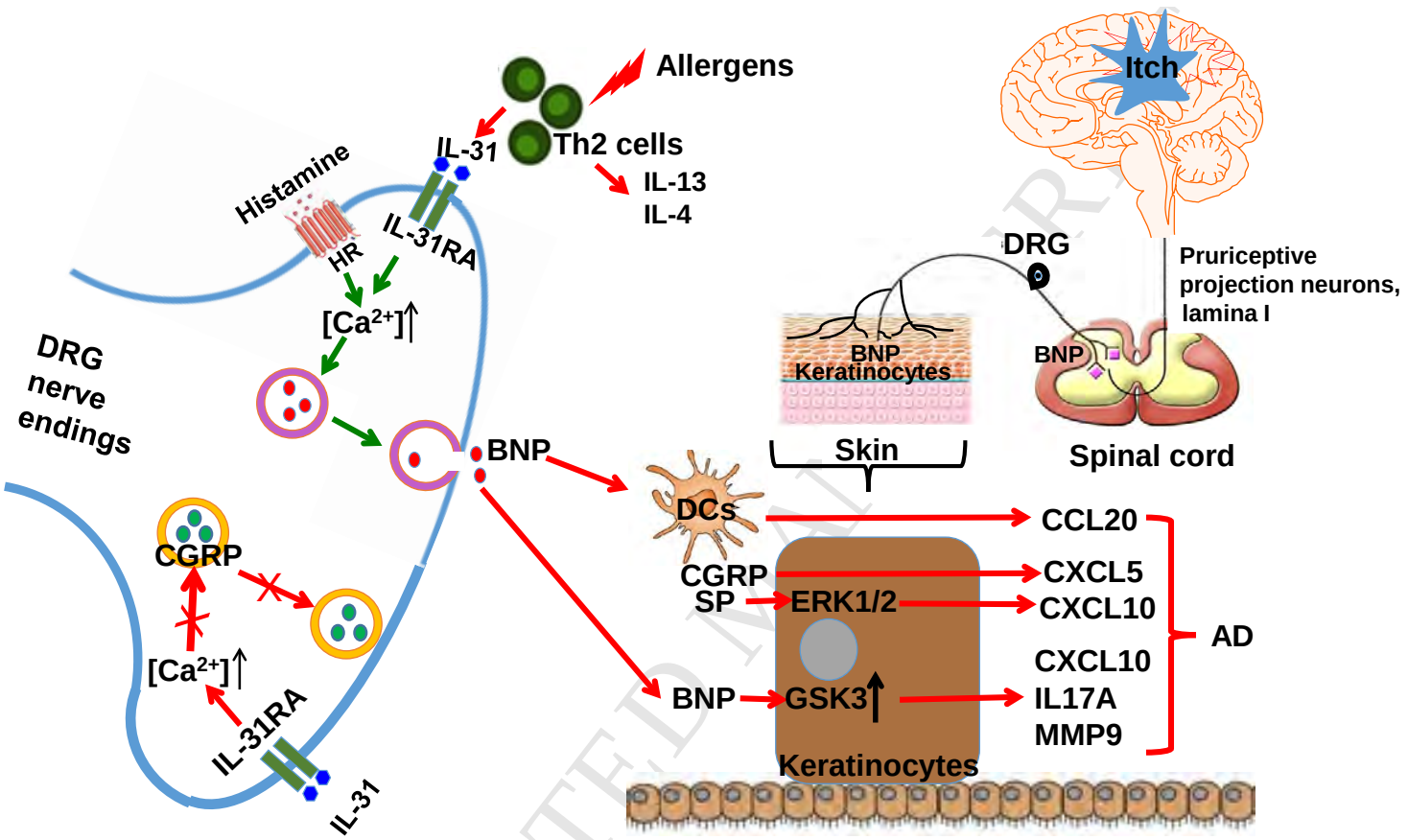


Fig. 8



Supplemental Materials and Methods

Materials

Skin samples from AD and healthy controls were bought from Tissue Solutions Ltd., Glasgow, Scotland. Enzyme immuno-assay (EIA) kits for CGRP and SP were bought from Bioquote; rabbit anti-syntaxin 1, rabbit anti-VAMP1 and 7 were bought from Synaptic Systems GmbH. SP, BNP, CGRP, histamine and ET-1, monoclonal antibody specific for CGRP (4901), rabbit anti-PGP9.5, ELISA kit for BNP, shRNA lentiviral particles were bought from Sigma-Aldrich. Donkey anti-rabbit Alexa-488 and anti-mouse Alexa-594, donkey anti-goat Alexa594 and anti-mouse Alexa 488 were supplied by Jackson ImmunoResearch. Ibidi GmbH provided the culture chambers. Rabbit anti-BNP antibody (G-011-23) was purchased from Phoenix Pharmaceuticals, Inc. and monoclonal anti-SNAP-25 (SMI 81) was bought from Sternberger Monoclonals Inc. Mouse monoclonal anti-PGP9.5 (Ab8189), -NPR2 (Ab55724), rabbit anti-NPR1 (Ab14356), -BNP (Ab19645), -NeuN antibody (Ab177487) and IL-17A ELISA kit were provided by Abcam; AL8697, goat anti-IL-31RA, mouse anti-OSMRβ, CCL20 ELISA kit, proteome profiler™ human XL cytokine array, human phospho-kinase array kits and IL-17A antibody (AF-317-NA) were bought from R&D Systems; SB216763 was purchased from Tocris; JNK-IN-8 was obtained from Axon Medchem. Rabbit anti-NPR1 antibody (NBP1-31333) was purchased from Novus Bio. Adult normal hKCs and hDCs and their culture medium were bought from Lonza. Human DRG paraffin sections were bought from Zyagen. Alexa 594 conjugated mouse anti-human Vimentin antibody, Alexa 488 conjugated mouse anti-human CD4 and Alexa 488 conjugated mouse anti-human CD80 were purchased from BioLegend. Mouse C57 DRG sections were purchased from Amsbio.

HDM application on Grhl3PAR2$^{/+}$ mice model

The Grhl3PAR2$^{/+}$ mice were maintained in C57BL6/J-129X1/SvJ mixed strain and used for the experiments as AD model. We used mite-extract ointment/house dust mite (HDM) (BiostirAD cat#303-34131) on the right cheek to induce severe skin lesion. For HDM treatment, 8 mice were shaved on cheek one day prior to application. On the application day, 4% SDS was always rubbed on shaved cheek area 2 h before HDM application. HDM was applied on right cheek twice a week with at least a 3 day resting period prior to the next application for 6 weeks. At the

end of the treatments we euthanized the mice and harvest lesional, perilesional and non-lesional skin for pathologic diagnosis using HE staining to measure the clinical score, and trigeminal ganglia were harvested for gene expression experiment.

RNA-Seq

For gene expression experiment, TGs were harvested from Grhl3PAR2$^{/+}$ mice. Total RNA was isolated from right TG (ipsilateral) and left TG (contralateral) with TRIzol Reagent (Life technologies cat#15596). Then mRNA library was made with Ovation Universal RNA-Seq System (NuGEN cat#0343). Samples were subsequently sequenced on the HiSeq2500 (illumine).

Quantitative real time PCR

DRGs were isolated from adult C57Bl6 mice (6-8 weeks old) and treated with 3 mg/ml collagenase (Sigma Aldrich) and 0.25 mg/ml trypsin (PAA) for 30 minutes. DRGs were triturated for dissociation and were plated onto cell culture dishes coated with poly-L-lysine (100 mg/ml; Sigma Aldrich) and laminin (5 mg/ml; Sigma Aldrich) in MEM supplemented with 10% horse serum, 1% penicillin/streptomycin, 1% vitamins, 1% N2-supplement and 2% B27-supplement. After overnight rest, DRG neurons were treated with 100 ng/ml murine IL-31 (ZymoGenetics) for 6 h. The quantitation of mRNA levels was performed by real time fluorescence detection using Absolute SYBR Green ROX mix (ABI).

The primers used for both DRGs and skin were as follows: m*Nppb* forward, 5´-gtcagtcgtttgggctgtaac-3´; m*Nppb* reverse, 5´-agacccaggcagagtcagaa-3´; m*Calca* forward, 5´-agcaggaggaagagcagga-3´; m*Calca* reverse, 5´- cagattcccacaccgcttag-3´;m*Tac1* forward, 5´-agcctcagcagttctttgga-3´; m*Tac1* reverse, 5´- tctggccatgtccataaagag-3´. Primer and probe specific for 18S RNA and glyceraldehyde 3-phosphate dehydrogenase (GADPH) were obtained from Life technologies (Darmstadt, Germany) and Eurofins Genomics (Germany). Target gene expression was analyzed on an ABI Prism 7000 supplemented with SDS 1.2.3 software and the expression profile was normalized using the 18S or GADPH expression. In the case of mouse tissue, the primers used were: mNPR1 forward, 5´- ttccacactggaggttctggct-3´; mNPR1 reverse, 5´- ctctgagaccagctccttttcc-3´.

BNP, CGRP and SP release assay

In order to investigate BNP, SP and CGRP release, DRG were isolated from postnatal d5 C57BL/6 mice and dissociated by collagenase I as detailed (1). Neurons were cultured in the presence of cytosine β-d-arabinofuranoside (AraC, Sigma) and nerve growth factor (NGF) 100 ng/ml for 7 DIV (days *in vitro*). Basal low potassium (LK) release buffer (mM; 22.5 HEPES, 135 NaCl, 3.5 KCl, 1 $MgCl_2$, 2.5 $CaCl_2$, 3.3 glucose and 0.1% BSA, pH 7.4) was added into each well, followed by a 30-min incubation at 37 °C (2). Cells were then stimulated for 30 min or as indicated in Figures by high potassium (HK, 60 mM K^+, isotonically balanced with NaCl), 1 µM histamine, or 300 nM IL-31 (Zymogenetics). Release of BNP, CGRP or SP was quantified using their ELISA or EIA kit.

Measurement of intracellular Ca^{2+} concentration

For the hDRG calcium measurement, cells in culture were loaded with 5 µM Fluo 8-AM (AAT Bioquest 21081) containing 0.1% Pluronic F-127 (Sigma P2443) for 20 min. Extracellular solution contained in mM: 145 NaCl, 3 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 glucose adjusted to pH 7.4 with NaOH. Fluo-8-loaded cells were excited at 480 nm and emission was collected at 520 nm with a pcoEDGE sCMOS camera (PCO) mounted on an inverted microscope (Olympus IX71). Following a baseline period of 60 s, ET-1 (30 nM); Histamine (10 µM); IL-31 (300 nM) were applied sequentially, for 60 s. each, followed by washout for 5 min. Images were acquired at 0.2 Hz. In the case of mDRGs, 3 µM Fluo 4-AM was used and images were acquired by a Zeiss LSM710 confocal microscope (Carl Zeiss MicroImaging) with argon laser at 2 sec intervals.

Immunofluorescence staining

For cellular localization of CGRP and BNP in cultured mDRGs, cells were processed by dual labelling overnight at 4°C with 1:100 rabbit anti-BNP antibody (G-011-23; Phoenix Pharmaceuticals, Inc.) and 1:200 mouse anti-CGRP antibody (clone 4901, Sigma). Secondary donkey anti-rabbit Alexa Fluor 488 (1:2000) and anti-mouse Alexa Fluor 594 (1:2000) were added for 1 h at room temperature.

Human DRG and skin paraffin sections (15µm) were deparaffinized, rehydrated before permeabilized in phosphate-buffered saline with 0.2% Triton X-100 (PBS-T) and then incubated

in PBS containing 5% normal donkey serum (blocking solution) at RT for 1 h. Specimens were then incubated with primary antibodies in blocking solution (4 °C, overnight). For human DRG sections, primary antibodies were used at the following dilutions: goat anti-IL-31RA 1:100 (R&D Systems); mouse anti-OSMRβ 1:100 (R&D Systems); mouse monoclonal PGP9.5 antibody 1:40 (Abcam) or rabbit anti-PGP9.5 1:1000 (Sigma); rabbit anti-NeuN antibody 1:500 (Abcam). The sections were washed in PBS and were incubated in donkey anti-goat Alexa 594 and donkey anti-mouse Alexa 488 (Jackson ImmunoResearch) or donkey anti-rabbit Alexa 488 diluted 1:2000 with blocking solution (RT, 1 h). For human skin sections (15µm), we used rabbit polyclonal to BNP (Abcam, 1:1000), rabbit polyclonal to NPR1 (Novus Bio, 1:500) or mouse monoclonal to NPR2 (Abcam, 1:80), rabbit anti-PGP9.5 (Sigma).

For hKC staining, cultured cells were fixed in 3.7% paraformaldehyde in PBS, permeabilized by 0.1% Triton X-100 and then blocked in 5% normal donkey serum (blocking solution) at RT for 1 h before samples were incubated with the primary antibodies overnight at 4 °C. Antibodies used are: rabbit anti-NPR1 1:500 (Abcam), rabbit anti-BNP 1:1000 (Abcam), mouse monoclonal to NPR2 1:80 (Abcam), mouse monoclonal to K14 1:200 (cytokeratin 14; Ab7800), goat anti-IL-17A 1:100 (R&D), Alexa 594 conjugated mouse anti-human Vimentin antibody 1:200, Alexa 488 conjugated mouse anti-human CD4 1:200 and Alexa 488 conjugated mouse anti-human CD80 1:200. The specimens were washed in PBS and were incubated in donkey anti-goat Alexa594 or donkey anti-goat 488 and donkey anti-mouse Alexa 488 or donkey anti-rabbit Alexa 488 (Jackson ImmunoResearch) diluted 1:2000 with blocking solution (RT, 1 h).

To check specificity of BNP and CGRP antibodies, rabbit anti-BNP antibody (G-011-23; Phoenix Pharmaceuticals, Inc.) was pre-incubated with or without mouse BNP-45 peptide (Phoenix Europe) at 1:10 molar ratio. Likewise, mouse anti-CGRP antibody (clone 4901, Sigma) was pre-incubated with or without rat α-CGRP peptide (Sigma) at 1:10 molar ratio. Antibodies alone or with their antigen mixtures were incubated at overnight at 4°C. Mouse C57 DRG sections purchased from Amsbio were formalin-fixed, permeabilised in PBS with 0.1% Triton X-100 and blocked in PBS containing 5% normal donkey serum. Immunofluorescence labelling was performed overnight at 4°C with 1:100 rabbit anti-BNP antibody, 1:200 mouse anti-CGRP antibody alone or antibody pre-incubated with its antigen (as specified in figure legends) in blocking solution. After extensive wash, secondary donkey anti-rabbit Alexa Fluor 488 (1:2000)

and anti-mouse Alexa Fluor 594 (1:2000) diluted in blocking solution were added for 1 h at room temperature.

After the final wash of the secondary antibody, specimens were mounted onto slides using prolong anti-fade reagents contain (4',6-diamidino-2-phenylindole) DAPI (ThermoFisher Scientific). Images were taken by a Zeiss LSM710 confocal microscope (Carl Zeiss MicroImaging) with argon and helium/neon lasers. Images were acquired by Zen software (Universal Imaging, Göttingen).

Lentivirus-mediated knockdown of SNARE proteins

At 7 DIV, mDRGs were incubated in medium containing shRNA lentiviral particles that specifically target to SNAP-25, VAMP1, or VAMP7, or non-targeted PLK0.1-puro particles (400 transducing units/well), and cultured as above. After 7–10 days in culture, cells were stimulated before being harvested in lithium dodecyl sulfate sample buffer and analysed by western blotting. Targeted genes/protein IDs and validated sequences for shRNA lentiviral transduction particles were as follows:

NM_011428,SNAP-25:
CCGGCATCAGGACTTTGGTTATGTTCTCGAGAACATAACCAAAGTCCTGATGTTTTT
G

NM_009496,VAMP1:
GTACCGGCATCGTGGTAGTGATTGTAATCTCGAGATTACAATCACTACCACGATGTT
TTTTG;

NM_011515,VAMP7:
CCGGGCACAAGTGGATGAACTGAAACTCGAGTTTCAGTTCATCCACTTGTGCTTTTT
G

Culture of human primary epidermal keratinocytes (hKCs) and human monocyte-derived dendritic cells (hDCs), cytokine antibody array and phosphor-kinase array

Human primary KCs were cultured in the KBM-Gold™ medium with KBM-Gold SingleQuot keratinocytes supplement (Lonza). All the release from hKCs was performed in hydrocortisone-free medium with or without stimulation compound. Human DCs were cultured by *in vitro*

differentiation of $CD14^+$ monocytes (subpopulation of peripheral blood mononuclear cells) using LGM-3™ Lymphocyte Growth Medium + 50 ng/ml GM-CSF + 50 ng/ml IL-4 (Lonza). Cells were maintained in the above medium for 3 d before use. Release was performed in plain LGM-3™ Lymphocyte Growth Medium in the presence of neuropeptides. After release, cell culture supernatant was collected for proteome profiler human cytokine antibody array according manufacturer protocol. Briefly, the array membranes were blocked and then incubated with the sample for overnight at 4 °C. The membranes were washed three times and then incubated in diluted primary antibodies for 1 h at room temperature. After washing, membranes were incubated with diluted HRP-conjugated streptavidin for 1 h at room temperature and membranes were developed using enhanced chemiluminescence reagent. Images were captured, densitometrically scanned. Each cytokine spot was analyzed using Image J software and the average of positive controls of each treatment was set to 100 and all cytokines of the treatment were compared to that. The resultant values from the treatment were calculated relative to the non-treated control to give the fold of increase.

For the kinase array, cells grown in T175 flasks were treated with or without neuropeptides for 8 min and cells were lysed for intracellular phosphor-kinase array according to manufacturer protocol.

Phospho-kinase inhibitors treatment

1 μM selective p38 MAPK inhibitor AL8697, 1 μM selective inhibitor of the α and β isozymes of GSK-3 SB216763 or 1 μM selective JNK1/2/3 inhibitor JNK-IN-8 was applied to the cultured hKCs 1 h prior to and during 24 h incubation with 1 μM BNP or 1 μM SP in serum free and hydrocortisone-free medium to release the cytokines. The supernatant was collected for assay of cytokines using antibody array or ELISA as detailed above.

Reference for Supplementary Methods

1. Malin SA, Davis BM, and Molliver DC. Production of dissociated sensory neuron cultures and considerations for their use in studying neuronal function and plasticity. *Nature protocols.* 2007;2(1):152-60.
2. Meng J, Ovsepian SV, Wang J, Pickering M, Sasse A, Aoki KR, Lawrence GW, and Dolly JO. Activation of TRPV1 mediates calcitonin gene-related peptide release, which excites trigeminal

sensory neurons and is attenuated by a retargeted botulinum toxin with anti-nociceptive potential. *The Journal of neuroscience : the official journal of the Society for Neuroscience.* 2009;29(15):4981-92.

Supplementary Figure Legends

Fig E1. Average *Nppb* value FPKM from contralateral cheeks of top 3 clinical scored Grhl3PAR2/+ mice was not significantly different from that of the WT mice. Statistical analysis demonstrated that *Nppb* FPKM from the non-treated contralateral cheeks of Grhl3PAR2/+ mice did not significantly differ from the values of Vaseline-treated ipsilateral cheeks or non-treated contralateral cheeks in WT mice. However, HDM induced significantly higher *Nppb* FPKM in ipsilateral cheeks compared with contralateral cheeks in Grhl3PAR2/+ mice. Average values of FPKM are plotted. P <0.05 indicates significance.

Fig E2. IL-31-induced *Nppb* mRNA synthesis in cultured mouse DRGs is time- and concentrationdependent.
A, RT-PCR analysis of *Nppb* mRNA synthesis in mDRGs upon treatment with various concentrations of IL-31 for 30 min. **B**, Time course of *Nppb* mRNA synthesis analysed by RT-PCR after treatment with 300 nM IL-31. Data are expressed relative to GAPDH and presented as mean ± S.E.M. n = 3 independent experiments. N.S.>0.05; ** P<0.01; *** P<0.001.

Fig E3. Antibody absorption test confirmed BNP and CGRP antibodies' specificities. A, Confocal images show that fluorescence signal of CGRP in mDRG sections was greatly reduced by pre-absorption of CGRP antibody with α-CGRP peptide. **B**, Immunofluorescence images showing staining pattern in mDRG sections using BNP antibody in comparison with its antigen absorbed control.

Fig E4. Characterization of cultured human primary keratinocytes (hKCs) using immunecytochemical staining. A, Immunofluorescene staining of BNP, NPR1 and NPR2 as well as proliferative keratinocyte marker Keratin 14 (K14) in hKCs. BNP was undetectable. NPR1 and NPR2 showed distinct distribution pattern. **B**, IL-17A immune-signal is detected in cultured hKCs. K14 and vimentin were expressed in all the cultured cells. Immuno-cell markers, CD4 and CD80 antibody staining signal is either absent or negligible.

Fig E5. Immunohistochemical dual staining of NPR1 and NPR2 in skin of human healthy control and patient with AD. Representative fluorescent images at low magnification (left column panels) and high magnification (right column panels) of NPR1 and NPR2 in healthy control (**A**) and AD patient skin (**B**). NPR1 and 2 are highly localized in some cells in the dermis. Specimens were counter-stained with DAPI.

Fig E6. RT-PCR analysis of *Nppb* and NPR1 mRNA levels in skin of IL-31Tg or WT mice. RT-PCR analysis of *Nppb* mRNA (**A**) or NPR1 mRNA (**B**) levels in skin isolated from lesional, nonlesional IL-31Tg or WT mice. Values were normalized to the housekeeping gene GADPH. The results (mean ± S.E.M.) are pooled data from multiple animals. Significant difference is indicated; N.S. P>0.05; * P<0.05; ** P<0.01, *** P<0.001.

Fig E1

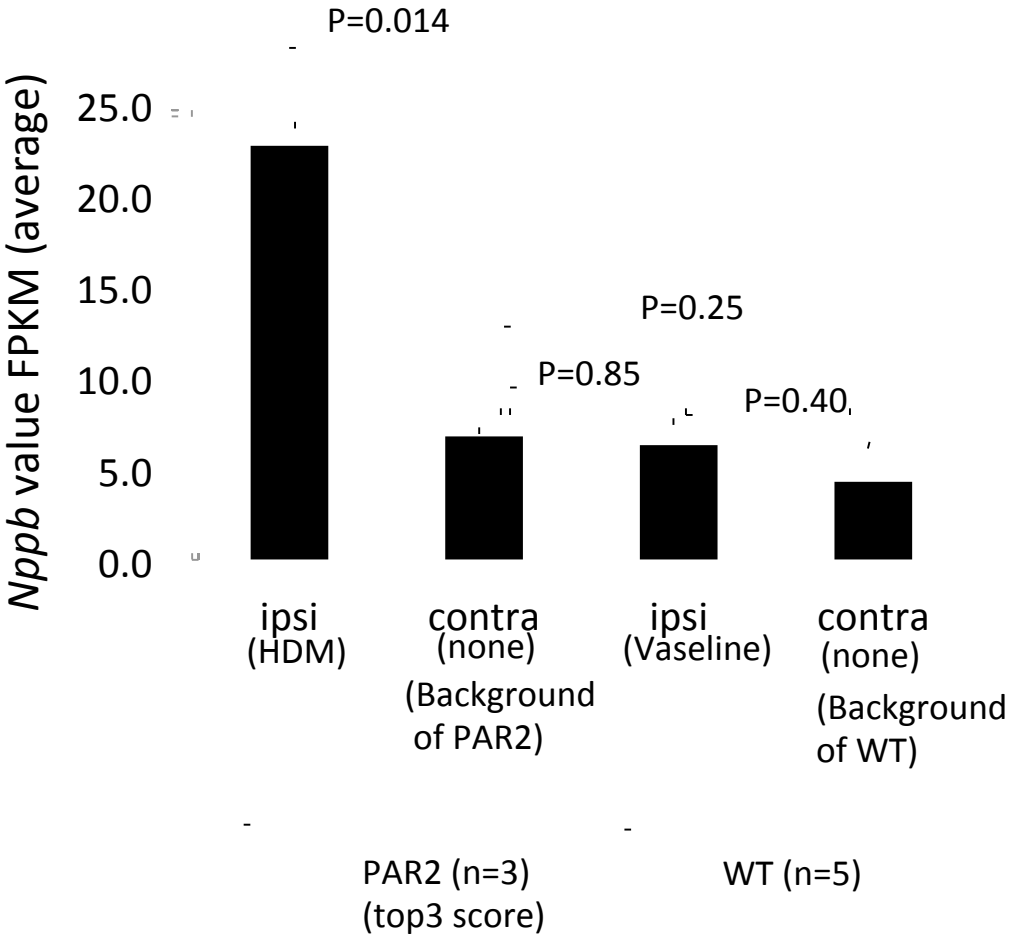


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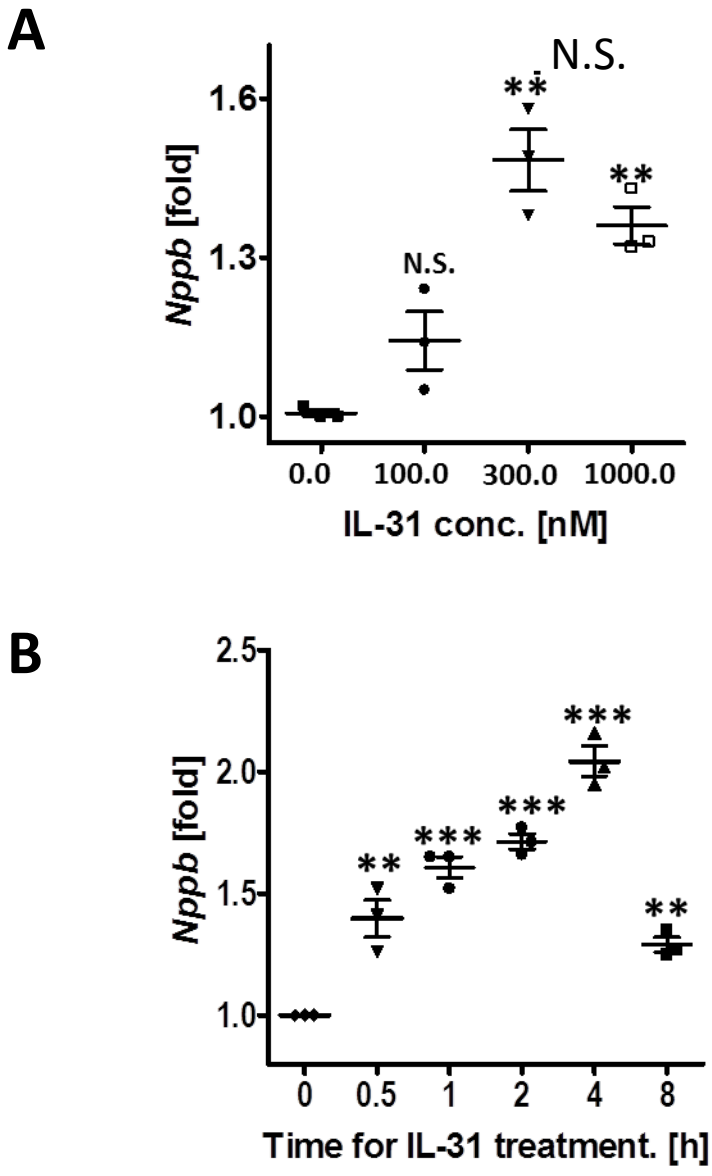
Fig E2



IL-31-induced *Nppb* mRNA synthesis in cultured mouse DRGs is time- and concentration-dependent. A, RT-PCR analysis of *Nppb* mRNA synthesis in mDRGs upon treatment with various concentrations of IL-31 for 30 min. **B**, Time course of *Nppb* mRNA synthesis analysed by RT-PCR after treatment with 300 nM IL-31. Data are expressed relative to GAPDH and presented as mean ± S.E.M. n = 3 independent experiments. N.S. $P>0.05$; ** $P<0.01$; *** $P<0.001$.

Fig E3

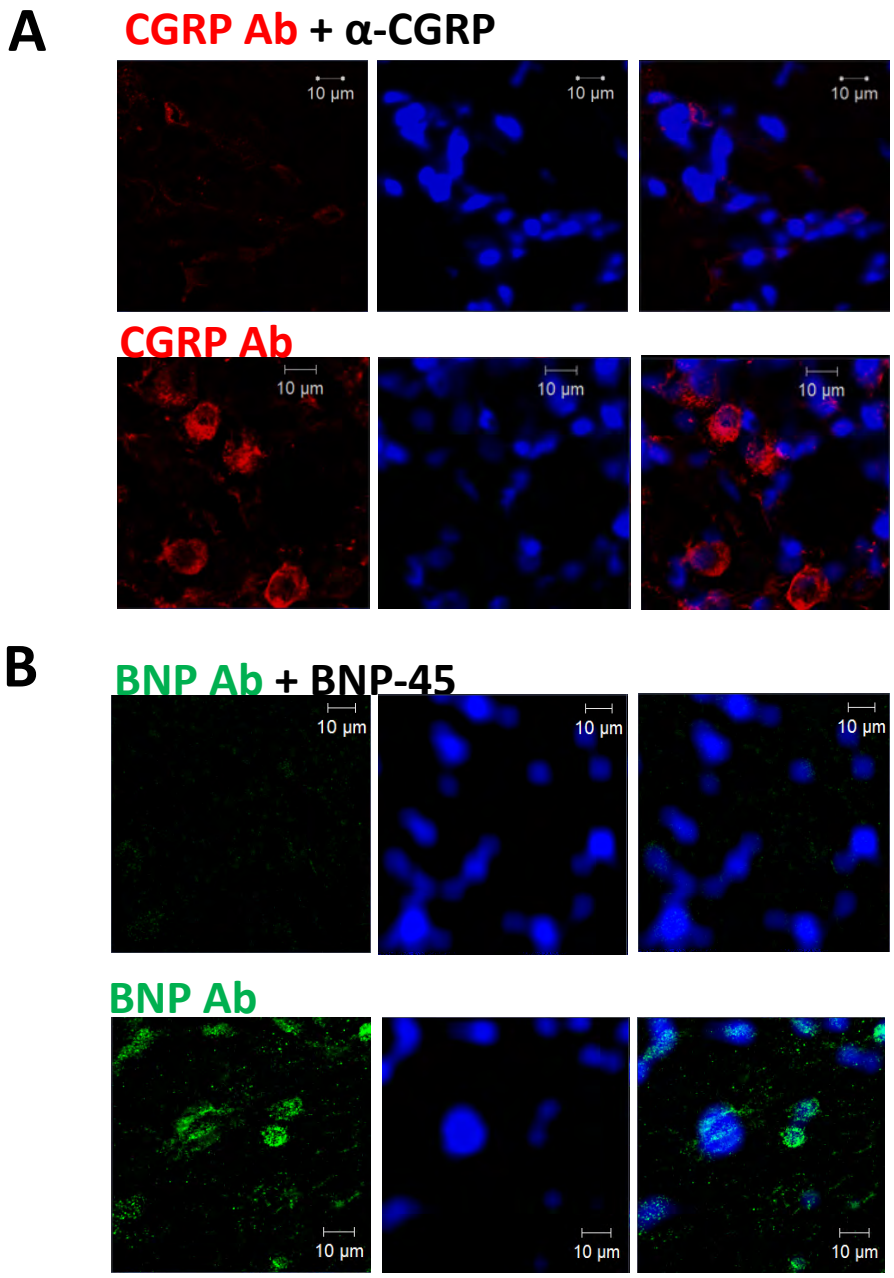


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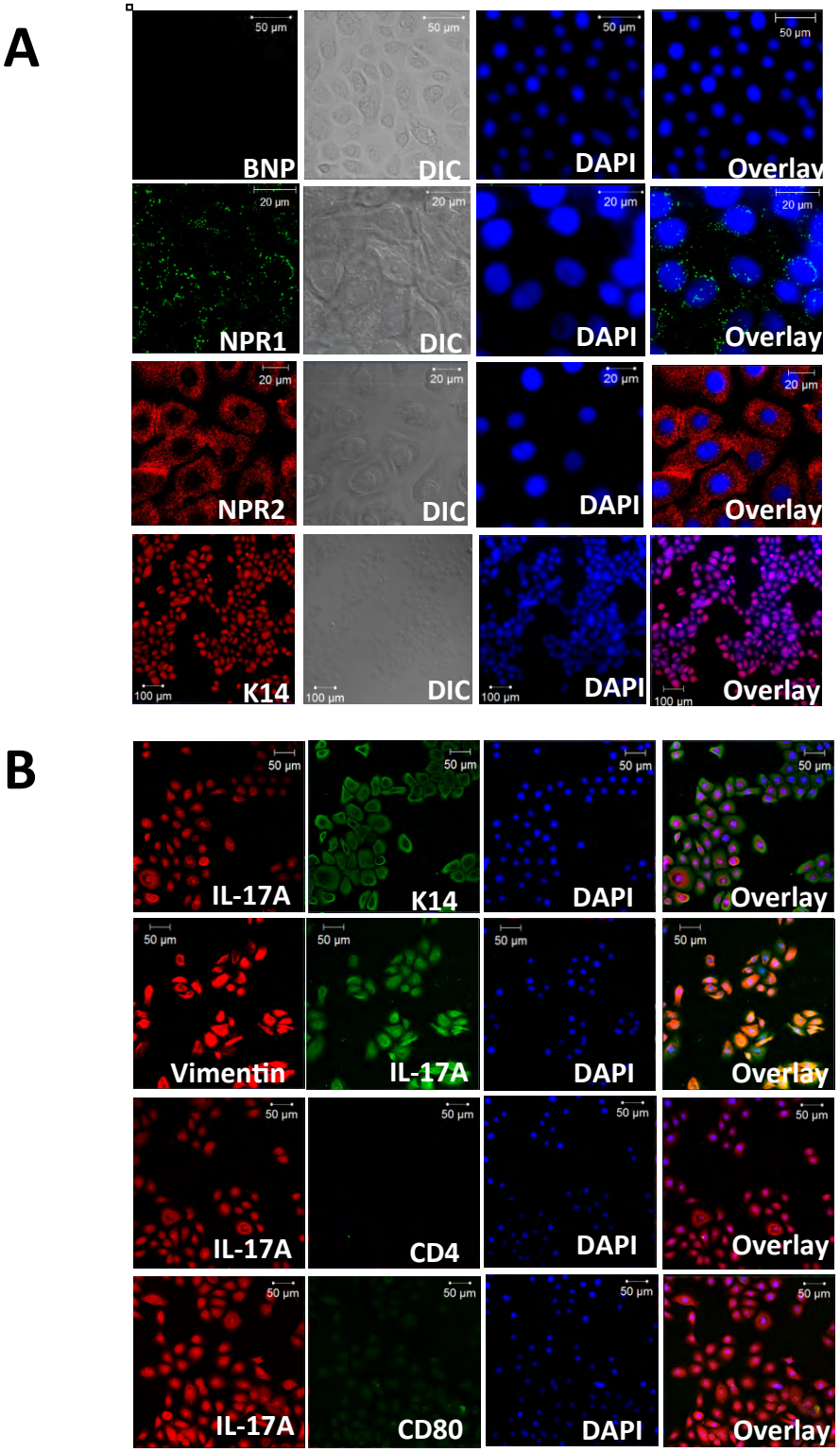
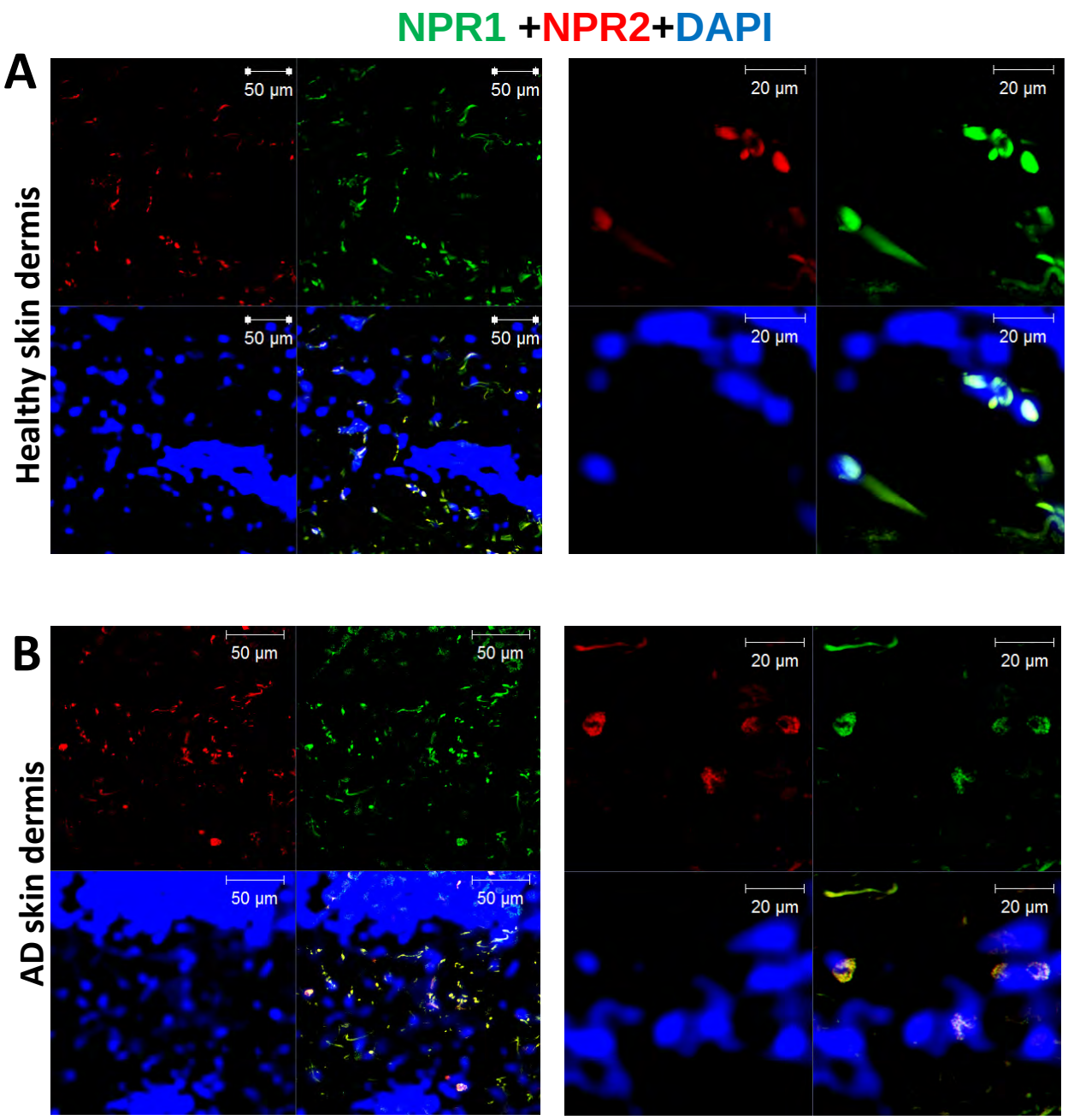
Fig E4



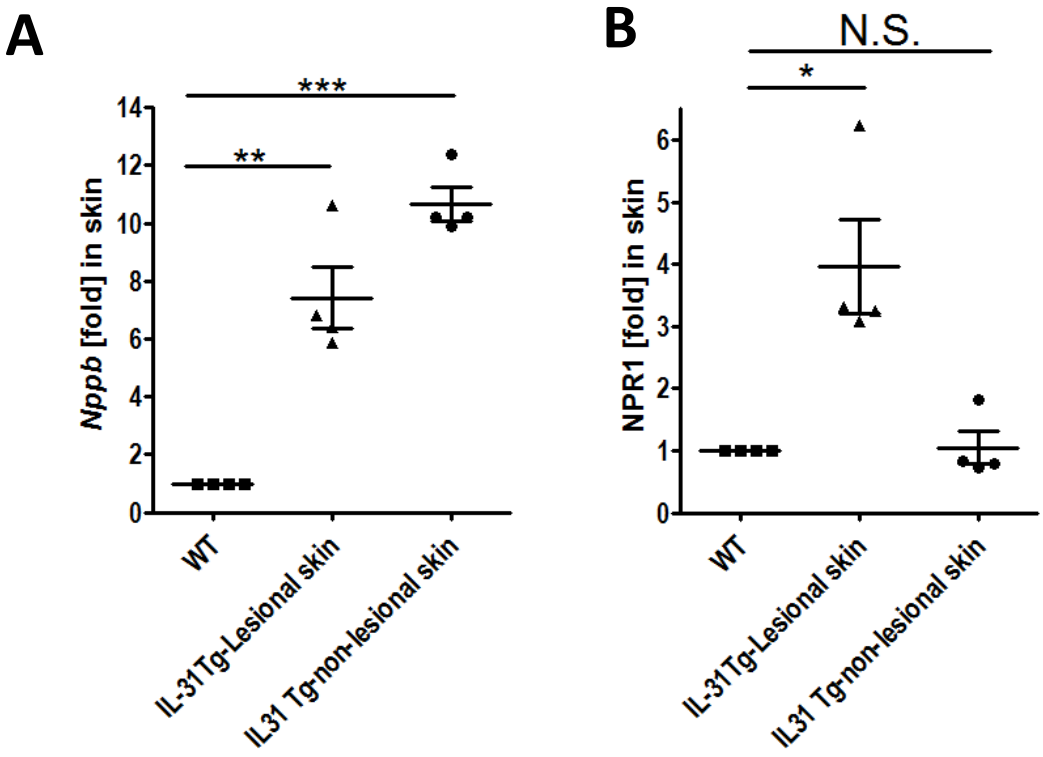
Characterization of cultured human primary keratinocytes (hKCs) using immune-cytochemical staining. A, Immunofluorescene staining of BNP, NPR1 and NPR2 as well as proliferative keratinocyte marker Keratin 14 (K14) in hKCs. BNP was undetectable. NPR1 and NPR2 showed distinct distribution pattern. **B**, IL-17A immune-signal is detected in cultured hKCs. K14 and vimentin were expressed in all the cultured cells. Immuno-cell markers, CD4 and CD80 antibody staining signal is either absent or negligible.

Fig E5



Immunohistochemical dual staining of NPR1 and NPR2 in skin of human healthy control and patient with AD. Representative fluorescent images at low magnification (left column panels) and high magnification (right column panels) of NPR1 and NPR2 in healthy control (**A**) and AD patient skin (**B**). NPR1 and 2 are highly localized in some cells in the dermis. Specimens were counter-stained with DAPI.

Fig E6



RT-PCR analysis of *Nppb* and NPR1 mRNA levels in skin of IL-31Tg or WT mice. RT-PCR analysis of *Nppb* mRNA (**A**) or NPR1 mRNA (**B**) levels in skin isolated from lesional, non-lesional IL-31Tg or WT mice. Values were normalized to the housekeeping gene GADPH. The results (mean ± S.E.M.) are pooled data from multiple animals. Significant difference is indicated; N.S. P>0.05; * P<0.05; ** P<0.01, *** P<0.001.