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Novel Insights into the TRPV3-Mediated Itch in Atopic Dermatitis

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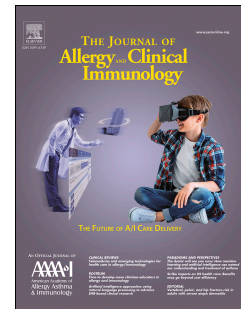
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29 Capsule Summary

30 This study discovered a novel neuro-epidermal BNP-TRPV3-Serpin E1-mediated pathway in
31 severe atopic dermatitis (AD). Serpin E1 was identified as a new itch-inducer. We propose
32 this pathway represents an important target for the treatment of AD.

33

To the Editor:

Chronic pruritus (itch) is a widespread and debilitating condition, associated with dermatological, systemic, neuropathic, or psychogenic disorders. The pathophysiological mechanisms underpinning the transduction and potentiation of this refractory pruritus remain unclear. Current therapeutics are largely ineffective.¹ Thus, we aim to address this gap in knowledge, specifically focusing on clinically relevant intercellular communication in human skin cells, murine models of acute and chronic itch, and samples from human atopic dermatitis (AD) and psoriasis (PS).

In conditions of chronic dermatological itch, such as AD and PS, certain members of the transient receptor potential (TRP) ion channel superfamily play an important role in the propagation of itch signalling. TRPV3 is a calcium-permeable cation channel which is abundantly expressed in epidermal keratinocytes (KCs). TRPV3 detects warm temperatures (> 33°C), is gated by a wide range of chemical stimuli, and plays an essential role in skin homeostasis and repair. Heat-induced activation of TRPV3 stimulates the release of a potent itch-inducer, thymic stromal lymphopoietin (TSLP), from cultured murine KCs.² In mice, intradermal injection of carvacrol, a TRPV3 agonist, elicits scratching behaviours. Gain-of-function mutations in TRPV3 have been confirmed in Olmsted Syndrome, a rare pruritic genodermatosis in humans,³ and induce AD-like inflammation in rodents. TRPV3 is upregulated in the skin of patients with AD.² Despite this, much remains unknown about the clinical relevance of TRPV3-linked pathways in human dermatitis and pruritus.

Herein, real-time PCR (RT-PCR) was used to quantify TRPV3 expression in the skin of the AD-like PAR2 (protease-activated receptor 2)-overexpressing mouse (Grhl3PAR2^{+/+}). TRPV3 transcripts were significantly increased in lesional Grhl3PAR2^{+/+} skin vs. age-matched wild-type (WT) controls (**Fig. 1A**). Moreover, relative TRPV3 levels were significantly higher in lesional Grhl3PAR2^{+/+} compared to nonlesional Grhl3PAR2^{+/+} (**Fig. 1A**), suggesting that TRPV3 expression is associated with the severity of dermatitis.

Human skin samples were then examined to evaluate the clinical relevance of these murine findings. Specimens were collected from patients with AD, both lesional (LAD) and nonlesional (NLAD), from patients with PS, both lesional (LPS) and nonlesional (NLPS), and from healthy controls (HC). All were analysed by RNA-seq, with data indicating the mean change in transcript level relative to HC. In LAD samples, TRPV3 was the only member of the TRPV family to be upregulated, with transcripts showing greater than a 2-fold

increase over HC levels (**Fig. 1B**). Similar to our murine model, this upregulation was absent in NLAD skin (**Fig. 1C**). TRPV3 transcripts were also increased in LPS vs. HC, but not in NLPS vs. HC (**Fig. 1D**). No significant difference was detected in the TRPV3 transcription level in LAD vs. LPS (**Fig. 1E**). Furthermore, immunohistochemical evaluation of LAD samples showed high levels of TRPV3 protein, revealing a striking increase when compared with HC (**Fig. 1F**). Importantly, this upregulation was primarily localised to the KC layer. Similarly, a recent report described enhanced TRPV3 immunosignal in LPS skin.⁴ Together, these findings provide a clear link between TRPV3 expression and lesional atopic dermatitis and psoriasis.

Previous reports from our group demonstrated the importance of the neuropeptide B-type natriuretic peptide (BNP) in IL-31-induced AD skin inflammation,^{5,6} building on earlier evidence of its role in itch transmission.⁷ As both NPR1 and NPR2 are expressed on human keratinocytes,^{5,6} we postulated that BNP may modify TRPV3 expression or activity. First, cultured primary human KCs (phKCs) were stimulated with BNP, Substance P (SP), or Calcitonin Gene-Related Peptide (CGRP) (all at 1 μ M). TRPV3 transcripts were measured by RT-PCR. Only BNP resulted in a significant increase in TRPV3 mRNA levels (**Fig. 1G**), suggesting that this AD-linked neuropeptide may be responsible for the upregulation of TRPV3 evident in our murine and human dermatitis samples.

Calcium imaging was then used to investigate if BNP could affect the functional activity of the TRPV3 cation channel. Here, phKCs were stimulated with one of two concentrations of drofenine (250 μ M or 500 μ M), a specific and potent activator of TRPV3, and the resulting calcium dynamics were recorded. In addition, a subset of phKCs were preincubated with BNP (1 μ M; 4 h) prior to stimulation with the lower concentration of drofenine (250 μ M) (**Fig. 1H**). The check time point (4 h) was chosen based on a previous study showing that in keratinocytes the TRPV3 sensitivity was altered by TGF α treatment at 3-5 h.⁸ Both concentrations of drofenine resulted in notable calcium fluctuations (**Fig. 1I**; representative traces). BNP pre-treatment significantly enhanced the drofenine-induced (250 μ M) calcium flux, as indicated by the area under the curve (AUC) (**Fig. 1J**). When expressed as peak calcium fluctuation ($(F\Delta/F0)_{\max}$) vs. the time at which this peak was reached ($\text{Time}_{\max}$), drofenine-responsive phKCs were found to cluster into two distinct subpopulations (**Fig. 1K and 1L**) (rapid and slow, with rapid responders found in the grey shaded area, **Fig. 1L**). Importantly, preincubation with BNP resulted in a striking increase in the percentage of rapid responders following drofenine (250 μ M) application (**Fig. 1K and**

Fig. 1L). It is postulated that this subset of phKCs express high levels of TRPV3. A separate experiment found that stimulation with BNP alone resulted in calcium fluctuations in just a small population (0.8%) of phKCs, with responders displaying low amplitude transients (**Fig. 1M**). Together, these data demonstrate that BNP can increase TRPV3-associated calcium responses, perhaps through sensitisation or upregulation of the TRPV3 channel on the cell surface.

Although keratinocyte-derived mediators are known to facilitate dermatitis, little is known about TRPV3-induced intercellular communication. To address this, phKC-expressed TRPV3 was activated with drofenine (500 μ M) and supernatant was analysed by cytokine array. Both Serpin E1 and TGF α were found to be significantly increased, $\sim$ 1.8 and $\sim$ 2 fold, respectively (**Fig. 2A**). To confirm that drofenine induced pharmacological effects is mediated by TRPV3, we performed the experiment of knockdown (KD) of TRPV3 using shRNA lentivirus. The latter reduced TRPV3 expression by $\sim$ 75% in phKCs compared to the non-targeted control virus treated cells (see **Fig. E1** in this article's Online Repository). KD of TRPV3 reduced drofenine-elicited Serpin E1 release (**Fig. 2B**) and the calcium transients, as well as the number of cells responding to drofenine (**Fig. 2C**), indicating that the drofenine-induced responses in phKCs were mediated by TRPV3. In addition, Serpin E1 release was found to be facilitated by a specific v-SNARE protein, namely vesicle associated membrane protein isoform 3 (VAMP3). Following targeted shRNA-mediated knockdown of VAMP3 in phKCs ($\sim$ 90%) (see **Fig. E2A** in this article's Online Repository), drofenine-induced Serpin E1 release was reduced by $\sim$ 62% (see **Fig. E2B** in this article's Online Repository). In contrast, TGF α release was not significantly altered (see **Fig. E2B** in this article's Online Repository), suggesting that Serpin E1, but not TGF α , is released via exocytotic vesicles. These data reveal a previously unknown intercellular pathway, mediated in part by VAMP3-dependent exocytosis.

To investigate whether these TRPV3-linked mediators are involved in human dermatitis, we analysed Serpin E1 and TGF α transcripts in AD and PS skin by RNA-seq. Interestingly, Serpin E1 was found to be significantly upregulated in LAD vs. HC, showing a striking 5.3-fold increase, whereas, TGF α levels were not significantly different from HC (**Fig. 2D**). Conversely, TGF α , but not Serpin E1, was significantly increased in LPS compared to both HC and NLPS (**Fig. 2E**). Notably, a 4-fold higher expression of Serpin E1 was observed in LAD vs. LPS (**Fig. 2F**). Immunohistochemical evaluation of HC and LAD skin revealed high levels of Serpin E1 (red) protein in LAD, but not HC skin (**Fig. 2G**, left

panel). The mean fluorescence intensity of Serpin E1 in LAD is greater than that of HC, revealed by analysis of regions of interest encompassing the epidermis of the skin (**Fig. 2G**, right panel). Importantly, Serpin E1 expression was primarily localised to the KC layer. Thus, TRPV3-linked mediators Serpin E1 and TGF α are differentially involved in human AD and PS, representing important disease-specific pathways. Together, these results strengthen the notion that TRPV3 and its downstream mediators are involved in human dermatitis.

Serpin E1 is primarily classified as an inhibitor of fibrinolysis. The findings presented herein describe a correlation between Serpin E1 expression and dermatitis. However, whether Serpin E1 could act as a pruritogen (itch-inducer) remained unknown. Firstly, the effect of Serpin E1 on mouse dorsal root ganglionic neurons (mDRGs) was analysed by using calcium imaging. A total of ~5.3% mDRGs were activated by Serpin E1, showing that Serpin E1 can directly activate a subpopulation of sensory neurons (**Fig. 2H**). In a mouse cheek model of itch, intradermal injection of Serpin E1 elicited significant site-directed scratching behaviour (**Fig. 2I**), with average scratching bouts peaking at 15 minutes (**Fig. 2J**). As a support to Serpin E1 effect on cheek sensory transmission, uPA receptor (U-PAR), known to bind Serpin E1,⁹ is present in cultured mouse trigeminal ganglionic neurons (TGNs) (see **Fig. E3** in this article's Online Repository). In contrast, Serpin E1 did not induce wiping (**Fig. 2K**), a behaviour that reflects pain associated responses, suggesting that it may represent a novel and specific itch-inducer. Of note, a selective, orally active inhibitor of Serpin E1 (TM5275) attenuated Serpin E1-elicited cheek scratching (**Fig. 2L**). Moreover, in a prolonged AD-like MC903-induced chronic pruritus mouse model which requires 7 days and longer for pruritus to be established, TM5275 also inhibited MC903-evoked itch behaviours at day 9 (**Fig. 2M**). Thus, Serpin E1 represents a novel pruritogen in mice and may promote dermatitis-associated pruritus in humans.

Overall, this study provides a clear link between TRPV3 and dermatitis (**Fig. 2N**). In AD conditions, IL-31 induces BNP synthesis and release from sensory neurons.⁵ BNP subsequently binds to NPR1 on KCs to upregulate TRPV3 transcripts. This could increase the surface expression of TRPV3, resulting in heightened TRPV3 activity and increased Serpin E1 release. Serpin E1 activates sensory fibers in the skin and promotes itch transduction. Our work reveals a disease-relevant neuro-epidermal pathway for TRPV3 upregulation and sensitisation. It also highlights the role of TRPV3-linked mediators in human dermatitis and pruritus, with Serpin E1 representing a novel itch-inducer (**Fig. 2N**). Based on these results,

we believe that targeting TRPV3 signalling will prove beneficial for the treatment of chronic
itch conditions.

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Figure legends

Figure 1. TRPV3 expression in skin is correlated with disease severity and BNP enhances TRPV3 activity. **A**, Fold change (FCH) of TRPV3 mRNA expression in lesional and nonlesional Grhl3PAR2⁺ skin vs. WT. **B**, FCH of TRPV family expression in human skin of LAD vs. HC. **C-D**, FCH of TRPV3 expression in human LAD and NLAD (**C**); LPS and NLPS (**D**). All relative to HC. **E**, Comparison of TRPV3 transcript level in LPS vs. LAD. **F**, Representative immuno-staining of TRPV3 (green) in human LAD and HC skin. Scale = 20 μ m. **G**, FCH of TRPV3 mRNA expression vs. control in phKCs. **H-L**, Effect of BNP pretreatment on TRPV3 channel activity in phKCs; Experimental design (**H**); Representative traces (Time vs. $F\Delta/F_0$) following Drofenine (Drof.) stimulation ($\pm$ BNP pretreatment) (**I**); Area under the curve (AUC) analysis (**J**); Temporal subpopulation analysis (**K**); Time_{max} vs. ($F\Delta/F_0$)_{max}, each dot representing the response profile of a single phKC (**L**). **M**, Representative traces following BNP stimulation and responder analysis. Data in **A**, **G**, **J** are mean $\pm$ SEM ($n \geq 3$); ns, not significant, * $P < 0.05$, ** $P < 0.01$, and *** $P < .001$; student t-test (**A**, **G**) or ANOVA (**J**).

Figure 2. Serpin E1 is released from keratinocytes following TRPV3 activation, is upregulated in LAD, promotes acute itch-like behaviour in mice and is involved in chronic scratching in the MC903-induced AD model. **A**, FCH of mediator release following Drof. (500 μ M; 1h) application vs. vehicle-treated phKCs. **B**, **C**, Effect of TRPV3 knockdown on Drof.-evoked Serpin E1 release (**B**); and calcium transients (**C**). **D-F**, FCH of

Serpin E1 and TGF α expression in human LAD vs. HC or NLAD (**D**); LPS vs. HC or NLPS (**E**); and LAD vs. LPS (**F**). **G**, Immuno-staining of Serpin E1 (red) in LAD and HC skin, and analysis of epidermal fluorescence intensity. Scale = 100 μ m. **H**, Traces following Serpin E1 stimulation of mDRGs and responder analysis. **I-K**, Effect of intradermal injection of vehicle or Serpin E1 in mice; Scratching bouts (**I**); Time-course of itch-like response (**J**); Wiping behaviour (**K**). **L-M**, Effect of vehicle or TM5275 on Serpin E1-elicited scratching (**L**) and MC903-induced chronic itch (**M**). **N**, A model for TRPV3-mediated itch and sensitisation in AD. Data in **A**, **B**, **I-M** are means $\pm$ SEMs ($n \geq 3$); ns, not significant, * $P < 0.05$, ** $P < 0.01$, and *** $P < .001$; student t-test.

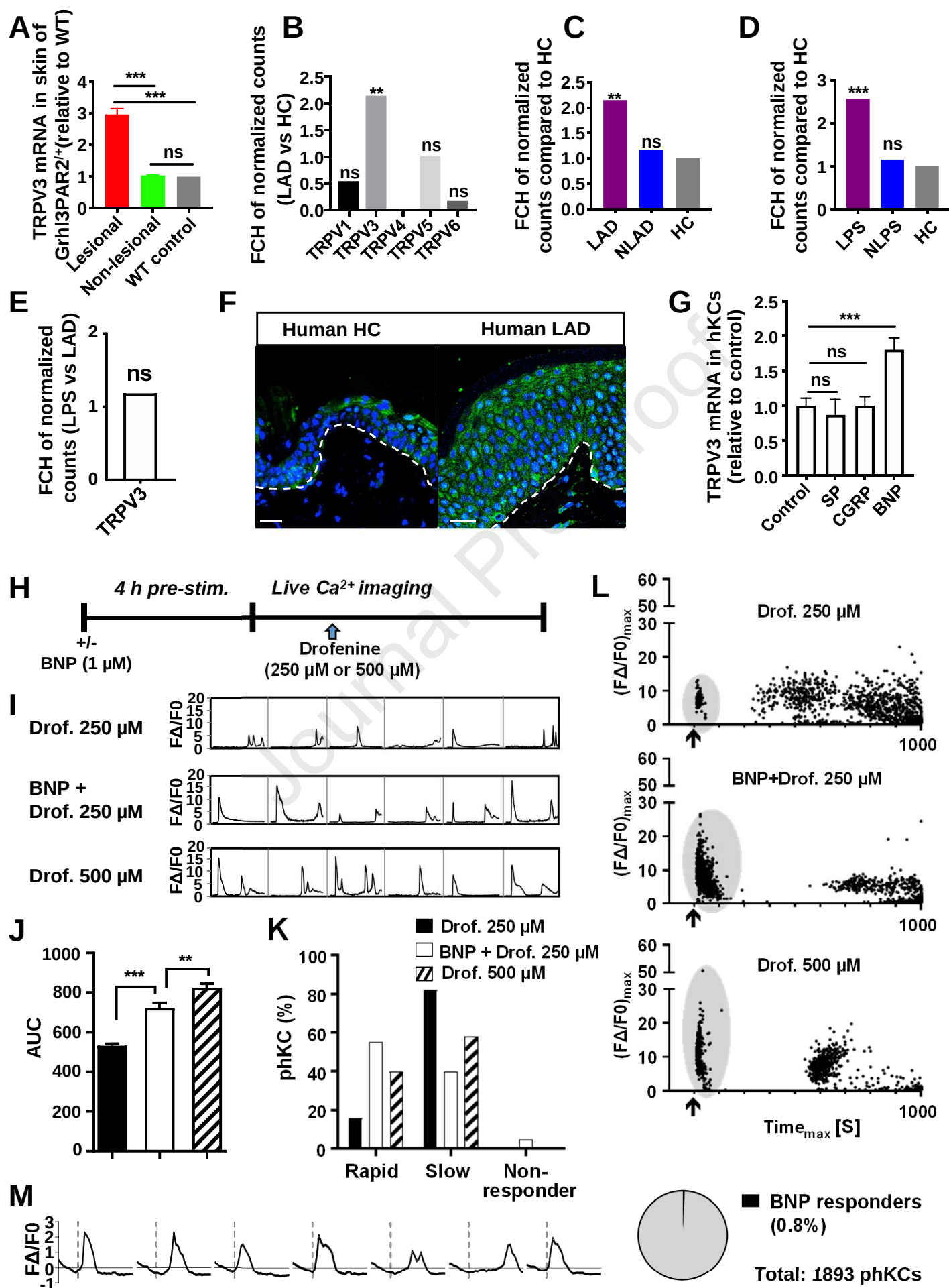
Fig. 1

Fig. 2