

Histological Characterization of Cutaneous Nerve Fibers Containing Gastrin-Releasing Peptide in NC/Nga Mice: An Atopic Dermatitis Model

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TO THE EDITOR

Histological observations have shown that unmyelinated nerve fibers originating from dorsal root ganglions are present at higher densities in the epidermis in several skin diseases such as atopic dermatitis (AD), suggesting that hyperinnervation is partly responsible for abnormal itch perception (Steinhoff *et al.*, 2003b; Ikoma *et al.*, 2006; Paus *et al.*, 2006). However, it has been difficult to histologically identify itch-specific fibers in the skin because there have been no itch-specific markers available. Recently, a study of gastrin-releasing peptide receptor (GRPR)-deficient mice has shown that the GRP/GRPR system is specifically involved in itch but not pain perception (Sun and Chen, 2007). Therefore, this study was conducted to immunohistochemically identify and characterize cutaneous nerve fibers containing GRP in NC/Nga mice using an AD model (Matsuda *et al.*, 1997).

Male NC/Nga mice (SLC Japan, Shizuoka, Japan) were maintained in specific pathogen-free (SPF) or air-uncontrolled conventional (Cnv) conditions. We used Cnv-NC/Nga mice of 8–12 weeks of age that manifested mild to severe skin lesions similar to AD, and SPF-NC/Nga mice with no AD as a control. All animal procedures were approved by the institutional Animal Care and Use Committee at Juntendo University Graduate School of Medicine. The immunohistochemical protocol for cutaneous nerve fibers was performed as described previously

(Tominaga *et al.*, 2007a). Cryosections (thickness 20 μ m) from dorsal neck skin were double-stained with anti-GRP and anti-product gene protein 9.5 (PGP9.5). The sections were observed with a confocal laser-scanning microscope DMIRE2 (Leica, Wetzlar, Germany).

In Cnv-NC/Nga mice, GRP⁺ PGP9.5⁺ fibers were observed in both the epidermis (Figure 1a) and the dermis (Figure 1b). They were mainly found in the dermis of SPF-NC/Nga mice, especially at the dermoepidermal junction (Figure 1c). Although the number of nerve fibers was significantly high in the epidermis and the dermis of Cnv-NC/Nga mice compared with that in SPF-NC/Nga mice (Figure 1d), the percentage of GRP⁺ fibers in PGP9.5⁺ fibers was approximately 2.4-fold higher in the epidermis of Cnv-NC/Nga mice, although this was not the case in the dermis (Figure 1e).

To further examine whether GRP⁺ fibers contain neuropeptides such as substance P (SP) and calcitonin-gene-related peptide (CGRP), which are involved in neurogenic inflammation and/or hypersensitivity of itch sensation (Stander and Steinhoff, 2002; Steinhoff *et al.*, 2003b), skin samples of NC/Nga mice were double-stained with antibodies against GRP, SP, and CGRP. In the epidermis and the dermis of Cnv-NC/Nga mice, almost all GRP⁺ fibers contained SP (Figure 1f) or CGRP (Figure 1i), as did those in the dermis of SPF-NC/Nga mice (Figure 1g and j). Many CGRP⁺ fibers in the skin of Cnv-NC/Nga (Supplementary Figure

S1a) and SPF-NC/Nga (Supplementary Figure S1b) mice also contained SP. To estimate the percentage of GRP⁺ SP⁺ or GRP⁺ CGRP⁺ fibers in PGP9.5⁺ fibers, the skin samples of NC/Nga mice were triple-stained with antibodies against GRP, PGP9.5, SP, and CGRP (Figure 1h and k). In comparison with SPF-NC/Nga mice, the percentages of GRP⁺ SP⁺ and GRP⁺ CGRP⁺ fibers in PGP9.5⁺ fibers were approximately 3.5-fold and 3.8-fold higher in the epidermis of Cnv-NC/Nga mice, respectively, although this was not the case in the dermis. GRP⁺ SP⁺ or GRP⁺ CGRP⁺ fibers were also observed in the skin of these mice (Supplementary Table S1), but they remained as uncharacterized nerve fibers in this study.

The cutaneous GRP⁺ fibers in Cnv-NC/Nga mice were immunohistochemically characterized using antibodies against itch-related molecules such as transient receptor potential vanilloid 1 (TRPV1), protease-activated receptor 2 (PAR-2), mu-opioid receptor (MOR), and tropomyosin-receptor-kinase A (Paus *et al.*, 2006). The GRP⁺ fibers expressed TRPV1, but some GRP⁺ TRPV1⁺ fibers were also observed in the atopic skin (Figure 2a). The GRP⁺ fibers also expressed PAR-2, MOR, or tropomyosin-receptor-kinase A in the atopic skin (Figure 2b–d).

In this study, we found that GRP⁺ fibers were present in mouse skin, and that the percentage of GRP⁺ fibers in PGP9.5⁺ fibers was markedly high only in the epidermis of atopic NC/Nga mice. Small- to medium-sized adult dorsal root ganglions neurons express GRP, and its receptor is present in the superficial dorsal horn.

Abbreviations: AD, atopic dermatitis; CGRP, calcitonin-gene-related peptide; GRP, gastrin-releasing peptide; MOR, mu-opioid receptor; PAR-2, protease-activated receptor 2; PGP9.5, product gene protein 9.5; SP, substance P; TRPV1, transient receptor potential vanilloid 1

Intrathecal injection of GRP₁₈₋₂₇ into wild-type mice induced scratching behavior, but did not affect pain sensitivity (Sun and Chen, 2007). Therefore, it is possible that GRP⁺ fibers are itch-specific fibers in the skin. The increase of epidermal GRP⁺ fiber density in Cnv-NC/Nga mice is probably caused by keratinocyte-derived nerve growth factor (Tominaga et al., 2007a). This is also supported by our data showing that GRP⁺ fibers expressed tropomyosin-receptor-kinase A, a receptor for nerve growth factor. In addition, the GRP⁺ fibers in the skin of NC/Nga mice contained SP or CGRP, and the nerve densities were significantly high in the epidermis of Cnv-NC/Nga mice compared with those in SPF-NC/Nga mice.

It is suggested that SP released from sensory nerve fibers indirectly induce pruritus via the activation of non-neuronal skin cells expressing cognate receptors (Paus et al., 2006). Although the role of sensory nerve-derived CGRP in the induction of pruritus remains controversial, it has also been shown to modulate itch sensation and inflammation (Bíró et al., 2007). Moreover, nerve growth factor is known to upregulate these neuropeptides in adult rat sensory neurons (Verge et al., 1995). Therefore, these findings raise the possibility that GRP⁺ fibers modulate the neuronal/non-neuronal interaction network to initiate and augment itch. This is strongly supported by our data showing that GRP was localized in cutaneous

TRPV1⁺ fibers, which respond to thermal and chemical stimuli (Bíró et al., 2007).

Our immunohistochemical data for Cnv-NC/Nga mice showed that the GRP⁺ fibers expressed PAR-2, which is involved in histamine-independent itch (Steinhoff et al., 2003a; Shimada et al., 2006). Recent studies have suggested a possible role of peripheral opioid systems in pruritus (Inan and Cowan, 2004; Bigliardi et al., 2007; Tominaga et al., 2007b). We also obtained noteworthy data showing that GRP was localized in some MOR⁺ fibers of mouse skin. This may provide direct evidence for the involvement of neuronal MOR in the induction and modulation of peripheral itch. Although

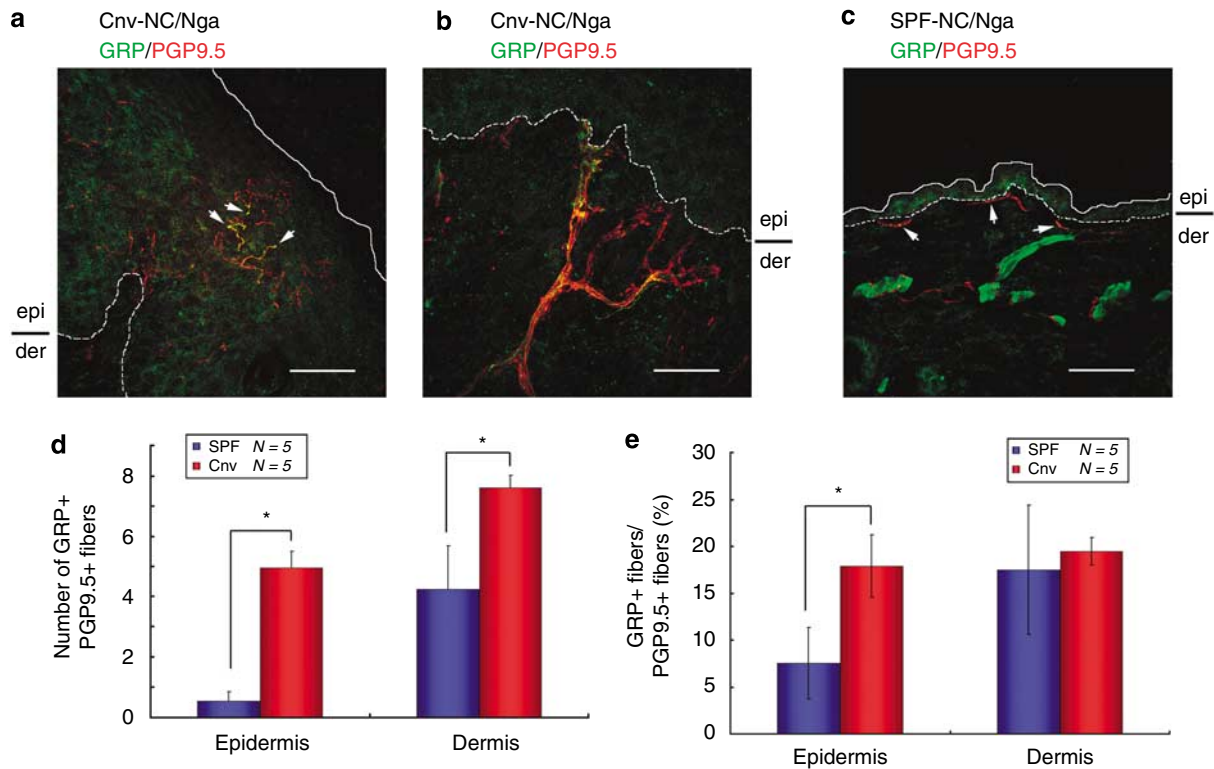


Figure 1. Immunohistochemical analyses of GRP⁺ fibers in the skin of NC/Nga mice. (a–c) Double-labeling of GRP (green) and PGP9.5 (red) in the skin of NC/Nga mice. A small proportion of PGP9.5⁺ fibers expressed GRP in both the epidermis (arrows in a) and the dermis (b) of Cnv-NC/Nga mice. GRP⁺ PGP9.5⁺ fibers were mainly observed in the dermis of SPF-NC/Nga mice (arrows), but they were occasionally present in the epidermis (c). (d) The number of GRP⁺ PGP9.5⁺ fibers was significantly high in both the epidermis and the dermis of Cnv-NC/Nga mice compared with that in SPF-NC/Nga mice. **P* < 0.05. (e) The percentage of GRP⁺ fibers in PGP9.5⁺ fibers was significantly high in the epidermis of Cnv-NC/Nga mice compared with that in SPF-NC/Nga mice, but was similar in the dermis of these mice. (f, g) Double-labeling of GRP (green) and SP (red) in the skin of NC/Nga mice. Almost all GRP⁺ fibers contained SP in both the epidermis and the dermis of Cnv-NC/Nga mice (arrows in f). They were mainly observed in the dermis of SPF-NC/Nga mice (arrows in g). (h) The percentage of GRP⁺ SP⁺ fibers in PGP9.5⁺ fibers was significantly high in the epidermis of Cnv-NC/Nga mice compared with that in SPF-NC/Nga mice, but was similar in the dermis of these mice. **P* < 0.05. (i, j) Double-labeling of GRP (green) and CGRP (red) in the skin of NC/Nga mice. Many GRP⁺ CGRP⁺ fibers (arrows) were observed in both the epidermis and the dermis of Cnv-NC/Nga mice (i). Some GRP⁺ CGRP⁺ fibers were also found in the skin (an arrowhead in i). In SPF-NC/Nga mice, GRP⁺ CGRP⁺ fibers (arrows) were mainly observed in the dermis (j). (k) The percentage of GRP⁺ CGRP⁺ fibers in PGP9.5⁺ fibers was significantly high only in the epidermis of Cnv-NC/Nga mice compared with that in SPF-NC/Nga mice. **P* < 0.05. In panels a, b, c, f, g, i, and j, yellow areas are double-labeled, and white and broken lines indicate the skin surface and the border between epidermis (epi) and dermis (der), respectively. Scale bars = 47.62 μm (a, b, c, f, g, i, and j).

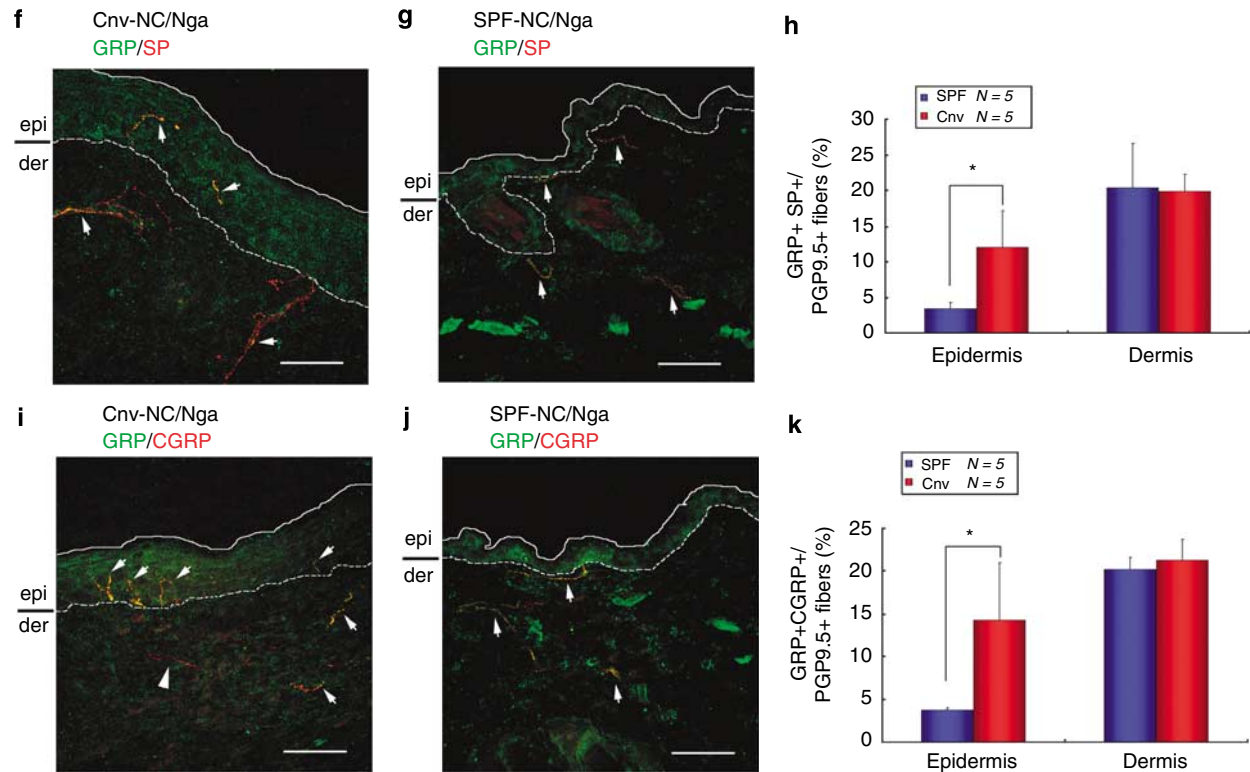


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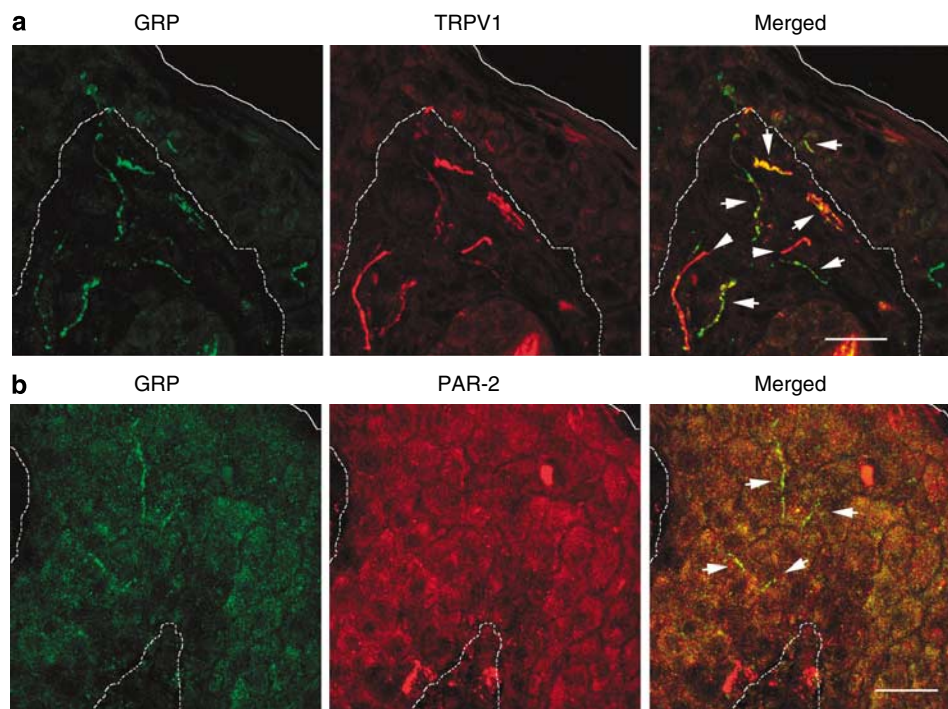


Figure 2. Immunohistochemical expression of itch-related molecules in cutaneous GRP⁺ fibers of atopic NC/Nga mice. Double-labeling of GRP with TRPV1 (**a**), PAR-2 (**b**), MOR (**c**), and TrkA (**d**) in the skin of NC/Nga atopic mice. (**a**) GRP (green) was localized in TRPV1⁺ (red) fibers in the skin (arrows). Some GRP⁺TRPV1⁺ fibers were also found in the skin (arrowheads). (**b**) GRP⁺ (green) fibers expressed PAR-2 (red) in the epidermis (arrows). (**c**) GRP (green) was present in a small proportion of MOR⁺ (red) fibers of the epidermis (inset) and the dermis. (**d**) GRP (green) was expressed in TrkA⁺ (red) fibers of the epidermis (an arrow), and GRP⁺TrkA⁺ fibers were also observed (arrowheads). Yellow areas in each panel are double-labeled. White and broken lines indicate the skin surface and the border between epidermis (epi) and dermis (der), respectively. Scale bars = 23.81 μ m.

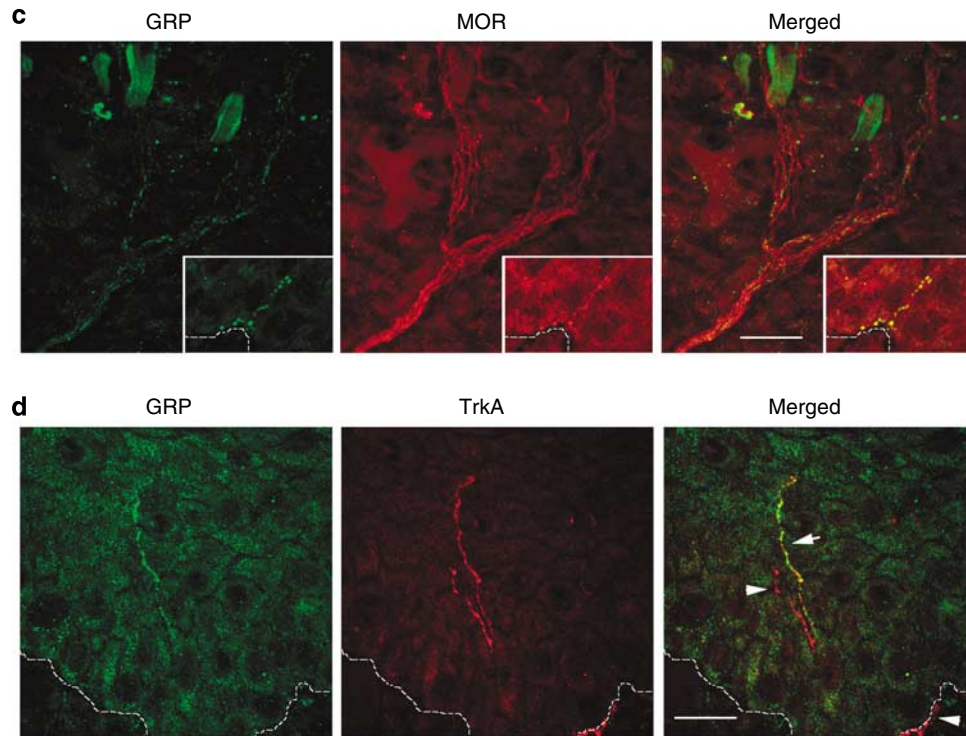


Figure 2. Continued.

we could not use immunohistochemical analysis to confirm whether GRP⁺ fibers express histamine receptors in this study because of the lack of antihistamine-receptor antibodies, the GRP/GRPR system in the spinal cord is strongly related to itch elicited by histamine-independent mechanisms such as the PAR-2 agonist and chloroquine (Sun and Chen, 2007). Taken together, these findings suggest the possibility that cutaneous GRP⁺ fibers respond to other pruritogens rather than histamine.

The GRP/GRPR system has also been documented to be expressed in normal human skin cells such as nerves, vessels, and keratinocytes, suggesting that it is important for modulation of various cell functions in the epidermis (Staniek *et al.*, 1996). Thus, future studies will be necessary to examine the expression of GRPR in the skin with allergic inflammation such as AD.

CONFLICTS 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 <http://www.nature.com/jid>

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