

Expression of Nerve Growth Factor in Itchy Skins of Atopic NC/NgaTnd Mice

Akane TANAKA¹⁾ and Hiroshi MATSUDA^{1)*}

¹⁾Laboratory of Veterinary Molecular Pathology and Therapeutics, Division of Animal Life Science, Graduate School, Institute of Symbiotic Science and Technology, Tokyo University of Agriculture and Technology, Fuchu, Tokyo 183–8509, Japan

(Received 24 March 2005/Accepted 20 May 2005)

ABSTRACT. Although the possible involvement of neurotrophic factors in itchy skins of atopic dermatitis has been predicted, the exact mechanism by which itch is induced remains unclear. Since nerve growth factor (NGF) has crucial effects on development and functions of sensory nerves, we determined production of NGF and extension of nerve fibers in skins of NC/NgaTnd mice with or without atopic dermatitis. NC/NgaTnd mice spontaneously develop atopic dermatitis-like skin lesions when they are raised in air-unregulated conventional circumstances. We quantified scratching behavior of NC/NgaTnd mice during the development of dermatitis using a novel analytical system and compared to clinical skin severity scores. A significant correlation between the severity of dermatitis and the increase in the number of scratches was identified, indicating that scratching behavior may associate with clinical skin conditions. NGF contents in the skin lesions of conventional NC/NgaTnd mice were significantly higher than those in SPF mice. Positive reactions for NGF were observed in keratinocytes and fibroblasts in affected skins of conventional NC/NgaTnd mice. Immunohistochemical analysis showed the extension of protein gene product 9.5-positive nerve fibers from the dermis toward the epidermis at the skin lesions. These results suggest that sensory nerves induced by NGF may contribute to development of itch, and that NGF produced at the affected site may provide abnormal skin sensitivity in atopic dermatitis.

KEY WORDS: itch, keratinocyte, mouse model, nerve fiber, neurotrophic factor.

J. Vet. Med. Sci. 67(9): 915–919, 2005

Itch is one of major clinical symptoms in atopic dermatitis [19]; therefore, reduction of itch is an important purpose to succeed in treatment of atopic dermatitis. Itch has been defined as an unpleasant skin sensation that provokes an urge to scratch [24]. Since both pain and itch are transmitted in the unmyelinated C-fibers, it was previously believed that itch was a kind of weak pain associated with a low frequency of discharge in nociceptive fibers [20]. Recent findings have revealed a specific pathway for itch and indicated its neurophysiological basis [1, 15]. Because the standard method to evaluate and quantify itch has not been established, we still know very few of the pathophysiological details of itch in various conditions including immunological and/or inflammatory dermatitis.

NC/NgaTnd mice, a model of human atopic dermatitis, spontaneously manifest atopic dermatitis-like skin lesions with marked elevation in plasma levels of total IgE when they are raised in air-unregulated conventional circumstances [6–8]. Itch is one of the most common symptoms that develop and exacerbate atopic dermatitis not only in human subjects but also in NC/NgaTnd mice [9]. Although various immunological and pathological approaches have taken place, the exact mechanism by which itch is induced in atopic subjects has been unclear.

Nerve growth factor (NGF) is a neurotrophic cytokine mandatory for the development and functions of peripheral and central nervous systems [4, 10, 17]. Further, NGF

exhibits various effects in periphery at an inflammatory condition by regulating sensory neurons and immune systems [2, 5, 13, 23]. Recently, neurogenic inflammation has provided a new point of view in understanding the pathogenesis of allergic disorders including atopic dermatitis [3, 12]. Plasma concentrations of NGF in patients with atopic dermatitis are significantly increased compared to those in control subjects, suggesting that NGF may play a crucial role in modulation of allergic responses [18]. An increase in expression of NGF was also found in skin lesions of psoriasis [14]. Because NGF stimulates extension and function of peripheral sensory neurons, NGF may directly or indirectly participate in provocation of itch in sensitive atopic skins. Further, NGF may contribute to constitution of skin hypersensitivity.

In the current study, we found that NGF contents in skins of conventional NC/NgaTnd mice with moderate to severe dermatitis were significantly higher than SPF mice. Extension of protein gene product (PGP) 9.5-positive nerves was obvious in affected sites of NC/NgaTnd mice with dermatitis. Further, major populations of cells expressing NGF in skin lesions were proliferating keratinocytes in the epidermis and fibroblasts in the dermis. These findings emphasize a possibility that over-production of NGF from affected keratinocytes and fibroblasts may contribute to extension of sensory nerves and may coordinately induce abnormal sensitivity of skins leading to itch in atopic dermatitis.

MATERIALS AND METHODS

Animals: NC/NgaTnd mice were maintained in air-uncontrolled conventional circumstances and provided with

* CORRESPONDENCE TO: MATSUDA, H., Laboratory of Veterinary Molecular Pathology and Therapeutics, Division of Animal Life Science, Graduate School, Institute of Symbiotic Science and Technology, Tokyo University of Agriculture and Technology, 3–5–8 Saiwai-cho, Fuchu, Tokyo 183–8509, Japan.

standard food and water *ad libitum* as described previously [6–8]. In this study, we used conventional NC/NgaTnd mice at the age of 8–17 wk that manifested mild to severe skin lesions similar to human atopic dermatitis. NC/NgaTnd mice maintained in the specific pathogen free (SPF) condition were used as a control with no atopic dermatitis. All experiments with animals were complied with the standards in the guidelines of the University Animal Care and Use Committee in Tokyo University of Agriculture and Technology.

Clinical skin severity scores and scratching analysis: Clinical features of dermatitis were scored at each point according to the criteria described previously [4, 10, 17]. Scratching behavior of the mice was recorded and analyzed using a SCLABA™ system [11] (Noveltec Inc., Kobe, Japan) at indicated age of weeks according to the manufacturer's instructions.

Measurement of NGF contents in affected skins of NC/NgaTnd mice by an ELISA: Skin samples were removed from the neck and dorsum of NC/NgaTnd mice, and were minced on ice. Five hundred μ l of lysis buffer (20 mM Tris, 137 mM NaCl, 1% nonidet P-40, 10% glycerol, and protease and phosphatase inhibitors, pH 8) was added to 100 mg of the skin samples. Samples were gently homogenized and collected supernatants were applied to an ELISA after an appropriate process of acid treatment by using NGF E_{max}™ immunoassay system (Promega Corp., Madison, WI, U.S.A.) according to the manufacturer's instruction.

Immunohistochemical analysis: To evaluate epidermal innervation, we performed anti-PGP 9.5 immunohistochemical analysis. PGP 9.5 is a ubiquitin carboxyl-terminal hydrolase, which is abundantly present particularly in axon of epidermal unmyelinated neurons [21, 22]. Dorsal skin samples were removed, fixed in 10% phosphate-buffered formalin, and embedded in paraffin. Five μ m-thick sections of dorsal skins were incubated with anti-PGP 9.5 antibody (Chemicon International Inc., Temecula, CA, U.S.A.) at 4°C overnight. After several wash, slides were incubated in Tris buffer containing skim milk to avoid non-specific reactions. Samples were incubated with horse-radish peroxidase (HRP)-conjugated anti-rabbit IgG antibody (Jackson ImmunoResearch, West Grove, PA) and positive reactions were visualized with 3–3'-diaminobenzidine tetrahydrochloride using FAST DAB™ (Sigma Chemicals, St. Louis) as a substrate. Five μ m-thick cryosections of dorsal skins fixed with 4% paraformaldehyde were incubated with anti-mouse 2.5S NGF antibody (Promega) at 4°C overnight. Positive reactions were visualized as described above.

Western blot analysis: Skin samples were homogenated in lysis buffer (50 mM Tris, 137 mM NaCl, 1% nonidet P-40, 10% glycerol, 1 mM EDTA, and protease and phosphatase inhibitors, pH 7.4), and supernatants were collected. Each lysate was mixed with sample buffer {125 mM Tris, 20% glycerol, 10% 2-mercaptoethanol, 4% sodium dodecyl sulfate (SDS), 0.01% bromophenol blue, pH 6.8}, and boiled for 7 min. Thirty μ g of total protein was applied to each lane of 12% SDS-polyacrylamide gel electrophoresis.

Separated proteins were transferred onto an Immobilon-P™ membrane (Millipore, Bedford, MA, U.S.A.), and blotted with anti-PGP 9.5 antibody and HRP-conjugated anti-rabbit IgG antibody. Positive reactions were visualized with an ECLplus™ detection reagent (Amersham Pharmacia, Arlington Heights, IL). Relative intensity of each band was analyzed by a digital image analysis using a Basic Quantifier (Genomic Solutions, Tokyo, Japan).

Statistical analysis: A two-tailed Student's *t* test was performed for statistical analysis of the data, and *p* < 0.05 was taken as the level of significance.

RESULTS

A correlation between scratching behavior and clinical skin conditions in NC/NgaTnd mice: Typical clinical features of conventional NC/NgaTnd mice were showed in Fig. 1A. A total of clinical skin severity scores for atopic dermatitis was defined as the sum of the individual scores graded as previously described for each of five symptoms (itch, erythema/hemorrhage, edema, excoriation/erosion and scaling/dryness) [6]. We analyzed scratching frequency using a SCLABA™ system and evaluated if obtained data relate to development and severity of clinical skin condition at each age of week. As shown in Fig. 1B, clinical aspects seen in conventional NC/NgaTnd mice began with itching behavior and turned worse every week. Increase in the scratching frequency and exacerbation of clinical aspects in conventional NC/NgaTnd mice were well correlated at each age of weeks.

Contents and localization of NGF in skins of NC/NgaTnd mice: We quantified NGF contents in skin homogenates of NC/NgaTnd mice by an ELISA. NGF contents were significantly higher in affected skins obtained from conventional NC/NgaTnd mice with dermatitis than those in normal skins from SPF mice (Fig. 2A). Immunohistochemical analysis revealed that NGF was mainly expressed in proliferating keratinocytes in the epidermis and fibroblasts in the dermis (Fig. 2B).

Expression of PGP 9.5-positive nerve fibers in the dermis of NC/NgaTnd mice: Because NGF is one of potent growth factors to peripheral sensory neurons, we examined whether extension of nerve fibers was induced in skin lesions. Marked hyperplasia and thickening of the epidermis were observed in the skin specimen obtained from conventional NC/NgaTnd mice (Fig. 3B). Positive reactions for PGP 9.5 were obvious in the dermis of conventional NC/NgaTnd mice with atopic dermatitis (Fig. 3B). Positive reactivity existed especially in the area close to the epidermis (Fig. 3B). On the other hand, the positive reaction was hardly observed in skins from SPF mice (Fig. 3A). We performed western blot analysis for quantification of PGP 9.5 in skins from conventional or SPF NC/NgaTnd mice. As shown in Fig. 4A, intensities of each PGP 9.5-positive band in skins from conventional NC/NgaTnd mice with atopic dermatitis were markedly stronger than those in skins from SPF mice. The relative intensity proved the level of significance (Fig. 4B).

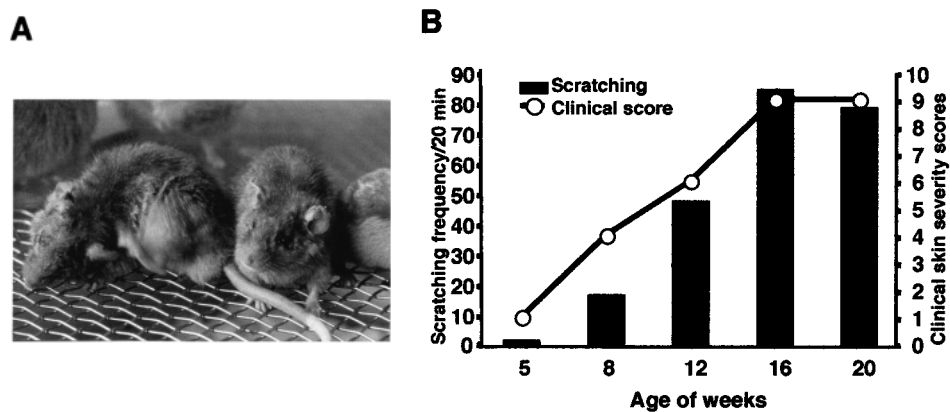


Fig. 1. (A) Typical clinical features of conventional NC/NgaTnd mice. (B) A correlation between clinical skin severity scores and scratching frequency. We analyzed the involvement of the increase of scratching behavior in exacerbation of dermatitis. There was a strong correlation between severity of dermatitis and the increase of scratching frequency.

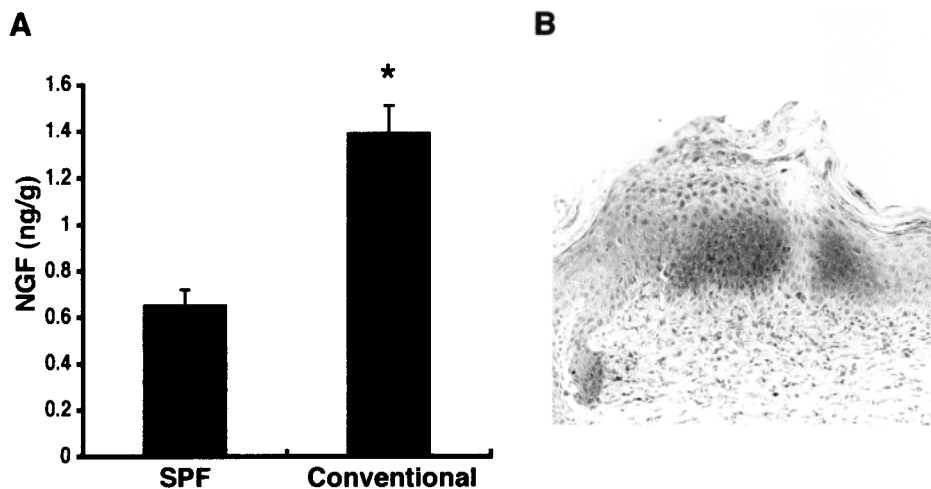


Fig. 2. (A) NGF contents in skins of NC/NgaTnd mice. We collected skins from both conventional and SPF mice, homogenized, and analyzed NGF contents using supernatants by an ELISA. NGF contents were significantly higher in affected skins from conventional NC/NgaTnd mice than those of normal skins from SPF mice. Each point represents means \pm SE of 8 mice. * $p < 0.05$, when compared with SPF mice. (B) Localization of NGF in the affected skin of the conventional mouse at 14 weeks of age. Positive reactions are obvious in proliferating keratinocytes and fibroblasts. The photograph represents typical immunohistochemical features of conventional NC/NgaTnd mice. Original magnification: $\times 160$.

DISCUSSION

Since NGF greatly contributes to neuronal development and functions [4, 10, 17], we attempted to determine association of NGF with hypersensitivity in skins of atopic dermatitis spontaneously appeared in NC/NgaTnd mice. In patients with atopic dermatitis, overextension of sensory nerve fibers may cause hypersensitivity of skins [16]. In the current study, we clearly demonstrated that PGP 9.5-positive nerve fibers were significantly increased in skins obtained from conventional NC/NgaTnd mice with atopic

dermatitis, whereas we detected a little positive reactivity of PGP 9.5 in skins from SPF mice. Results indicate the existence of numerous nerve fibers in affected skins of atopic mice. Plasma concentrations of NGF have reported to be increased in human patients with atopic dermatitis and the phenomenon has been proposed as a marker representing disease severity [18]. Expression of NGF in affected skins was significantly increased in conventional NC/NgaTnd mice with mild to severe atopic dermatitis compared to NGF contents in mice with no dermatitis, suggesting possible involvement of NGF not only in inflammatory process but

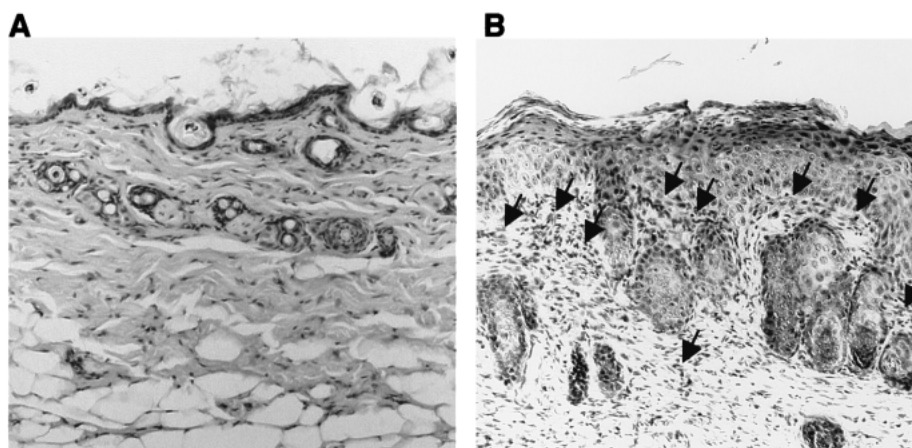


Fig. 3. Immunohistochemical examination of the conventional NC/NgaTnd mice at 14 weeks of age with anti-PGP 9.5 antibody. Positive reactions (indicated with arrows) are obvious in the skin of the conventional NC/NgaTnd mouse (B), but not in the SPF mouse (A). The photograph represents typical immunohistochemical features of conventional and SPF NC/NgaTnd mice. Original magnification: $\times 80$.

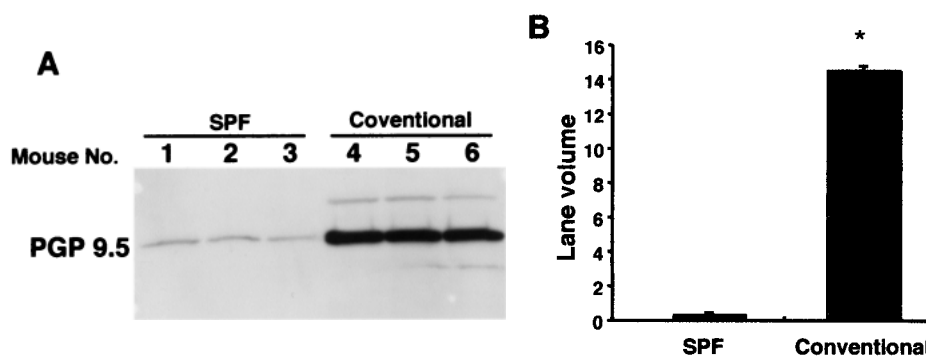


Fig. 4. Western blot analysis of PGP 9.5 in skins of conventional and SPF NC/NgaTnd mice (A). Images were digitalized and relative intensity was analyzed by a Basic Quantifier (Genomic Solutions) (B). * $p < 0.05$, when compared with SPF mice.

also in extension of sensory nerve fibers. NGF produced from proliferating keratinocytes and fibroblasts in atopic skins may invite sensory nerves closely to the epidermis, and may cause itch indirectly.

Increase in the number of scratching behavior and exacerbation of clinical skin symptoms were closely correlated, suggesting that development of scratching behavior may play a critical role in pathogenesis of atopic dermatitis [9]. Because scratching behavior results from itch [9, 11], investigation concerning itch may provide a new aspect of the disease and may contribute much to establishment of novel therapy for atopic dermatitis. A chain of reaction at skin lesions may bring serious exacerbation of atopic dermatitis. The scratching leads to further irritation of the skin, producing more itching, thus, initiating a vicious itch-scratch-circle [19, 24]. A typical example of this is atopic dermatitis, resulting from the patient's own scratching [19, 24]. NC/NgaTnd mice are also a useful animal model to investigate

mechanism of itch [9]. Further study must take place; however, our results may provide possible involvement of neurotrophic factors in itch in patients with atopic dermatitis.

ACKNOWLEDGEMENTS. This work was supported by the Grant for Practical Application of University R&D Results under the Matching Fund Method from The New Energy and Industrial Technology Development Organization (NEDO).

REFERENCES

1. Andrew, D. and Craig, A.D. 2001. Spinothalamic lamina I neurons selectively sensitive to histamine: a central neural pathway for itch. *Nat. Neurosci.* **4**: 72–77.
2. Bonini, S., Lambiase, A., Bonini, S., Angelucci, F., Magrini, L., Manni, L. and Aloe, L. 1996. Circulating nerve growth factor levels are increased in humans with allergic diseases and asthma. *Proc. Natl. Acad. Sci. U.S.A.* **93**: 10955–10960.

3. Huang, C.H., Kuo, I.C., Xu, H., Lee, Y.S. and Chua, K.Y. 2003. Mite allergen induces allergic dermatitis with concomitant neurogenic inflammation in mouse. *J. Invest. Dermatol.* **121**: 289–293.
4. Liuzzi, A., Angeletti, P.U. and Levi-Montalcini, R. 1965. Metabolic effects of a specific nerve growth factor (NGF) on sensory and sympathetic ganglia: enhancement of lipid biosynthesis. *J. Neurochem.* **12**: 705–708.
5. Matsuda, H., Koyama, H., Sato, H., Sawada, J., Itakura, A., Tanaka, A., Matsumoto, M., Konno, K., Ushio, H. and Matsuda, K. 1998. Role of nerve growth factor in cutaneous wound healing: accelerating effects in normal and healing-impaired diabetic mice. *J. Exp. Med.* **187**: 297–306.
6. Matsuda, H., Watanabe, N., Geba, G.P., Sperl, J., Tsudzuki, M., Hiroi, J., Matsumoto, M., Ushio, H., Saito, S., Askenase, P.W. and Ra, C. 1997. Development of atopic dermatitis-like skin lesion with IgE hyperproduction in NC/Nga mice. *Int. Immunol.* **9**: 461–466.
7. Matsumoto, M., Itakura, A., Tanaka, A., Fujisawa, C. and Matsuda, H. 2001. Inability of IL-12 to down-regulate IgE synthesis due to defective production of IFN-gamma in atopic NC/Nga mice. *J. Immunol.* **167**: 5955–5962.
8. Matsumoto, M., Ra, C., Kawamoto, K., Sato, H., Itakura, A., Sawada, J., Ushio, H., Suto, H., Mitsuishi, K., Hikasa, Y. and Matsuda, H. 1999. IgE hyperproduction through enhanced tyrosine phosphorylation of Janus Kinase 3 in NC/Nga mice, a model for human atopic dermatitis. *J. Immunol.* **162**: 1056–1063.
9. Mihara, K., Kuratani, K., Matsui, T., Nakamura, M. and Yokota, K. 2004. Vital role of the itch-scratch response in development of spontaneous dermatitis in NC/Nga mice. *Br. J. Dermatol.* **151**: 335–345.
10. Toschi, G., Dore, E., Angeletti, P.U., Levi-Montalcini, R. and de Haen, C. 1966. Characteristics of labeled RNA from spinal ganglia of chick embryo and the action of a specific growth factor (NGF). *J. Neurochem.* **13**: 539–544.
11. Orito, K., Chida, Y., Fujisawa, C., Arkwright, P.D. and Matsuda, H. 2004. A new analytical system for quantification scratching behaviour in mice. *Br. J. Dermatol.* **150**: 33–38.
12. Ostlere, L.S., Cowen, T. and Rustin, M.H. 1995. Neuropeptides in the skin of patients with atopic dermatitis. *Clin. Exp. Dermatol.* **20**: 462–467.
13. Ransohoff, R.M. and Tredst, C. 2000. Surprising pleiotropy of nerve growth factor in the treatment of experimental autoimmune encephalomyelitis. *J. Exp. Med.* **191**: 1625–1630.
14. Raychaudhuri, S.P. and Raychaudhuri, S.K. 2004. Role of NGF and neurogenic inflammation in the pathogenesis of psoriasis. *Prog. Brain Res.* **146**: 433–437.
15. Schmelz, M., Schmidt, R., Bickel, A., Handwerker, H.O. and Torebjork, H.E. 1997. Specific C-receptors for itch in human skin. *J. Neurosci.* **17**: 8003–8008.
16. Tobin, D., Nabarro, G., Baart de la Faille, H., van Vloten, W.A., van der Putte, S.C. and Schuurman, H.J. 1992. Increased number of immunoreactive nerve fibers in atopic dermatitis. *J. Allergy Clin. Immunol.* **90**: 613–622.
17. Toschi, G., Dore, E., Angeletti, P.U., Levi-Montalcini, R. and de Haen, C. 1966. Characteristics of labeled RNA from spinal ganglia of chick embryo and the action of a specific growth factor (NGF). *J. Neurochem.* **13**: 539–544.
18. Toyoda, M., Nakamura, M., Makino, T., Hino, T., Kagoura, M. and Morohashi, M. 2002. Nerve growth factor and substance P are useful plasma markers of disease activity in atopic dermatitis. *Br. J. Dermatol.* **147**: 71–79.
19. Wahlgren, C.F. 1991. Itch and atopic dermatitis: clinical and experimental studies. *Acta. Derm. Venereol. Suppl.* **165**: 1–53.
20. Wall, P.D. and Cronly-Dillon J.R. 1960. Pain, itch, and vibration. *Arch. Neurol.* **2**: 365–375.
21. Wilkinson, K. D., Lee, K., Deshpande, S., Duerksen-Hughes, P., Boss, J. M. and Pohl, J. 1989. The neuron-specific protein PGP 9.5 is a ubiquitin carboxyl-terminal hydrolase. *Science* **246**: 670–673.
22. Wilson, P. O., Barber, P. C., Hamid, Q. A., Power, B. F., Dhillon, A. P., Rode, J., Day, I. N., Thompson, R. J. and Polak, J. M. 1988. The immunolocalization of protein gene product 9.5 using rabbit polyclonal and mouse monoclonal antibodies. *Br. J. Exp. Pathol.* **69**: 91–104.
23. Woolf, C.J., Safieh-Garabedian, B., Ma, Q.P., Crilly, P. and Winter, J. 1994. Nerve growth factor contributes to the generation of inflammatory sensory hypersensitivity. *Neuroscience* **62**: 327–331.
24. Yosipovitch, G., Greaves, M.W. and Schmelz, M. 2003. *Itch*. *Lancet* **361**: 690–694.