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Neurotropin inhibits the increase in intraepidermal nerve density in the acetone-treated dry-skin mouse model

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Summary

Epidermal hyperinnervation is considered one cause of sensitization to itch, and is thought to regulated by keratinocyte-derived axonal guidance molecules, including nerve growth factor (NGF) and semaphorin (Sema)3A. Neurotropin (NTP) shows antipruritic effects in allergic disease and is also used for pain relief. Using cultured rat dorsal root ganglion neurones, we previously found that NTP inhibited NGF-induced neurite outgrowth. However, no such inhibitory effect has been shown *in vivo*. We therefore assessed the effects of intraperitoneal administration of NTP on nerve density and expression of NGF and Sema3A mRNAs in the epidermis of acetone-treated mice showing epidermal hyperinnervation. We found that NTP significantly reduced intraepidermal nerve growth in these acetone-treated mice. NTP significantly upregulated epidermal Sema3A mRNA but had no effect on expression of epidermal NGF mRNA. These findings indicate that NTP may reduce intraepidermal nerve density by inducing expression of Sema3A in the epidermis.

Itch (pruritus) is a sensation of discomfort that leads to scratching. It often accompanies a variety of dermatological and systemic diseases. One itch mediator, histamine, acts as a pruritogen in humans, and is used as an experimental itch-causing substance. Histamine type 1 receptor (H1R) antagonists (antihistamines) are therefore used to treat many types of itch, including those resulting from renal and liver disease, and from several skin diseases, such as atopic dermatitis (AD) and xerosis. However, antihistamines are often ineffective in patients with chronic itch.[1]

Epidermal hyperinnervation is considered one cause of intractable itch, suggesting that this area of the skin is susceptible to stimulation and is sensitive to itching.[1] The sprouting of epidermal nerve fibres associated with pruritus is seen in patients with AD, xerosis and allergic contact eczema, and also in experimental animal models.[1,2] Epidermal innervation is regulated by a fine balance between nerve elongation factors, such as nerve growth factor (NGF), amphiregulin and gelatinase, and nerve repulsion factors, such as semaphorin (Sema)3A and anosmin-1, produced by epidermal keratinocytes.[1] Therefore, NGF and Sema3A have been regarded as therapeutic targets in patients with pruritus. We previously showed that commonly used clinical therapies, such as emollients and ultraviolet light-based therapies, have inhibitory effects on intraepidermal nerve fibres, and that these effects may be due to imbalances between NGF and Sema3A.[3]

Neurotropin (NTP), a nonprotein extract isolated from the inflamed skin of rabbits inoculated with vaccinia virus, is widely used in Japan and China to treat various chronic pain conditions.[4] In clinical studies in Japan, NTP has been shown to have antipruritic effects in patients with eczema, dermatitis and urticaria,[5] and in those undergoing haemodialysis.[6] We also found that NTP inhibits NGF-induced neurite outgrowth of dorsal root ganglion (DRG) neurones *in vitro*.[7]

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To expand these observations, we examined whether NTP affects intraepidermal nerve density and the epidermal expression of NGF and Sema3A mRNA in a mouse dry-skin model. We describe the possible inhibitory effects of NTP on the pruritus accompanying epidermal hyperinnervation.

Report

All animal procedures were approved by the Institutional Animal Care and Use Committee of the Juntendo University Graduate School of Medicine. Male 10-week-old ICR mice (SLC Japan, Shizuoka, Japan) were maintained in the experimental animal facility of Juntendo University Graduate School of Medicine.

The protocol for cutaneous barrier disruption has been described previously.[3] Briefly, the hair over the rostral back of the mice was shaved at least 3 days before acetone treatment, and each shaved area was treated with acetone-soaked cotton balls for 5 min. Each mouse was injected intraperitoneally with 200 NTP units (NU) per kg of NTP (experimental group) or saline (control group), immediately and 8, 16, 24, 32 and 40 h after acetone treatment.

Because epidermal nerve density in acetone-treated mice peaks at 48 h after acetone treatment,[3] skin samples were collected at that time, and analysed by immunohistochemistry and quantitative reverse transcription (qRT)-PCR (Fig. 1a).

The numbers of epidermal nerve fibres were assessed immunohistochemically using an antibody to protein gene product (PGP)9.5 at 1 : 4000 dilution (Biomol International Corp., Plymouth Meeting, PA, USA), with Alexa 488-conjugated secondary antibodies (Life Technologies Corp., Carlsbad, CA, USA) used at 1 : 300 dilution. The numbers of PGP9.5-positive nerve fibres penetrating into (Fig. 1b, arrow) and within (Fig. 1b, arrowheads) the epidermis in areas of 1.6×10^5 μm in size in the images of nine sections (20 μm thick) from each mouse were counted by hand. In these analyses, each typical line structure with a minimum length of 12.5 μm was counted as one fibre, and counting was based on intraepidermal nerve-fibre counting rules.[8]

Following treatment with trypsin, epidermal sheets were separated from the skin using forceps, and qRT-PCR analysis was performed as described previously.[2] The primers used to assay NGF and Sema3A mRNA (Perfect Real Time Support System, TaKaRa, Kyoto, Japan) were designed to meet specific criteria.

All statistical analyses were performed using Prism software (version 5; GraphPad Software Inc., La Jolla,

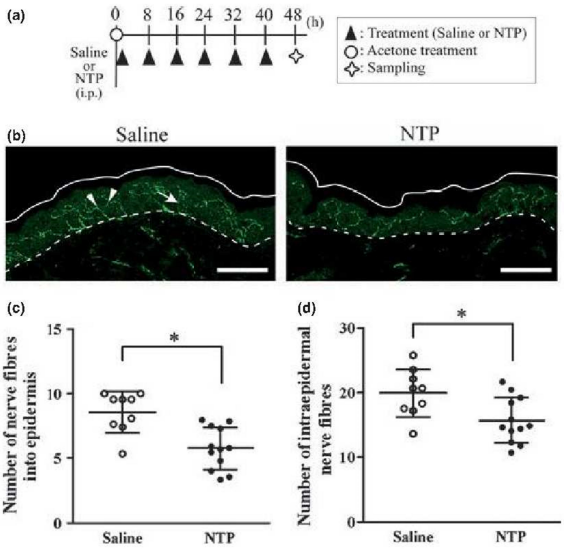

Figure 1 (a) Mice were injected with saline or neurotropin (NTP) immediately and 8, 16, 24, 32 and 40 h after acetone treatment. (b) Distribution of the intraepidermal protein gene product (PGP)9.5-immunoreactive fibres in mice treated with saline or NTP. White and broken lines indicate the skin surface and the border between the epidermis and dermis, respectively. The arrow indicates penetrating nerve fibres and the arrowheads indicate the branching neurones. Scale bars, 50 μm. (c,d) Numbers of PGP9.5 positive nerve fibres (c) penetrating into and (d) lying within the epidermis in NTP-treated and control mice. All values represent the mean ± SD of 9–12 animals. *$P < 0.05$.

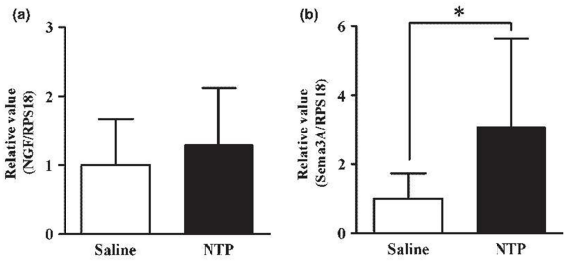

Figure 2 (a,b) Effects of neurotropin (NTP) on epidermal levels of (a) nerve growth factor (NGF) and (b) semaphorin (Sema)3A mRNA levels: NGF mRNA levels were similar in NTP-treated and control mice, whereas Sema3A mRNA levels were significantly higher in NTP-treated than in control mice. All values represent the means ± SD of seven animals. *$P < 0.05$

CA, USA). The differences between means were analysed by the Student *t*-test.

NTP administrations after acetone treatment significantly reduced the numbers of nerve fibres penetrating into (Fig. 1c) and within (Fig. 1d) the epidermis, compared with saline-treated mice. When we assessed the expression levels of NGF and Sema3A mRNA in the epidermal sheets from saline-treated and NTP-treated skin samples, we found that the expression of NGF mRNA was similar in the two groups (Fig. 2a), whereas the expression of Sema3A mRNA was significantly higher in the NTP than in the saline group (Fig. 2b).

NTP contains a number of small molecules, including nucleic acids, amino acids and sugars (Nippon Zouki Pharmaceutical Company, unpublished observations). Vaccinia virus-infected cells were recently shown to generate RNA species by activating interferon-β gene transcription, which modulates the expression of numerous cellular microRNAs.[9] Moreover, endogenous microRNA was shown to regulate axonal guidance.[10] Therefore, NTP may contain some effectors that bind certain molecules involved in the induction of Sema3A expression in the epidermis.

In conclusion, we found that NTP inhibited acetone-induced increases in intraepidermal nerve density by inducing expression of Sema3A in the epidermis of acetone-treated mice. Previously, using cultured DRG neurones, we reported that NTP inhibited NGF-induced neurite outgrowth.[7] Thus, in addition to having a direct effect on nerve growth *in vitro*, NTP may have an indirect effect *in vivo*. In this study, although the effect of NTP on scratching behaviour was not measured, our previous studies have shown that epidermal hyperinnervation can be correlated with pruritus.[1] Therefore, NTP may be a possible treatment to relieve pruritus accompanying epidermal hyperinnervation, and there is support for this idea from several clinical studies of NTP treatment for pruritus.[5]

Learning points

- Neurotropin induces Sema3A expression in the epidermis of acetone-treated mice and may inhibit nerve growth.
- Neurotropin may act through both direct and indirect mechanisms to regulate intraepidermal nerve density.
- This inhibitory effect raises the possibility of an effective treatment for pruritus accompanying epidermal hyperinnervation.

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CPD questions

Learning objective

To demonstrate an understanding of the up-to-date mechanism about treating intractable itch involving epidermal hyperinnervation.

Question 1

Which of the following is a repulsive factor for intraepidermal nerve fibres?
a) Histamine.
b) Nerve growth factor.
c) Amphiregulin.
d) Semaphorin 3A.
e) Neurotropin.

Question 2

Which of the following is **not** a pharmacological action of neurotropin (NTP)?
a) Normalization of the expression balance of epidermal axonal guidance molecules.
b) Relief of pain.
c) Inhibition of nerve growth factor-induced neurite outgrowth of dorsal root ganglion neurones.
d) Relief of itch.
e) Increase in epidermal nerve growth factor levels.

Instructions for answering questions

This learning activity is freely available online at http://www.wileyhealthlearning.com/ced.

Users are encouraged to
- Read the article in print or online, paying particular attention to the learning points and any author conflict of interest disclosures
- Reflect on the article
- Register or login online at http://www.wileyhealthlearning.com/ced. com and answer the CPD questions
- Complete the required evaluation component of the activity

Once the test is passed. you will receive a certificate and the learning activity can be added to your RCP CPD diary as a self-certified entry.

