

RESEARCH ARTICLE

Decreased innervation of eczematous skin in NC/Nga atopic mice during chronic mild stress

Louise Lönndahl¹, Sol-Britt Lonne-Rahm¹, Klas Nordlind¹, Elvar Theodorsson², and Husameldin El-Nour¹

¹Karolinska Institutet, Department of Medicine Solna, Dermatology and Venereology Unit, Karolinska University Hospital, Stockholm Sweden, and ²IBK/Clinical Chemistry, University Hospital, Linköping, Sweden

Abstract

Background and aim: A connection between chronic mild stress and altered innervation in the skin of an atopic mouse strain, NC/Nga, was studied.

Material and methods: We used three groups of mice, stressed control (SC, stressed but not immunized with a mite antigen), non-stressed eczematous (NSE, not stressed but immunized) and stressed eczematous (SE, stressed and immunized).

Results: There was a decrease of protein gene product (PGP) 9.5 positive nerve fibers in SE compared to SC mice, in both epidermis and dermis, also in SE compared to NSE mice. Also a decrease in growth associated protein (GAP)-43 positive nerve fibers was seen in SE compared to SC, in both epidermis and dermis.

Conclusion: Chronic mild stress in atopic mice leads to decreased innervation in eczematous lesions.

Keywords: Stress; atopic eczema; innervation; PGP 9.5; GAP-43

Introduction

Atopic dermatitis (AD) is a common multifactorial chronic eczematous skin disorder that often starts in early childhood, affecting 10–20% children worldwide and 1–3% of the adult population [1]. This disease often has a remitting/flaring course, and is exacerbated by social, environmental and biological triggers. Psychological stress is known to aggravate the symptoms of AD [2, 3]. Nevertheless, the exact mechanism by which psychological stress deteriorates AD is not yet known. The patients, from our treatment perspective, are more able to handle acute compared to chronic stress.

The definition of acute and chronic stress is related to the duration of the stress. Acute stress lasts for minutes to hours whereas chronic stress persists several hours a day for weeks or months. In general, acute stress has been found to be immunoenhancing compared to chronic stress which is immunosuppressive. An important

marker for chronic stress is dysregulation of the cortisol/corticosterone rhythm [4].

The cross-talk between neuroendocrine and immune systems is well documented [5]. Stress can stimulate the hypothalamic-pituitary-adrenal axis (HPA) to release stress hormones, such as cortisol, that may influence the pathogenesis of immune and cutaneous diseases. Furthermore, stress is known to cause mast cell activation and degranulation [5]. Slominski et al. [6] showed the cutaneous synthesis and release of adrenocorticotrophic hormone (ACTH) by keratinocytes and melanocytes. ACTH has been suggested to play a regulatory role in skin inflammatory diseases [7].

It has been reported that the innervation of the lesional skin from patients with AD is increased [8, 9]. In addition, the diameter of the nerves was also larger [10]. This implicates that nerve fiber growth could be a part in the pathogenesis of AD. Acute stress has been reported to increase the innervation in humans [11] and mice [12].

Address for Correspondence: Louise Lönndahl, Karolinska Institutet, Department of Medicine Solna, Dermatology and Venereology Unit, Karolinska University Hospital, SE-171 76 Stockholm, Sweden. Tel: 46-8-51772158; Fax: 46-8-51777851. E-mail: louise.lonndahl@ki.se

(Received 00 00 0000; revised 00 00 0000; accepted 00 00 0000)

It is possible to examine innervation and axonal growth by using PGP 9.5 (protein gene product 9.5), which is ubiquitin carboxyl-terminal hydrolase that is abundantly present in unmyelinated epidermal C fibers and also most of the cutaneous nerves [13], and GAP-43 (growth associated protein-43) that detects newly generated nerve fibers.

There are different animal models where an AD-like disease may be obtained [14]. The most common used of these models is the NC/Nga mouse that upon immunization suffers from both macroscopic and microscopic AD-like skin lesions [15, 16]. These animals constitute a good model for studying the processes that take place in the skin, and for comparisons with AD in humans.

It is of interest to investigate whether chronic mild stress [17] could lead to a change in the innervation of the affected skin in AD. In the present study we examined the expression of PGP 9.5 and GAP-43 positive nerve fibers in atopic mice exposed to chronic mild stress.

Material and Methods

Animals

Six-week-old female NC/Nga mice (Charles River Laboratories, Solna, Sweden) were used.

Chronic stress

The chronic mild stress procedure used was earlier described by Lanfumey et al. [18]. The mice were kept in a Scantainer box type 50-SCNT-Z11 (Scanbur AS, Køge, Denmark) and we used different stressors such as reversed light/dark cycle, confinement to small cages, one period of continuous overnight illumination, overcrowding of the animals, one overnight period of wet soil, repeated periods of cage-tilting (30°) and short periods of food and water deprivation.

Immunization

The mice were divided into 3 groups (8 mice per group). One group was stressed and immunized (stressed eczematous, SE). They were subject to chronic mild stress for 8 weeks. In addition to a continuation of the stress, eczema was induced, in parallel for an additional 4 week period, by painting their ears with a mite antigen, *Dermatophagoides pteronyssinus* (Allergon, Ängelholm, Sweden).

A non-immunized control group was stressed (stressed control, SC), being painted on their ears using the solvent, whereas a second immunized group was relieved from stress (non-stressed eczematous, NSE), being kept outside the box and also painted on their ears with the

mite antigen for the last 4 weeks. They were maintained on a 12-hr light/dark cycle under controlled temperature between 18 and 22°C.

Processing of samples

After 12 weeks the animals were sacrificed and the ear diameters were immediately measured using a calliper (Kroeplin, Schluchtern, Germany). Blood samples were collected at 10 a.m. and the plasma corticosterone was measured using a Corticosterone RIA kit (RS 490 11) from IBL (Hamburg, Germany) following the instructions of the manufacturer.

Tissue samples of the ears were subject to immunohistochemistry in order to analyze a change in innervation. For that purpose the ears were fixed in 4% formalin with 0.2% picric acid for 2 h at 4°C. They were then rinsed with 0.1 mol/L phosphate buffer containing 10% sucrose for at least 24 h. Tissues were then embedded in Tissuettek (Sakuro Finetek, Zoeterwoude, The Netherlands) and sectioned (14 µm thick) using a Dittes cryostat (Heidelberg, Germany). The cryosections were mounted on SuperFrost/Plus glass slides (Menzel-Glaser, Freiburg, Germany) and stored at -70°C until the application of immunohistochemistry using an immunofluorescence (IF) technique.

Immunohistochemistry

Polyclonal rabbit antibodies directed against PGP 9.5 (dilution 1:10 000; RA95101, UltraClone, Cambridge, England) and GAP-43 (1:3 000; AB5220, Chemicon, Temecula, CA, USA) were used. The sections were incubated with the primary antibodies at 4°C overnight.

On the next day the secondary biotinylated goat anti-rabbit antibody (BA 1000; Vector, Burlingame, CA, USA), diluted 1:200 in 1% bovine serum albumin (BSA)/phosphate buffered saline (PBS), was applied and incubated for 40 min. This step was followed by adding fluorochrome Cy2-labelled streptavidine (1:2 000; PA 42001, Amersham, Biosciences, Uppsala, Sweden), for 40 min, in order to visualise the primary antibodies.

As negative controls the primary antibodies were omitted or normal rabbit serum (X0936; Dako, Glostrup, Denmark) was used replacing the primary antibody, which gave a negative staining for the nerve fibers. Lastly, the sections were rinsed in PBS and mounted with Kaiser's glycerol gelatin.

Microscopy

For examining and photographing the PGP 9.5 sections a Nikon Eclipse E 800 (Yokohama, Japan) microscope equipped for epifluorescence was used. Cy2-fluorescent elements were visible with a B-1E filter cube (excitation

at 465–495 nm). The PGP 9.5 stained sections were then analysed using a software program (see *Image analysis*). Twenty-four pictures, representing four sections, were taken per mouse. All areas of the sections were represented except the ends.

GAP-43 positive nerve fibers were counted manually for practical reasons due to the low contrast between the positive nerves and the surrounding tissue, which influenced the threshold settings for detection of the fluorescence. Nerve fibers that crossed the dermoepidermal junction were included in the layer in which most of the nerve fiber was found. Fibers that were clearly connected to hair follicles or glands were not included. From the GAP-43 stained sections 2 areas per section (8 per mouse) were counted. The microscopic evaluation was performed by one observer (LL).

Image analysis

The image analysis was performed on the PGP 9.5 stained nerve fibers using the software program Easy Image Analysis (Bergström Instruments, Solna, Sweden). Images from microscopic fields for the ear sections (1 cm long) were stored by using a digital video camera system (Nikon DXM 1200, Yokohama, Japan) attached to the Nikon Eclipse E 800 microscope and connected to a PC. The results were given in area fraction (ratio of specifically stained fluorescent/nonfluorescent area).

Statistical analysis

For statistical analysis the Student's t-test was used. A *p* value of <0.05 was regarded as significant.

Results

Corticosterone levels

At the termination of the experiment the SE group showed lower corticosterone levels, 496.4 ± 221.5 (mean $\pm$ SD) ng/ml, compared to the other groups, 738.6 ± 232.1 , in the NSE and 805.2 ± 144.4 ng/ml, in the SC.

Degree of inflammation

The diameter of the ears was larger in the SE group, 0.49 ± 0.11 mm, compared to the NSE, 0.36 ± 0.13 mm ($p < 0.01$) and the SC, 0.23 ± 0.00 mm ($p < 0.001$).

PGP 9.5

Epidermis

There was a decrease ($p < 0.01$) in the PGP 9.5 positive nerve fibers (Figure 1A–C) in the SE (area fraction,

0.8 ± 0.3 , mean $\pm$ SD) compared to the NSE group (2.4 ± 1.2) (Figure 2). Also the SE compared to the SC group (2.5 ± 1.2) showed a decrease ($p < 0.01$) of these fibers.

Dermis

There was a decrease of the PGP 9.5 positive nerve fibers ($p < 0.01$) in the SE (1.3 ± 0.3) compared to the NSE group (2.9 ± 1.2) (Figure 3). Also the SE compared to the SC group (3.4 ± 0.9) showed a decrease ($p < 0.001$).

GAP-43

Epidermis

There was a decrease ($p < 0.005$) in the GAP-43 positive epidermal fibers (Figure 4A,B) in the SE (40.7 ± 20.5 fibers per section) compared to the SC group (75.9 ± 20.4). Also the NSE (48.1 ± 28.8) compared to the SC group showed a decrease ($p < 0.05$). There was no difference in the stressed eczema group, SE, compared to the NSE group (Figure 5).

Dermis

There was a decrease ($p < 0.05$) in the number of GAP-43 positive dermal fibers in the SE (108.6 ± 29.7 fibers per section) compared to the SC group (133.5 ± 31.8). The value for the NSE group was 122.9 ± 27.5 .

Discussion

In the present study there was a decrease in PGP 9.5 positive nerve fibers in the SE compared to the NSE mice, in both epidermis and dermis. Also a decrease in GAP-43 positive nerve fibers was seen in the SE compared to the SC group in both the epidermis and dermis.

An increase in innervation has been shown in some studies of AD [8–10]. Since stress is known to make the symptoms of AD worse it might have been expected that the innervation would have been increased instead of being decreased in response to stress, in our present study. In this respect the skin innervation has been noted to increase in mice exposed to sonic stress for 24 h [12] and also in humans 24 h after acute social stress [11]. Yet, in a human study of AD patients with a history of worsening during chronic stress it was found that the number of nerve fibers was decreased in involved compared to non-involved eczematous skin [19].

A possible explanation for our present results could be that the stress situation used was a chronic one. Initially stress might increase the innervation, but with time it might instead lead to a decrease. It is possible that there has been a mechanical injury of the nerve fibers caused by the scratching, giving a slight nerve damage. This

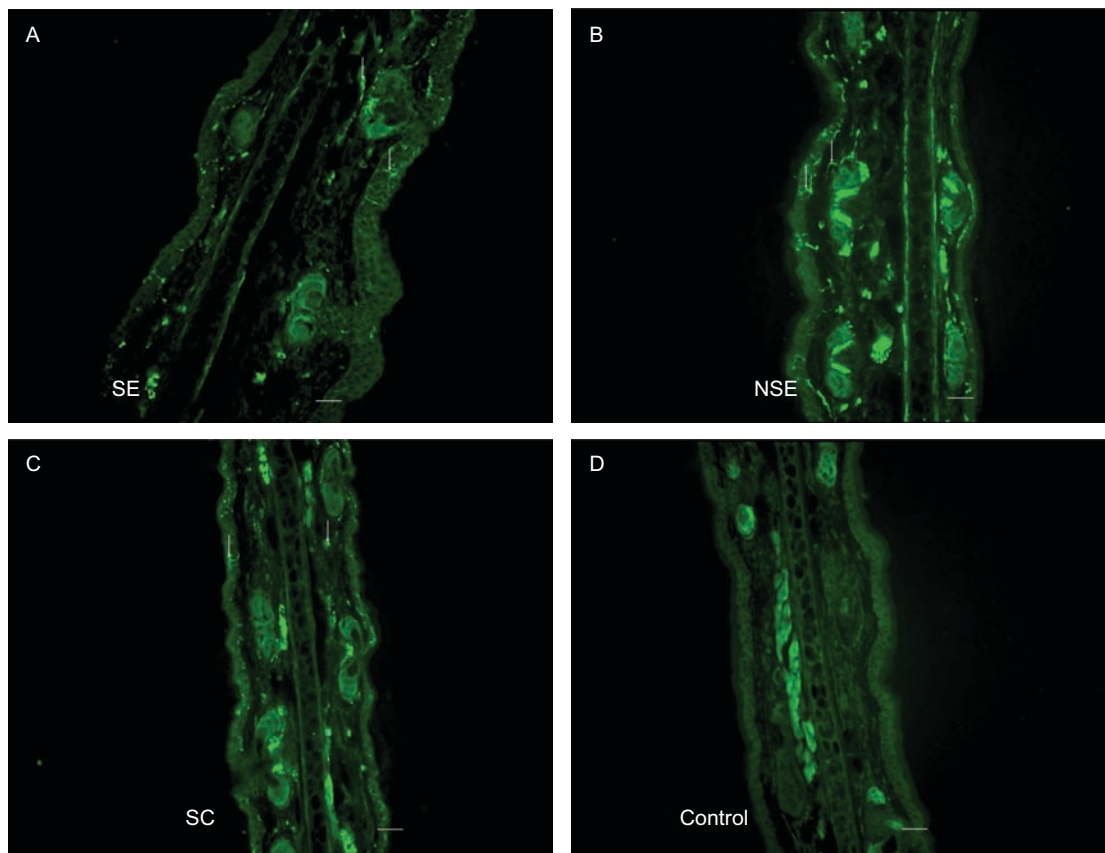


Figure 1. Expression of PGP 9.5 positive nerves (arrows) in the epidermis and dermis of stressed eczema (SE) (A), non-stressed eczema (NSE) (B) and stressed control (SC) (C) groups. A negative control, where the primary antibody has been omitted, is seen in (D). Scale bars 20 μ m.

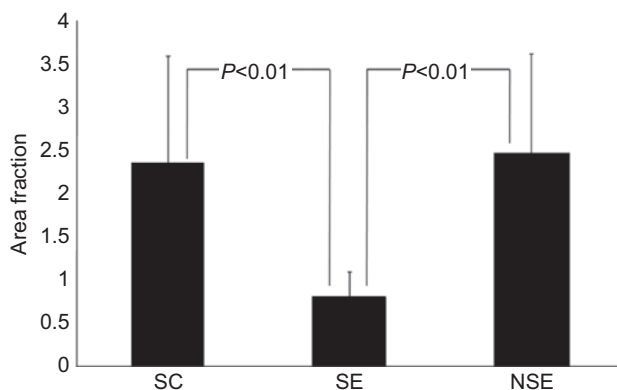


Figure 2. Graph showing area fraction (mean $\pm$ SD) of PGP 9.5 fibers in the epidermis of the different mice groups.

scratching per se might be due to central effects of the chronic stress, lowering the threshold for perception of pruritus.

One should also consider a modulation in the function of the keratinocytes as a result of the chronic inflammation in the skin, which worsens by chronic stress. Keratinocytes have a trophic effect on sensory neurons that is mediated by soluble factors among which nerve growth factor (NGF) is believed to play an important role

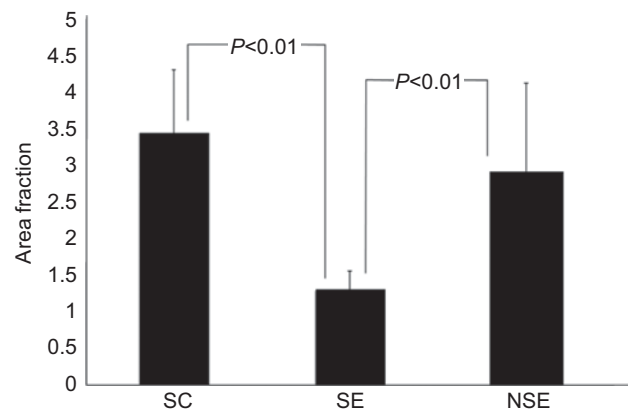


Figure 3. Graph showing area fraction (mean $\pm$ SD) of PGP 9.5 fibers in the dermis of the different mice groups.

[20]. One could speculate on a stress-related decrease of the innervation mediated by the central and peripheral nervous systems, presumably caused by a combination of the physical, in the form of eczema, with keratinocyte malfunction, and psychological stress, decreasing the neurogenesis. This may further lead to a dysregulation of the functions of factors such as neurotrophins, NGF and brain-derived neurotrophic factor (BDNF).

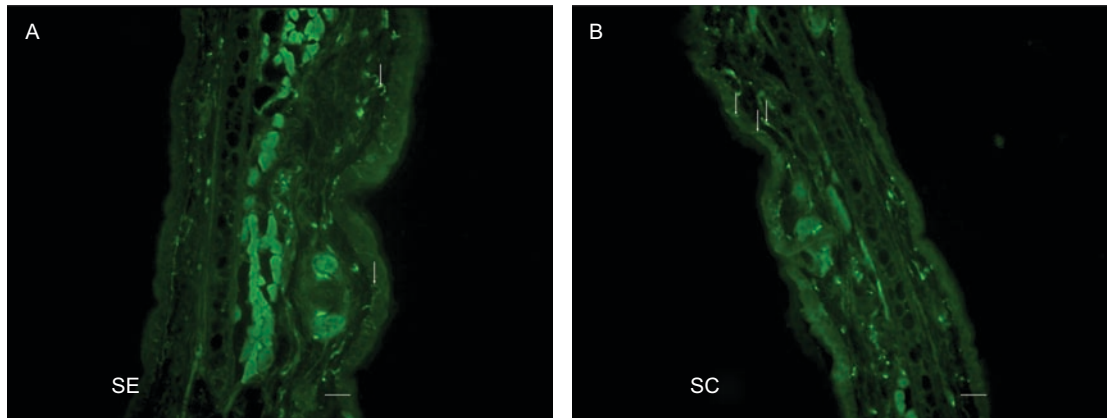


Figure 4. Expression of GAP-43 positive nerves (arrows) in the epidermis and dermis of stressed eczema (SE) (A) and stressed control (SC) (B) groups. Scale bars 20 μ m.

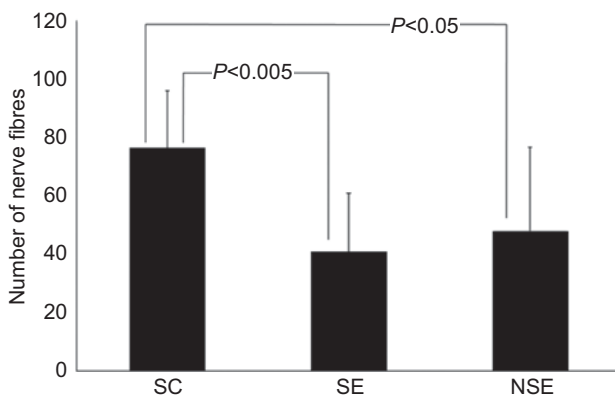


Figure 5. Graph showing number (mean $\pm$ SD) of GAP-43 fibers in the epidermis of the different mice groups.

In the present study there was no difference in the GAP-43 expression between the SE and NSE groups. This might indicate that the chronic mild stress does not affect the newly formed nerve fibers. In a rat model it has been shown that chronic psychosocial stress and chronic restraint stress lead to suppression of neurogenesis of the hippocampus as measured by the bromodeoxyuridine (BrdU) labeling method [21]. Yet, after chronic restraint stress GAP-43 expression was shown to be only slightly decreased in specific areas of the hippocampus or not decreased when analyzing the total hippocampus [21, 22].

However, one should consider possible differences in the GAP-43 expression in the central compared to the peripheral nervous systems. It should also be taken under consideration that GAP-43 might not be sensitive enough when studying effect of stress on neuronal tissue.

In conclusion, chronic mild stress can cause a decrease in preformed cutaneous nerves in eczematous skin. The slowdown of the process of neuronal regeneration in the eczematous skin of atopic mice could be due to the inflammation per se.

Acknowledgements

The technical assistance of Anna-Lena Kastman is gratefully acknowledged.

Declaration of interest: The present study was supported by grants from the Welander/Finsen Foundation and Karolinska Institutet. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

1. Leung DY, Bieber T. Atopic dermatitis. *Lancet* 2003;361 (9352):151–160.
2. Katsuno M, Aihara M, Kojima M, Osuna H, Hosoi J, Nakamura M, et al. Neuropeptides concentrations in the skin of a murine (NC/Nga mice) model of atopic dermatitis. *J Dermatol Sci* 2003;33 (1):55–65.
3. Wright RJ, Cohen RT, Cohen S. The impact of stress on the development and expression of atopy. *Curr Opin Allergy Clin Immunol* 2005;5 (1):23–29.
4. Dhabhar FS. Stress, leukocyte trafficking, and the augmentation of skin immune function. *Ann N Y Acad Sci* 2003;992:205–217.
5. Bhatia V, Tandon RK. Stress and the gastrointestinal tract. *J Gastroenterol Hepatol* 2005;20 (3):332–339.
6. Slominski A, Botchkareva NV, Botchkarev VA, Chakraborty A, Luger T, Uenalan M, et al. ACTH production in C57BL/6 mouse skin. *Ann N Y Acad Sci* 1999;885:448–450.
7. Slominski A, Wortsman J, Luger T, Paus R, Solomon S. Corticotropin releasing hormone and proopiomelanocortin involvement in the cutaneous response to stress. *Physiol Rev* 2000;80 (3):979–1020.
8. Tobin D, Nabarro G, Baart de la Faille H, Van Vloten WA, Van der Putte SC, Schuurman HJ. Increased number of immunoreactive nerve fibers in atopic dermatitis. *J Allergy Clin Immunol* 1992;90 (4 Pt 1):613–622.
9. Pincelli C, Fantini F, Massimi P, Girolomoni G, Seidenari S, Giannetti A. Neuropeptides in skin from patients with atopic dermatitis: an immunohistochemical study. *Br J Dermatol* 1990;122 (6):745–750.
10. Urashima R, Mihara M. Cutaneous nerves in atopic dermatitis. A histological, immunohistochemical and electron microscopic study. *Virchows Arch* 1998;432 (4):363–370.
11. Kleyen CE, Schneider L, Saraceno R, Mantovani C, Richards HL, Fortune DG, et al. The effects of acute social stress on epidermal

- Langerhans' cell frequency and expression of cutaneous neuropeptides. *J Invest Dermatol* 2008;128 (5):1273–1279.
12. Peters J, Kuhlmei A, Tobin DJ, Müller-Röver S, Klapp BF, Arck PC. Stress exposure modulates peptidergic innervation and degranulates mast cells in murine skin. *Brain, Behav Immun* 2005;19 (3):252–262.
 13. Wilkinson D, Lee KM, Deshpande S, Duerksen-Hughes P, Boss JM, Pohl J. The neuron-specific protein PGP 9.5 is a ubiquitin carboxyl-terminal hydrolase. *Science* 1989;246 (4930):670–673.
 14. Jin H, He R, Oyoshi M, Geha RS. Animal models of atopic dermatitis. *J Invest Dermatol* 2009;129 (1):31–40.
 15. Sasakawa T, Higashi Y, Sakuma S, Hirayama Y, Sasakawa Y, Ohkubo Y, et al. Atopic dermatitis-like skin lesions induced by topical application of mite antigens in NC/Nga mice. *Int Arch Allergy Immunol* 2001;126 (3):239–247.
 16. Gutermuth J, Ollert M, Ring J, Behrendt H, Jacob T. Mouse models of atopic eczema critically evaluated. *Int Arch Allergy Immunol* 2004;135 (3):262–276.
 17. Vollmayr B, Mahlstedt MM, Henn FA. Neurogenesis and depression: what animal models tell us about the link. *Eur Arch Psychiatry Clin Neurosci* 2007;257 (5):300–303.
 18. Lanfumey L, Pardon M-C, Laaris N, Joubert C, Hanoun N, Hamon M, et al. 5-HT_{1A} autoreceptor desensitization by chronic ultramild stress in mice. *NeuroReport* 1999;10 (16): 3369–3374.
 19. Lonne-Rahm S-B, Rickberg H, El-Nour H, Mårin P, Azmitia EC, Nordlind K. Neuroimmune mechanisms in patients with atopic dermatitis during chronic stress. *Eur Acad Dermatol Venereol* 2008;22 (1):11–18.
 20. Ulmann L, Rodeau J-L, Danoux L, Content-Audonneau J-L, Pauly G, Schlichter R. Trophic effects of keratinocytes on the axonal development of sensory neurons in a coculture model. *Eur J Neurosci* 2007;26 (1):113–125.
 21. Rosenbrock H, Koros E, Bloching A, Podhorna J, Borsini F. Effect of chronic intermittent restraint stress on hippocampal expression of marker proteins for synaptic plasticity and progenitor cell proliferation in rats. *Brain Res* 2005;1040 (1-2): 55–63.
 22. Kuroda Y, McEwen BS. Effect of chronic restraint stress and tianeptine on growth factors, growth-associated protein-43 and microtubule-associated protein 2 mRNA expression in the rat hippocampus. *Mol Brain Res* 1998;59 (1):35–39.

Copyright of Immunopharmacology & Immunotoxicology is the property of Taylor & Francis Ltd and its content may not be copied or emailed to multiple sites or posted to a listserv without the copyright holder's express written permission. However, users may print, download, or email articles for individual use.