DEPARTMENT OF MEDICINE, SOLNA
Karolinska Institutet, Stockholm, Sweden

TACHYKINERGIC MECHANISMS IN ATOPIC DERMATITIS

Louise Lönndahl

Karolinska Institutet

Stockholm 2015

Published by Karolinska Institutet. Printed by Universitetsservice US AB.

ISBN 978-91-7676-117-5

Karolinska Institutet

Institutionen för Medicin, Solna

Tachykinergic Mechanisms in Atopic Dermatitis

AKADEMISK AVHANDLING
som för avläggande av medicine doktorsexamen vid Karolinska Institutet offentligen försvaras i Welandersalen- Karolinska Universitetssjukhuset Solna, B2:00.

Fredagen den 13:e November 2015, klockan 9.00

av

Louise Lönndahl
MD

Huvudhandledare:
Professor Klas Nordlind
Karolinska Institutet
Institutionen för Medicin, Solna
Enheten för Dermatologi och Venereologi

Bihandledare:
Docent Mikael Holst
Karolinska Institutet
Institutionen för kvinnors och barns hälsa
Enheten för Barnendokrinologi

MD/PhD Husameldin El-Nour
Karolinska Institutet
Institutionen för Medicin, Solna
Enheten för Dermatologi och Venereologi

Docent Sol-Britt Lonne-Rahm
Karolinska Institutet
Institutionen för Medicin, Solna
Enheten för Dermatologi och Venereologi

Fakultetsopponent:
Professor Laurent Misery
University of Brest
Department of Dermatology, Brest University Hospital, Brest, Frankrike

Betygsnämnd:
Professor Magnus Lindberg
Örebro Universitet
Institutionen för hälsovetenskap och medicin

Professor Björn Meister
Karolinska Institutet
Institutionen för neurovetenskap

Docent Peter Thelin Schmidt
Karolinska Institutet
Institutionen för Medicin, Solna
Enheten för internmedicin

Stockholm 2015

ABSTRACT

Atopic dermatitis (AD) is an often severely itching, chronic, inflammatory skin disorder and may worsen due to stress and anxiety. Tachykinins have been suggested to influence the level of inflammation as well as being involved in pruritus, stress and anxiety.

The aim of the study was to investigate the role of tachykinins, including substance P and neurokinin (NK) A, in the pathogenesis of AD, pruritus and worsening during stress.

Initially an animal model was used to investigate innervation, substance P and the neurokinin (NK)-1 receptor (R) in relation to eczema and stress using an immunohistochemistry method. Chronic mild stress seemed to decrease innervation in eczematous skin lesions and we recorded a decrease in substance P positive fibres in stressed eczematous skin compared to in stressed control. In addition, a tendency of increased levels of NK-1R m-RNA in skin of stressed compared to non-stressed eczematous mice was apparent using PCR. A decrease of substance P immunoreactivity was detected in the medial hippocampus of the brain in stressed compared to in non-stressed eczematous mice.

Tachykinin expression was then examined in the skin of AD patients, and possible correlations to clinical and psychodemographic parameters were investigated. The numbers of substance P- and NKA positive nerve fibres and also of NKA positive dermal cells were increased in lesional compared to non-lesional skin. In addition, there was a correlation between the NK1-R positive cells, and the level of acanthosis and inflammation, in the lesional skin. There was also a correlation between the depression score and the number of the NK-1R positive cells in lesional as well as in non-lesional skin.

Finally, the effect of an NK-1R antagonist, aprepitant, was evaluated in adult patients with moderate-severe AD, compared to a standard topical treatment in an open randomized trial. In both the treatment group and the control group significant improvement was evident both regarding extent of disease and pruritus. However, there was not any significant additional improvement for any of these parameters in the aprepitant-treated patients compared to controls.

In conclusion, tachykinins seem to have a role in the pathogenesis and stress-worsening of AD. However, this role seems to be complex and further investigations are needed to fully understand these connections.

LIST OF PUBLICATIONS

I. **Lönndahl L**, Lonne-Rahm SB, Nordlind K, Theodorsson E, El-Nour H. *Decreased innervation of eczematous skin in NC/Nga atopic mice during chronic mild stress.* Immunopharmacol Immunotoxicol. 2010; 32:147-152.

II. **Grip L,** Lonne-Rahm SB, Holst M, Johansson B, Nordlind K, Theodorsson E, El-Nour H. *Substance P alterations in skin and brain of chronically stressed atopic-like mice.* J Eur Acad Dermatol Venereol. 2013; 2:199-205.

III. **Lönndahl L**, Rasul A, Lonne-Rahm SB, Holst M, Johansson B, El-Nour H, Radu-Djurfeldt D, Nordlind K. *Tachykinergic markers in atopic dermatitis.* Manuscript submitted.

IV. **Lönndahl L**, Holst M, Bradley M, Killasli H, Heilborn J, Hall MA, Zou L, Nordlind K. *The substance P antagonist aprepitant shows no additive effect compared to standardized topical treatment alone, in atopic dermatitis patients.* In manuscript.

TABLE OF CONTENTS

1 BACKGROUND 1
1.1 Atopic dermatitis – clinical aspects 1
1.2 Pathogenesis of atopic dermatitis 3
1.3 The nervous system and the skin 5
1.4 Immunology of the skin 5
1.5 Interaction between the nervous and immune systems 6
1.6 Stress and neuro-endocrino-immunology 6
1.7 Tachykinins, neurogenic inflammation and atopic dermatitis 9
1.8 Atopic dermatitis, pruritus and substance P 12
2 AIMS 15
3 MATERIALS AND METHODS 17
3.1 Animal studies (I-II) 17
3.1.1 Mouse model (I-II) 17
3.1.2 Chronic mild stress (I-II) 17
3.1.3 Immunization (I-II) 18
3.1.4 Samples (I-II) 18
3.1.5 RT-PCR (II) 19
3.1.6 Immunohistochemistry (I-II) 19
3.1.7 Microscopy (I-II) 20
3.2 Human studies (III-IV) 22
3.2.1 Study design (III) 22
3.2.2 Study design (IV) 22
3.2.3 Patients (III-IV) 23
3.2.4 Extent of disease (III-IV) 24
3.2.5 Pruritus (III-IV) 24
3.2.6 Psychodemographic measurements (III-IV) 24
3.2.7 Skin sample processing (III) 25
3.2.8 Immunohistochemistry (III) 25
3.2.9 Microscopy (III) 26
3.2.10 Statistics (I-IV) 26
3.2.11 Ethical permission (I-IV) 27
4 RESULTS 29
4.1 Animal studies (I-II) 29
4.1.1 Study I 29
4.1.2 Study II 30
4.2 Human studies (III-IV) 33
4.2.1 Study III 33
4.2.2 Study IV 36
5 DISCUSSION 39
5.1 Animal studies (I-II) 39
5.1.1 Skin (I-II) 40
5.1.2 Brain (II) 42

5.2 Human studies (III-IV) 42
5.2.1 Cross sectional study (III) 42
5.2.2 Treatment study (IV) 45
6 METHODOLOGICAL CONSIDERATIONS 49
6.1 Chronic mild stress studies (I-II) 49
6.2 Image analysis (I-II) 49
6.3 Immunohistochemical methods (II-III) 50
6.4 Treatment study (IV) 50
7 CONCLUSIONS 53
8 FUTURE PERSPECTIVES 55
9 SAMMANFATTNING PÅ SVENSKA 57
10 ACKNOWLEDGEMENTS 59
11 REFERENCES 61

LIST OF ABBREVIATIONS

ACTH	Adrenocorticotropic hormone
AD	Atopic dermatitis
CRH	Corticotrophine-releasing hormone
GAP-43	Growth associated protein-43
HAD	Hospital Anxiety and Depressive scale
HPA	Hypothalamic pituitary adrenal axis
Ig	Immunoglobulin
IDEC	Inflammatory dendritic epidermal cell
IFN	Interferon
IIT	Intention to treat
IL	Interleukin
MADRS-S	Montgomery-Åsberg Depression Rating Scale-Self assessment
MCP-1	Monocyte chemotactic protein 1
NGF	Nerve growth factor
NNA	Neuropeptides and neurotrophines axis
NKA	Neurokinin A
NK-1R	Neurokinin 1 receptor
NSE	Non-stressed eczematous
PAR	Protease-activated receptor
PGP 9.5	Protein gene product 9.5
PP	Per protocol
PPT	Preprotachykinin
RT-PCR	Reverse transcription polymerase chain reaction
SA	Sympathetic axis
SE	Stressed eczematous
SC	Stressed control
SCORAD	SCORing of Atopic Dermatitis
SPF	Specific pathogen free
SSP	Swedish Universities Scales of Personality
T_H	T helper
TSLP	Thymic stromal lymphopoietin
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRP	Transient receptor potential
VAS	Visual analogue scale

1 BACKGROUND

1.1 ATOPIC DERMATITIS – CLINICAL ASPECTS

Atopic dermatitis (AD) is a common chronic inflammatory skin disease. The disease often appears in early childhood. It´s incidence has increased during past decades in industrialized countries. A lot of data concerning AD prevalence and trends has been drawn from the International Study of Asthma and Allergies in Childhood (ISAAC) (1). This study revealed that over 20% of children are affected by AD in some countries, but that the prevalence varies greatly throughout the world. The prevalence in the adult population is usually estimated to be 2-3%, but it is probably higher in industrialized countries. Studies have reported a prevalence varying between 0.3–14.3% (2-5), indicating that this skin disease truly is also a problem in adults.

AD is characterized by eczematous lesions that in infants tend to localise to extremities and cheeks, while in elder children the eczema is often evident in the flexures of arms, legs and sometimes in the gluteal region. In adults the lesions often are located to neck, flexures, hands and face.

The histological features of acute AD include epidermal intercellular oedema and dermal perivascular infiltrates of inflammatory cells including lymphocytes, dendritic cells and a few eosinophils. During the chronic phase the lesional skin exhibits a thickened epidermis with a hypertrophied upper layer. The eczematous lesions are often characterized by a severe pruritus and this often affects the night sleep and quality of life. Psychological stress is known to be a factor that could trigger and worsen the eczema, and an exacerbation of the eczema is also in itself a stressor for the patient, often resulting in impaired quality of life and sleep disturbances. *Chronic mild stress* is a stress form that describes the everyday stressors and seems to be relevant from a

patient perspective. Chronic mild stress is connected to and not always easy to differentiate from anxiety and depression.

Typically the AD is associated with asthma and allergies, as a part in the atopic march or atopic triad (including AD, food allergy, allergic rhinitis, and asthma), and then often combined with high immunoglobulin (Ig)-E levels. One suggestion has been to distinguish "true" AD (extrinsic form) from non-IgE associated dermatitis (non-atopic dermatitis, intrinsic form)(6). However, the development of IgE-antibodies, sensitisation and in some patients development of autoimmunity, has been suggested to be part of the development in different stages of AD. The other clinical characteristics of dry skin are also present in both types. Bieber (7) suggests that the development of AD has three phases; the initial non-atopic form in infancy when sensitization has not yet occurred, the second phase when sensitization to food or environmental allergens (in 60-80%) of patients takes place (transition to true AD) and lastly the development of autoantibodies in some patients as a result of skin damage due to scratching and inflammation. Thereby the division into two types of eczema has not yet become standard clinical practice.

There are several diagnostic tools for AD and one of the most commonly used is Williams' criteria (8) which include an itchy skin condition plus three or more of the following: history of flexural involvement, a history of asthma/hay fever, a history of a generalized dry skin, onset of rash under the age of 2 years, or visible flexural dermatitis. One well established way to evaluate the severity of disease is by using objective SCORing of Atopic Dermatitis (SCORAD). The SCORAD assessment can also include an evaluation of pruritus (using visual analogue scale, VAS) and sleep disturbance (subjective SCORAD).

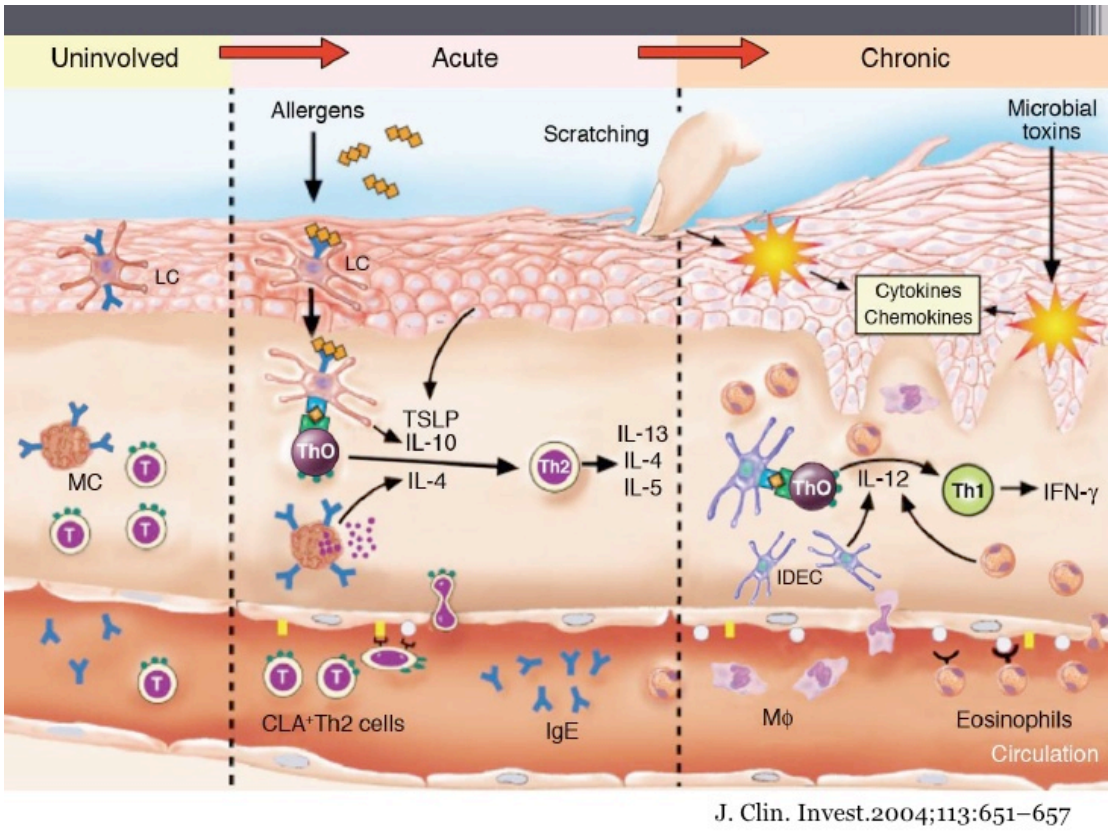
Regarding treatment there are currently rather limited ways of treating AD. The basal treatments for patients with mild-moderate symptoms include steroid crèmes,

moisturisers and topical calcineurin inhibitors (e.g. tacrolimus, pimecrolimus). The next step is often to add treatment with phototherapy and for patients suffering from severe AD the systemic treatments available are either methotrexate, systemic cyclosporine or azathioprine. There have been and are currently several trials that aim to evaluate the effect of specific immunomodulatory therapies in AD. Trials targeting e.g. interleukin (IL)-5, CD-20 IgE and tumor necrosis factor (TNF)-α have been performed without homogenous results and good clinical response (9). However, there are several ongoing trials evaluating new therapies and currently dupilumab (targeting IL-4 and IL-13) seem to be one of the most promising (10).

1.2 PATHOGENESIS OF ATOPIC DERMATITIS

The pathogenesis of the disease is complex and not fully understood but involves a disturbance in the skin barrier, abnormal IgE levels and a triggered inflammatory response with cytokine release (Fig. 1).

Regarding the skin barrier, a mutation in important genes such as the gene encoding filaggrin, has been reported (11). Filaggrin is the major source of several major components of the natural moisturizing factors in the stratum corneum. The defect could also be acquired due to a downregulation by T-helper (T_H)-2 cytokines at the protein level. Other defects of the skin barrier have been studied in AD, such as tight junction formation (reviewed in Wollenberg *et al.* (12)). Defects in barrier function and permeability increase the exposure to allergens and exotoxins. The abnormal IgE response in AD is mediated by T_H2 cells. During the acute phase of AD Langerhans cells are activated by allergens binding to IgE and high-affinity IgE receptor (FceR1). Allergen proteins are presented to T-cells and a T_H2 profile is induced. These T_H2 cells regulate and enhance type 1 hypersensitivity responses.



J. Clin. Invest.2004;113:651–657

Fig. 1. Immunologic pathways in AD. AD inflammation is associated with increased Th2 cells in acute skin lesions, but chronic AD results in the infiltration of inflammatory IDECs, macrophages (Mφ), and eosinophils. IL-12 production by these various cell types results in the switch to a Th1-type cytokine milieu associated with increased IFN-γ expression.

Cytokines released by the T_H2 cells (e.g. IL-3, IL-4, IL-5 and IL-10) stimulate this response in several ways. These include activation and migration of mast cells and other inflammatory cells (eosinophils and neutrophils), which in turn are capable of causing significant tissue injuring by releasing toxic enzymes, oxygen radicals and cytokines (13). In chronic AD lesions there is a tilted balance towards a T_H1 response and a modulation of the neuropeptides (see further below). The monocytes recruited to the skin differentiate into inflammatory dendritic epidermal cells (IDECs), which in their turn produce IL-1, IL6 and TNF-α as well as IL-12 and IL-18. These interleukins contribute to the switch and the tilted balance to T_H1/ T_H0 cells which are evident in the chronic phase of the disease (7).

1.3 THE NERVOUS SYSTEM AND THE SKIN

The skin is the largest organ in the human body, and it has important functions in protection and interaction with the outside environment. Cells forming the skin and nervous system have a common origin in the ectoderm during the embryonal development. The skin is richly innervated with a dense network of nerve fibres present in both dermis and the epidermis. There are different types of nerve fibres connecting the nervous system with the skin, sensory nerve fibres and autonomic nerve fibres. The sensory nerve fibres originate from the dorsal root ganglia and are divided into subgroups (Aβ, Aδ and C nerve fibres) depending on diameter and speed of signal transduction. Aβ fibres are myelinated and the largest (thereby transducing signals the fastest), whereas C fibres are not myelinated and smaller and transmit signals more slowly. Aδ fibres are thinly myelinated, they send impulses faster than unmyelinated C fibres, but more slowly than the thicker Aβ fibres. The nerve fibres in the skin transduct stimuli from mechanoreceptors, thermoreceptors and nociceptors that are located in the epidermis and dermis. The autonomic nerve fibres constitute a minor part of the nerves innervating the skin. These fibres innervate blood and lymphatic vessels, glands and hair follicles, and the large numbers of nerves in the dermis may even allow vessels to be exposed to neuropeptides without specific innervation (14).

1.4 IMMUNOLOGY OF THE SKIN

As mentioned in the previous section, the skin, with its various immune cells, plays a central part in our defence against microorganisms and other environmental dangers. The epidermis and the keratinocytes form the outer layer. The skin hosts microbes normally colonizing the skin, also forming a defence to foreign microbes. The skin contains a number of different immune cells. In the epidermis there are Langerhans

cells and skin-homing T-cells. The keratinocytes are also immunologically active and have the ability to mount an immune response by releasing proinflammatory mediators. The underlying dermis contains a variety of immune cells including dendritic cells, lymphocytes, mast cells and macrophages. When damage or infection occurs the recruitment of other immune cells like eosinophils, monocytes and neutrophils takes place.

1.5 INTERACTION BETWEEN THE NERVOUS AND IMMUNE SYSTEMS

There is a close relationship between the nervous and immune systems. Interactions between nerves and immune cells play an important role in skin homeostasis and disease. The mechanisms underlying this dynamic relationship are not yet fully understood.

Neuropeptides, including substance P, are predominantly released by sensory C-fibres that pass through the epidermis in a three-dimensional network with free endings. These neuropeptides are capable of directly modulating the functions of many cells including keratinocytes (15), mast cells (16), Langerhans cells (17, 18), dermal microvascular endothelial cells (19), lymphocytes (20) and macrophages (21) For example substance P, at physiological serum concentrations, has an enhancing effect on both interferon (IFN)-γ and IL-4 synthesis in stimulated lymphocytes (22).

1.6 STRESS AND NEURO-ENDOCRINO-IMMUNOLOGY

When a psychologically or a physiologically stressful situation occurs the body responds via the systems described above. This has different effects on inflammatory processes in the body. The interactions between the systems are complex and only partly explored.

Several chronic inflammatory skin diseases like AD, psoriasis, urticaria and rosacea etc. are reported to be worsened by stress. The stress response has developed from the immune system and remains tightly connected and is the oldest response to environmental changes (23).

In chronic inflammatory skin diseases there are three different systems that could be considered to influence the process, by different pathways. The hypothalamic pituitary adrenal (HPA) axis via cortisol, the sympathetic (SA) axis via release of adrenalin and noradrenalin, and the third axis, also known as the neuropeptides and neurotrophines axis (NNA) (23), and which includes the release of neurotransmittors such as substance P as a direct neurological response to stress, both centrally and in the periphery.

The HPA axis is considered to be the most important pathway in regulating peripheral inflammation due to the strongly immunomodulatory actions of glucocorticoids. Stressors of both psychological and physiological origin activate the HPA axis. It has been shown that peripheral inflammation can activate neurons in the hypothalamus through the activation of proinflammatory cytokines and also by ascending sensory pathways (reviewed in Maier (24) and Rosenkranz (25)). Another study also suggests that peripheral inflammation can have a direct effect on the brain through toll-like receptor (TLR)-4 (26). Neurons in the hypothalamus stimulate the release of corticotrophine-releasing hormone (CRH) to the anterior pituitary, which subsequently leads to the systemic release of adrenocorticotrophic hormone (ACTH). The adrenal cortex responds by releasing glucocorticoids.

There is no conclusive evidence that corticosteroids are indeed increased or decreased during chronic inflammatory diseases. It has been suggested that a lowered level of glucocorticoids, e.g. a lowered activity of the HPA axis, would be a part of the development of inflammatory and autoimmune diseases. This is considered to be due to chronic stress or chronic inflammation that impairs the system (27-29). There have also

been several contradictory reports, demonstrating normal or increased levels of ACTH and glukocorticoids in animal models of chronic inflammation (reviewed in Harbuz *et al.* (30) and Rosenkranz (25)). The impact of inflammation on the HPA axis has been suggested to mediated by substance P, in a rat model (31).

The SA axis is the autonomic response to stressors. It is a reflex that increases heart rate, blood pressure and the release of adrenaline and noradrenaline from peripheral nerve endings. The possibility to regulate bloodflow also affects inflammation *per se*. These catecholamines modulate inflammation through interaction with sensory nerves and immune cells.

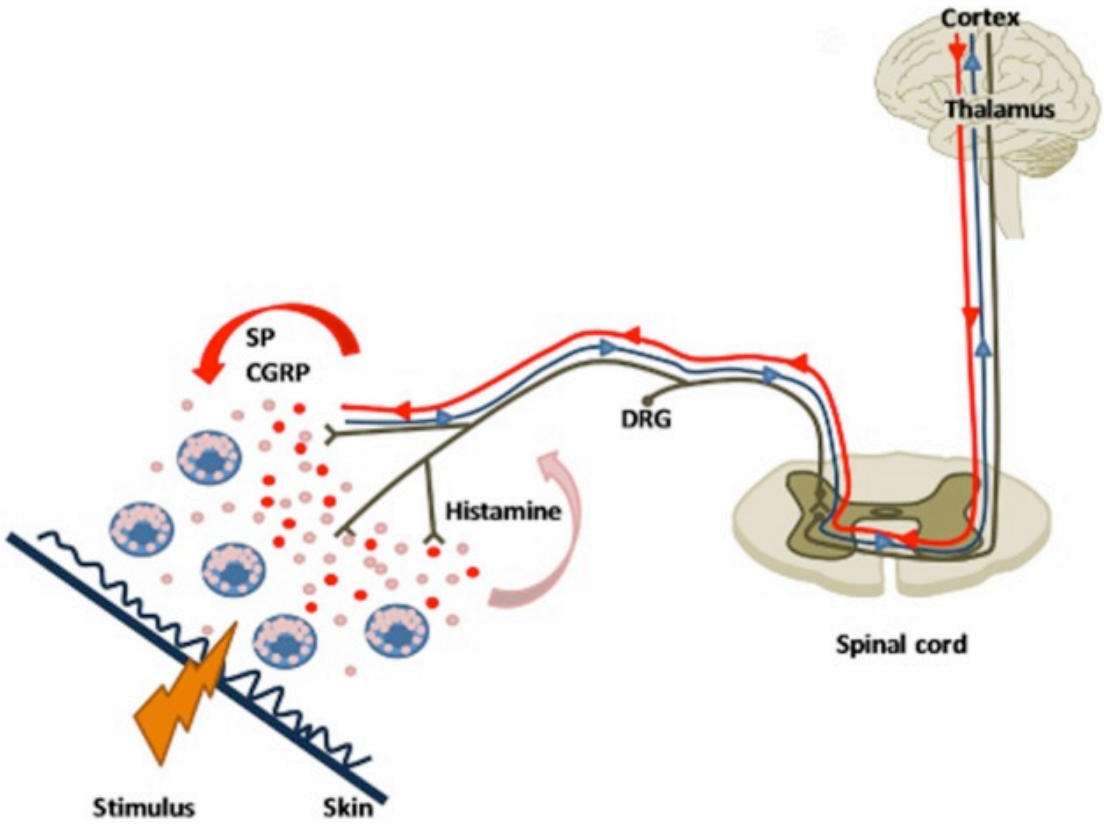
The effect of adrenaline and noradrenaline is generally considered to be anti-inflammatory due to their inhibitory effects on immune cells and the ability to activate CRH-releasing neurones in the hypothalamus. The effect can, however, vary depending on the dose of and the type of adrenergic receptor stimulated, and also during which step of the immune response the cells are exposed to the neurotransmitter (reviewed in Besedovsky and Ray (32), and Rosenkranz (25)). In this context it should also be mentioned that a defect parasympathetic system has been suggested to link chronic stress and itch (33). This imbalance in the autonomic system results in a prolonged activation of the HPA axis as well as modulation of the descending regulating system and could cause both central and peripheral sensitization, potentiating itch.

The third axis, the NNA axis, is not as well described and understood as the other systems mediating stress. Neurotrophines and neuropeptides are released both centrally and peripherally as a response to physical or psychological stress (reviewed in Liezmann *et al.* (23)). It has also been shown that neuropeptides can suppress activation of the HPA axis (23, 34). In the periphery the release of neuropeptides mediate proinflammatory processes (see below in the section Neurogenic inflammation).

As discussed above, when considering the development and exacerbation of chronic inflammatory diseases, the basal stress levels seem to be of importance. The immune system responds to acute stress by increased pro-inflammatory cytokines such as IFN-γ, resulting in a fast tissue damaging cellular response. Conversely, chronic stress (for example in the form of critical life events or increased every day life stress) is different and has other effects on the immune system. Chronic stress increases the basal cortisol levels and shifts the immune response from cellular to humoral, and the production of other interleukins like IL-4 which is a T_H 2 cytokine, increases. This is thought to facilitate the development of atopic and autoimmune disease, summarized in Liezmann *et al.* (23). Buske-Kirschbaum *et al.* has in several articles, see (35) demonstrated a blunted response of the HPA axis in patients with atopic disease (AD and asthma). This blunted response has, as described above been linked to chronic inflammatory disease (e.g., autoimmune and allergic disease).

1.7 TACHYKININS, NEUROGENIC INFLAMMATION AND ATOPIC DERMATITIS.

Neurogenic inflammation is inflammation mediated by the release of inflammatory neuropeptides like substance P and calcitonin gene-related peptide (CGRP) from afferent neurons. Once released these mediators induce the release of histamine from adjacent mast cells. In turn, histamine binding to nerve cells evokes the release of substance P and CGRP. Thus, a bidirectional link between histamine release from mast cells and neuropeptides is established (Fig. 2).



Br J Pharmacol. 2013;170:38-45

Fig. 2. Neurogenic inflammation and itch. Histamine release from mast cells after a local stimulus activates neurons communicating with the thalamus and cortex (blue line). Next, this creates activation in opposite direction (red line) and induces the release of mediators from sensory endings, like SP and CGRP. The SP and CGRP released causes further mast cell degranulation, resulting in vasodilatation (flare) and the recruitment of other inflammatory mediators.

Focusing on the neurogenic inflammation in the pathogenesis of AD, tachykinins are presumably important mediators. Substance P is the most abundant tachykinin peptide in mammals and is widely distributed in the peripheral and the central nervous system (CNS) of many species (36). Three types of tachykinin receptors (R) have been identified to date in mammals: neurokinin (NK)-1, NK-2 and NK-3 receptors. Although all endogenous tachykinins can bind to all three receptor types, substance P has the highest affinity for NK-1R (37). Neurokinin (NK)-A is encoded from the same precursor gene, preprotachykinin (PPT)-A, and this peptide has also been suggested to be involved in the inflammatory response, acting mainly through the NK-2R.

As mentioned above, stress is a possible trigger for worsening and possible development of AD. The exact mechanisms underlying this are not yet known, but the release of substance P could be one reason (38). Data indicate that substance P could play an important role for stress induction and worsening of AD. It has been reported that stress-induced (*acute* sound stress) worsening of AD is caused by substance P-dependent neurogenic inflammation, and also shifts the cytokine profile towards T_H2 (39). This further emphasizes the importance of stress in development of allergic diseases.

An altered number of substance P positive nerve fibres and levels of substance P have been reported in the lesional skin of human AD, summarized in Salomon and Baran (40). It has also been demonstrated that the number of mast cells containing substance P was increased in AD patients (41) and such an increase has also been noted in NC/Nga mice (see below) (42). Substance P mediates the degranulation of mast cells. The mast cells in turn contain substances such as histamine, cytokines and substance P that have different physiological effects. Substance P is also able to specifically bind to keratinocytes and to stimulate them to release cytokines such as IL-1, which in turn can enhance inflammation(43). Other cell types that contain and can release substance P are granulocytes, monocytes, activated lymphocytes(44, 45) and keratinocytes (44)

Substance P upregulates TLR-2 in mast cells and potentiates signalling pathways associated with TLR-2. This suggests that the immune response to bacterial infections in the skin involves the production of substance P and amplification of TLR activation (26) In addition, substance P modifies immune responses of atopic T cells to *Dermatophagoides farinae* (in patients sensitized to this mite antigen), by promoting proliferation and altering cytokine profiles, indicating that it might modulate the clinical manifestations of AD (46). This modulation of the cytokine expression could

also, in theory, influence the response to *Staphylococcus aureus*. The release of substance P in the skin has been suggested to contribute to the priming of the immune response, so that bacterial infections can be cleared (26). One could speculate that there is an excessive release of substance P in the skin as a result of inflammation and stress, and a following blunted response to substance P. This blunted response to substance P has been suggested in AD (47, 48). This could, in turn, contribute to an impaired immunological response and a subsequent reduced defence to microbial infections. Suppression of the innate immune system could explain the colonisation with *Staphylococcus aureus* in more than 70% of AD patients (49).

NKA has not, to the best of our knowledge, previously been studied in AD *per se* but this neuropeptide has been suggested to be involved in skin inflammation through its actions on cell types such as keratinocytes (15). An addition, it has been reported that NKA activates dendritic cell-mediated T_H1 immune response via NK-2R in a mouse model of asthma (50).

In this way tachykinins, substance P and NKA can all mediate an immune response, suggesting their involvement in the pathophysiology of AD and especially the worsening of AD during stress.

1.8 ATOPIC DERMATITIS, PRURITUS AND SUBSTANCE P

Pruritus is one of the main symptoms in AD and the point prevalence of chronic itch has been estimated to be around 87-100% (51). The pruritus is part of the vicious cycle of AD resulting in scratching, increased inflammation and impaired skin barrier, creating worse inflammation and pruritus. The mechanisms involved in pruritus in AD is complex and there is no single cause (reviewed in Mollanazar *et al.* (51)). Substance P is a potent mediator of pruritus and can act both through histamine-dependent and histamine-independent pathways. The nerve fibres that are responsible for mediating

itch are sensory afferent nerve fibres of C -type. It should be noted, however, that pruritus can be induced through several mechanisms, including disruption of the skin barrier and an upregulated inflammatory response, resulting in the release of itch-provoking substances like proteases, histamine, neuropeptides and interleukins. An increase of innervation (reviewed in Tominaga and Takamori (52)) and substance P positive nerve fibres in lesional skin in AD (summarized by Salomon and Baran (40)) have been reported. This could result in an increased ability to react to pruritogenic stimuli, and also in an increased release of substance P. There is also a possibility of a peripheral sensitisation as well as an activation of centres related to pruritus in the CNS (reviewed in Mollanazar *et al*. (51) and Yosipovitch and Papoui (53)).

As mentioned above, substance P is an important pruritic mediator in humans and mice (54, 55). When substance P is released it binds to NK-1R on mast cells, keratinocytes and cutaneous nerve endings, resulting in the release of pruritogenic mediators. There are several receptors that have the ability to mediate itch. Of these, the transient receptor potential (TRP) superfamily is a class involved in sensory perception, including itch, as well as other various sensory modalities (e.g. chemical and thermal) as reviewed in Mollanazar *et al*. (51). Several subfamilies of the TRP superfamily are expressed in normal human skin and have been implicated in itch: TRPV1, TRPA1, and TRPM8. Histamine causes activation of TRPV1 and result in the perception of itch, in contrast to TRPA1 that seems to be activated by non-histaminergic mediators. Another family that mediates itch via non-histaminergic pathways are the protease-activated receptors (PAR), PAR 2 and PAR4. As the name suggests these receptors are activated by proteases, released during inflammation in the skin. In this context, substance P has the ability to interfere in the different pathways mediating itch. For example substance P causes mast-cell degranulation and the release of histamine, resulting in activation of the histaminergic pathway.

Furthermore, the release of proteases from mast cells and other inflammatory cells can activate non-histaminergic pathways by the receptors described above. The binding of substance P to NK-1R on nerve endings in the dorsal horn could also activate neurons transducing itch, indicating substance P to be an important mediator at different levels (56). Substance P is, as described above, a proinflammatory peptide and can thereby also indirectly worsen the itch by worsening the inflammation, resulting in a local increase of all the pruritogenic mediators mentioned above. In addition, an increased inflammation affects the skin barrier, and a damaged skin barrier *per se* seems to be able to cause itch, possible by the TRPA1R (57). Keratinocytes are also able to mediate itch via sensory nerve fibres and factors like an impaired skin barrier, epidermal inflammation and the release of substance P itself can affect the keratinocytes. Consequently, substance P can induce pruritus, even though it might be indirectly (51).

Furthermore, it has been shown that the scratching behaviour in a mouse strain is inhibited by a NK-1R antagonist (55). Studies have been made using a NK-1R antagonist, aprepitant (Emend ®), on different types of pruritic conditions in humans with promising results (58).

2 AIMS

The aim of the study was to investigate the role of tachykinins in the pathogenesis of AD, pruritus and worsening during stress. This has been the focus of the following studies:

I. To examine nerve fibres and axonal growth in the skin in a mouse model of AD and chronic mild stress, and to determine if any modulation in eczematous skin and/or modulation is due to chronic mild stress.

II. To examine a possible connection between chronic mild stress and changes in the expression of substance P and its receptor NK-1 in the skin and stress-related brain regions, in the same mouse model.

III. To study the expression of tachykinins; substance P, NKA and the NK-1R, in human AD skin, as well as possible correlations to clinical and psychodemographic parameters.

IV. To examine the effect of a substance P antagonist in patients with moderate/severe AD.

3 MATERIALS AND METHODS

3.1 ANIMAL STUDIES (I-II)

3.1.1 Mouse model (I-II)

One often-used animal model to obtain an AD-like lesion is the NC/Nga mouse. Itching dermatitis, being similar to AD in humans, arises spontaneously at the age of 8 weeks in NC/Nga mice kept in a non-sterile environment. In sublines of this strain eczema is developed by challenging the skin with a mite antigen (59). These animals constitute a good model for studying the processes in AD that takes place in the skin, and for comparative studies of human pathogenesis (60) .

3.1.2 Chronic mild stress (I-II)

A modification of the chronic mild stress procedure described by Lanfumey *et al.*(61) was used. A Scantainer box type 50-SCNT-Z11 (Scanbur AS, Köge, Denmark) was used to home the mice and 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 were employed. Chronic mild stress was maintained for 8 weeks, at which point the immunization protocol was initiated and stress maintained for another 4 weeks, after which the mice were sacrificed.

3.1.3 Immunization (I-II)

The mice were divided into 3 groups (8 mice per group). One group was stressed and immunized (stressed eczematous, SE). A non-immunized control group was stressed (stressed control, SC), with the mice being painted on the ears using the vehicle only, whereas a second immunized group was not exposed to stress (non-stressed eczematous, NSE). Eczema was induced (also in the NSE group) by painting their ears with a mite antigen, *Dermatophagoides pteronyssinus* (Allergon, Ängelholm, Sweden), which had been dissolved in phosphate buffered saline (PBS) and 0.5% Tween 20 at a concentration of 10 mg/ml. All mice were maintained on a 12 h light/dark cycle with a temperature between 18 and 22^{o}C.

3.1.4 Samples (I-II)

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

Tissue samples from the ears and brains were subjected to reverse transcription polymerase chain reaction (RT-PCR) and immunohistochemistry in order to analyse changes in expression of substance P or NK-1R. For RT-PCR samples were snap-frozen on dry ice and for immunohistochemistry they were fixed in 4% formalin with 0.2% picric acid for 2 h at 4°C. The immunohistochemistry samples were then rinsed with 0.1 mol/L phosphate buffer containing 10% sucrose for at least 24 h. Tissues were next embedded in Tissue-tek (Sakura Finetek, Zoeterwoude, The Netherlands) and 14 µm thick skin and sagittal brain cryostat (Microm, Heidelberg, Germany) sections were

mounted on glass slides (SuperFrost Plus, Menzel-Gläser, Freiburg, Germany) and stored at -70°C.

3.1.5 RT-PCR (II)

RT-PCR was used to determine PPT-A and NK-1R mRNA from mice skin and brain.

Total RNA was extracted from the skin and brain samples (Aurum Total RNA Fatty and Fibrous Tissue Kit, Bio-Rad, Sundbyberg, Sweden). First strand cDNA for PCR was generated from 1µg of total RNA using the manufacturer´s kit (Bio-Rad). Primers for amplification were as follows: PPT-A (289 bp) sense ACCTGCTCCACTCCTGCACCG CGGCCAAG, antisense GAACTGCTGAGGCTTGGGTCTTCGGGCGAT, NK-1R (440 bp) sense TTCCCCAACACCTCCACCAA, antisense AGCCAGGACCCAGATGACAA. PCRs were performed in a thermal cycler (GeneAmp PCR System 2700, Applied Biosystems, Stockholm, Sweden) using experimental conditions that had been set up in previous experiments. After migration the bands corresponding to the amplified products (characterized on the basis of their molecular weights) were photographed and their sizes analysed (Image J program).

3.1.6 Immunohistochemistry (I-II)

Sections were prepared as described above followed by immunohistochemistry for protein gene product (PGP) 9.5, growth associated protein (GAP)-34, substance P, NK-1R and tryptase. Antibodies were incubated for 40 min with 10% normal goat serum (S-100, Vector, Burlingame, CA, USA), in order to minimize non-specific protein binding. The sections were then incubated with rabbit polyclonal antibodies directed against PGP 9.5 (dilution 1:10 000; RA95101, UltraClone, Cambridge,

England), GAP-43 (1:3 000; AB5220, Chemicon, Temecula, CA, USA), substance P (T-4107; 1:10 000, Bachem, St Helens, England), NK-1R (NB 300-101; 1:5 000, Novus Biologicals, Littleton, CO, USA) or tryptase (1:10 000) a kind gift from Prof. I. Harvima, Kuopio, Finland) at 4°C overnight. Thereafter, incubation with biotin-labeled goat anti-rabbit (BA-1000, 1:200) as the secondary antibody was performed for 40 min at room temperature followed by treatment with the fluorochrome Cy2-labelled streptavidine (PA42001; 1:2 000, Amersham Pharmacia Biotech, Uppsala, Sweden), also for 40 min at room temperature. All antibody solutions were diluted in PBS containing 1 % bovine serum albumin (BSA) (A9418, Sigma-Aldrich, Stockholm, Sweden) prior to use.

Acetylcholinesterase histochemistry was used to confirm the location of amygdala, and to match it with the expression of substance P and NK-1R on adjacent sections.

As negative controls, either the primary antibodies were omitted or normal rabbit serum (X0936; Dako, Glostrup, Denmark) was used instead of the primary antibody. In addition, pre-adsorbtion of substance P and NK-1R antibodies was performed using 10^{-6} mol/L of substance P (H1890, Bachem) and 4 µg/ml of NK-1R (ab92818, Abcam, Cambridge, UK), respectively. These control experiments resulted in a loss of, or a substantially decreased, signal.

Finally, the sections were rinsed in PBS and mounted with Kaiser´s glycerol gelatin (Merck, Darmstadt, Germany).

3.1.7 Microscopy (I-II)

Immunoreactivities were observed and documented by epifluorescence (Zeiss Axioskop 2 MOT microscope, Carl Zeiss, Stockholm, Sweden).

3.1.7.1 Study I

The PGP 9.5 stained sections were analysed using a software program (see *Image analysis 2.I.7.3).* 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).

3.1.7.2 Study II

Substance P-labelled brain sections were subjected to image analysis (see *Image analysis 2.I.7.3)*. Regarding prefrontal cortex and amygdala, two selected areas per mouse were analysed and the mean was calculated, while one area per mouse was analysed for the CA1 and CA3 regions of hippocampus.

Substance P positive nerve fibres in the skin were counted manually due to the low contrast between positive nerves and surrounding tissue, which prohibited satisfactory signal-to-noise discrimination with our image analysis program. From the substance P-labelled skin sections 2 areas (microscopic fields) per section (8 per mouse) were counted. The same number of areas was chosen for tryptase-labelled sections. Mast cell degranulation was assessed using a semiquantative method (low = 1, moderate = 2, high = 3). The skin sections immunolabelled for NK-1R were also analysed using a semi-quantitative method, where the intensity (as above) of the immunoreactivity was microscopically evaluated on coded slides by one observer (LG).

3.1.7.3 Image analysis (I-II)

Appropriate software (Easy Image Analysis, Bergström Instruments, Solna, Sweden) was applied to analyse the amount of substance P immunoreactivity in digital images of the brains. Results are presented as area fraction (ratio of specifically immunoreactive/ non-fluorescent area).

3.2 HUMAN STUDIES (III-IV)

3.2.1 Study design (III)

A cross-sectional study was performed to investigate the expression of tachykinins substance P, NKA and the NK-1R in human skin of AD, as well as possible correlations to clinical and psychodemographic parameters.

3.2.2 Study design (IV)

An open randomized trial was conducted with an active treatment period of 7 days in order to investigate the effect of aprepitant on extent of disease and pruritus in AD, and also to investigate if there would be any effect on depression and anxiety. All participants received oral and written information about the study and voluntarily signed an informed consent. The study was conducted during the period from October 2013 to March 2015.

Randomization was performed using a randomization list with no stratification. The nurses who evaluated SCORAD in the patients were unaware of which treatment group the patients belonged to.

The patients received 80 mg of aprepitant daily for 7 days in addition to topical treatment with a moisturizer and a moderately strong steroid cream (hydrocortisone

butyrate; Locoid ®). The control group received only the topical treatment. The patients were monitored regarding extent of the disease (SCORAD), their degree of pruritus, as well as anxiety and depressive scores (see below) using Montgomery-Åsberg Depression Rating Scale-Self assessment (MADRS-S).

3.2.3 Patients (III-IV)

3.2.3.1 Study III

Twenty-eight adult AD patients, 18 females and 10 males, mean age of 29.5 years (range 19-48 years) were included in the study. The patients were recruited among referred patients to our clinical department. To be included in the study the patients should have an ongoing AD according to Williams *et al. (8)* and they should not receive systemic therapy (including phototherapy and antihistamines) during the study, or within one month prior to inclusion. All patients included, except one, fulfilled all procedures; one female did not complete the questionnaires.

Ten healthy individuals, 5 males and 5 females (mean age 37.7 years, range 20–61 years), with no previous atopic manifestations such as hay fever, asthma or AD, were recruited as controls for the histochemical parameters.

3.2.3.2 Study IV

Forty-one adult patients between 20-50 years of age, both females (n=23) and males (n=18), were included in the study. The patients attended our out-patient unit at the Department of Dermatology, Karolinska University Hospital, Solna, Sweden. The patients had a moderate – severe (measured as SCORAD >20) AD, and diagnosis was determined according to Williams´ criteria (8). To be included they needed to be otherwise healthy. Except for contraceptives they should have no other medication, such as regular asthma and allergy medication. Other exclusion criteria were dark skin

and skin infections. Patients who were pregnant, breast-feeding or planning to become pregnant were also excluded.

3.2.4 Extent of disease (III-IV)

The extent of the disease was assessed using SCORAD (62). Both objective and subjective SCORADs were included.

3.2.5 Pruritus (III-IV)

The degree of pruritus was assessed using a visual analogue scale (VAS) in which patients rate their own pruritus for the last three days using a scale 0-10 (0 = no pruritus, 10 = worst imaginable pruritus). In addition, in study IV, the patients registered scratching movements per day using a manual counter (Clas Ohlson, Insjön, Sweden). The patients were asked to register either a scratching movement or in the case of several movements, an episode.

3.2.6 Psychodemographic measurements (III-IV)

In study III SSP (Swedish Universities Scales of Personality) (63), a 91-item questionnaire, was used to evaluate patients personality traits. The questionnaire was completed by the patients themselves and then analysed regarding somatic trait anxiety, psychic trait anxiety, stress susceptibility, impulsivity, trait irritability, lack of assertiveness, verbal trait aggression and physical trait aggression. Absolute scale values were calculated.

To assess the level of depressive symptoms in study III and IV, MADRS-S (64) was used, describing the patient´s current psychological status.

In study IV, to assess depression and anxiety the Hospital Anxiety and Depressive scale, HAD (65) was used. This is a simple self-assessment questionnaire that reflects the patient's mood. It assesses anxiety and depression simultaneously.

3.2.7 Skin sample processing (III)

Biopsies, 3 mm in diameter, were taken from lesional skin (characterized by dryness, papules, and often lichenification) of the bend of the arm, and from non-lesional skin from the sacral region. Biopsies from healthy control persons were also taken from the sacral region. No topical steroids should have been used for at least 14 days on these areas prior to inclusion in the study. The biopsies were then fixed and sectioned as for the mouse tissue specimens described above (1.1.3)

3.2.8 Immunohistochemistry (III)

The immunohistochemistry process was performed for substance P and NK-1R as described in the previous section (1.1.5). Regarding NKA the antibody used was T-445; (1:5 000, Bachem)

Double staining was performed in order to characterize the cellular phenotype expressing substance P, NKA and NK-1R. The sections were incubated with the primary antibodies, followed by secondary biotinylated antibody as above and Alexa SAV 555 red or Alexa 488 green (1:1 000, Life Technologies, Stockholm, Sweden). Monoclonal antibodies specific for CD3 (555916; 1:25, BD Pharmingen, Franklin Lakes, NJ, USA) or tryptase (MAB1222 ;1:1 000, Chemicon) were then added, followed by Alexa 488 green or Alexa 594 red (1:1 000). The substance P positive cells were mostly positively stained for CD3, indicating that majority of cells were lymphocytes. NKA positive cells were mostly positive for tryptase. The majority of

NK-1R positive cells were positively stained with tryptase, indicating these cells to be mast cells.

3.2.9 Microscopy (III)

General histopathological changes, hyperkeratosis, acanthosis and degree of inflammation (infiltration of inflammatory cells in the dermis) were graded semi quantitatively, 0-3 (0 = normal appearance, 1 = mild, 2 = moderate and 3 = strong).

Sections were counted manually by one observer (LL). For the absolute numbers of substance P and NKA positive cells and nerve fibres, and NK-1R positive (mostly dendritic) cells, the mean value per section was calculated and standardized to 2.5 fields of vision. Four sections per biopsy were analysed. The semi-quantitative evaluations for NK-1R cellular epidermal infiltration and NKA degranulation were graded 0-3 (0 = negative, 1 = mild, 2 = moderate and 3 = strong). NK-1R cell degranulation was graded 0-1 (0= none, 1= positive).

3.2.10 Statistics (I-IV)

3.2.10.1 Study I-II

For statistical analysis the Student´s t-test or the Mann-Whitney test were used depending on distribution of the data. In addition, Chi-square was used to analyse semiquantitative data. A *p*- value of < 0.05 was considered statistically significant.

3.2.10.2 Study III

The numerical calculations for substance P, NKA and NK-1R were calculated using Student's t-test or Mann-Whitney test (depending on distribution of the values). For semiquantitative evaluation Chi-square was used. Correlations between the different

parameters were measured using Spearman´s or Pearson´s tests (depending on distribution of the values). Differences were considered to be statistically significant when $p < 0.05$.

3.2.10.3 Study IV

Statistical analysis was performed for all patients included in the study (intention to treat, IIT), as well as on values for patients that would continue in the study as planned by protocol (per protocol, PP). Lost values would be included in the IIT-analysis using the principle last value carried forward. Statistical comparisons in order to test differences between two independent groups were made by use of the Student's t-test for uncorrelated means, after validation for normal distribution by use of the Shapiro Wilk test. The dimension of the study was calculated to provide 80% power to detect a difference in SCORAD-improvement of 3 points at the 5% significant level (2-sided).

3.2.11 Ethical permission (I-IV)

Ethical permissions were obtained for all of the studies. Number N440/04 for study I-II, and 2010/800-31/3 for study III. The protocol for the fourth study was approved both by the local ethics committee, number 2013/1158-31/3, and by the Medical Products Agency.

4 RESULTS

4.1 ANIMAL STUDIES (I-II)

4.1.1 Study I

4.1.1.1 Combination of chronic stress and eczema results in decreased corticosterone levels and increased degree of inflammation

To estimate levels of chronic stress, corticosterone levels were determined. At the termination of the experiment the SE group exhibited lower corticosterone levels, 496.4 ± 221.5 (mean±SD) ng/ ml, compared to the other groups, NSE 738.6 ± 232.1, and SC 805.2 ± 144.4 ng/ml.

To estimate the degree of inflammation the diameter of the ears in millimetre was used. The results showed that 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 ($p < 0.001$), indicating a higher degree of inflammation.

4.1.1.2 Chronic mild stress has an additive effect in eczematous skin and results in decreased innervation

To investigate the impact that inflammation and stress could have on innervation, expression of PGP 9.5 was analysed. In the epidermis there was a decrease ($p < 0.01$) in the PGP 9.5 positive nerve fibres in the SE, measuring area fraction, 0.8 ± 0.3, compared to the NSE group (2.4 ± 1.2) and the SC group (2.5 ± 1.2). This was also true for the dermis where there was a decrease of the PGP 9.5 positive nerve fibres in the SE (1.3 ± 0.3) compared to the NSE group (2.9 ± 1.2, $p < 0.01$) and the SC group (3.4 ± 0.9, $p < 0.001$).

4.1.1.3 Inflammation decreases newly formed nerve fibres in skin

To investigate newly formed nerve fibres analysis of GAP-43 was performed. In the epidermis the results revealed a decrease ($p<0.01$) in the GAP-43 positive fibres in the SE (40.7 ± 20.5 fibres per section) compared to the SC group (75.9 ± 20.4). The NSE (48.1 ± 28.8) compared to the SC group also showed a decrease ($p<0.05$). There was no difference in the SE group compared to the NSE group. In the dermis similar results were observed with a decrease ($p < 0.05$) in the number of GAP-43 positive dermal fibres in the SE (108.6 ± 29.7 fibres per section) compared to the SC group (133.5 ± 31.8). The value for the NSE group was 122.9 ± 27.5.

4.1.2 Study II

4.1.2.1 RT-PCR in skin – chronic mild stress further increases NK-1R in eczematous skin.

To analyse the expression of substance P and NK-1R, on RNA-level, PCR was performed.

No difference in the PPT-A mRNA signals could be determined between the three groups.

An increased NK-1R signal ($p < 0.05$) was found in SE (0.95, median; 0.22 quartile deviation), compared to SC (0.71; 0.17) mice. A strong tendency towards an increase ($p = 0.06$) was also found in SE compared to NSE (0.73; 0.45) mice.

4.1.2.2 Immunohistochemistry in skin - chronic mild stress further decreases expression of substance P and increases mast cell activity in eczematous skin

To further evaluate the impact of stress and inflammation in skin investigation of expression of substance P, NK-1R and mast cells in skin was performed. A significantly lower ($p < 0.05$) number of substance P positive fibres/area in dermis and epidermis was evident in the SE group (0.8; 0.3), compared to SC (1.4; 0.9) group. The corresponding value for the NSE group was (1.6; 1.5), suggesting that the lower number of substance P fibres in the SE group was caused by a combination of mild stress and immunization.

The mean intensity of NK-1R immunoreactivity was higher in epidermis of NSE and SE mice (NSE: 2.2 ± 0.7, mean ± SD, SE: 2.0 ± 0.8), compared to SC mice (1.7 ± 0.5), although these differences did not reach statistical significance. Neither NK-1R immunoreactive inflammatory cells nor nerve fibres were observed.

An increase of tryptase immunoreactive mast cells/area in dermis in SE (89 ± 18) compared to both SC (52 ± 17) and NSE (63 ± 26) mice ($p < 0.001$ and $p < 0.05$, respectively), was observed. Mast cells also showed increased ($p < 0.05$) degranulation in SE (2.8 ± 0.7) compared to both NSE (1.8 ± 0.9) and SC (1.6 ± 0.7) mice.

4.1.2.3 RT-PCR in brain – no differences found

No significant differences or stable trends in RNA expression levels were found in the three groups for PPT-A or NK-1R.

4.1.2.4 Immunohistochemistry in brain – combination of chronic mild stress and eczema decreases substance P expression in hippocampus

In order to investigate the expression of substance P and NK-1R in the central nervous system, and discover possible modulations of inflammation and chronic mild stress, analysis was made of prefrontal cortex, amygdala and hippocampus.
Substance P immunoreactivity was abundantly evident in the prefrontal cortex. In the amygdala the most prominent substance P activity occurred in the central nucleus which prompted further analysis. We recorded more immunoreactivity in the medial compared to the lateral hippocampus, and hence decided to analyse that part of the hippocampus. The CA1 and CA3 areas of medial hippocampus were analysed separately.

In CA1 a tendency toward a decrease ($p = 0.08$) in area fraction of substance P positive nerve fibres and neuronal cells was apparent in SE, 3.2 (median); 3.9 (quartile deviation), compared to NSE (7.8; 2.9) mice. The area fraction was 5.2; 5.4 in SC mice. In CA3, a decrease ($p < 0.05$) of substance P positive nerve fibres and neuronal cells was seen in SE (0.6; 0.7) compared to NSE (1.8; 1.5) mice. In addition, a tendency toward a decrease ($p = 0.06$) in SE compared with SC (1.1; 0.5) mice was noted.

In the prefrontal cortex there was no significant difference between the groups in terms of amounts of substance P positive nerve fibres and neuronal cells, the area fractions being (0.90; 1.35), (1.33; 0.35), (1.07; 0.85), for SE, NSE and SC, respectively.

In the amygdala there was no significant difference between the groups with regard to amounts of substance P immunoreactive nerve fibres. The area fractions were (3.84; 3.17), (7.90; 3.43), (4.13; 2.95), for SE, NSE and SC, respectively.

NK-1R immunoreactivity was not detected in prefrontal cortex, amygdala or hippocampus, in the different groups. NK-1R positive nerve fibres/cells were, however, observed in other parts (e.g. striatum) of the brain.

A few tryptase positive mast cells could be observed throughout cerebrum, particularly located around vessels.

4.2 HUMAN STUDIES (III-IV)

4.2.1 Study III

4.2.1.1 Extent of disease

The extent of the disease was assessed using SCORAD. Both objective and subjective SCORAD were included. The mean objective SCORAD among included patients was 42.3 ± 11.5 (mean ± SD) and subjective SCORAD 51.6 ± 13.4.

4.2.1.2 Pruritus

The degree of pruritus was assessed using VAS, in which the patients rated their own pruritus, for the last three days. The pruritus intensity among the patients was 5.2 ± 2.4.

4.2.1.3 Psychodemographic data

Psychodemographic data were analysed in order to establish correlations to clinical or histological parameters. The score for somatic trait anxiety was 15.1 ± 4.3, psychic trait anxiety 15.2 ± 3.8, and stress susceptibility 16.3 ± 4.1. The depression score (measured

using MADRS-S) was 8.0 ± 6.5. Impulsivity score was 17.0 ±3.9, verbal trait aggression being 13.5 ± 4.2, and physical trait aggression 12.7 ± 4.7.

4.2.1.4 General histopathological findings – increased inflammatory signs in lesional skin

The skin samples were analysed in order to investigate the histological appearance. The results showed that the degree of acanthosis was elevated ($p < 0.001$), 2.1 ± 0.8, in lesional skin compared to non-lesional, 0.6 ± 0.8, skin, and control, 0.6 ± 0.5, skin ($p = 0.001$). The degree of inflammation was also elevated ($p < 0.001$) in lesional, 2.4 ± 0.7, compared to non-lesional, 1.1 ± 0.8, skin, and there was also a strong tendency ($p = 0.06$) to increase compared to control skin, 0.3 ± 0.5.

4.2.1.5 Immunohistochemistry – increased expression of substance P nerve fibres in lesional skin

The skin samples were further stained for tachykinins and NK-1R in order to establish any differences between AD and control skin and to investigate if there would be any correlations to psychodemographic or clinical parameters.

The immunohistochemical analysis revealed an increase ($p < 0.01$) in substance P positive fibres in lesional, 2.1 ± 2.3, compared to non-lesional, 0.7 ± 1.0, skin, and an increase ($p < 0.05$) in control 1.4 ± 1.1 compared to non-lesional skin. Regarding substance P positive cells, the values for lesional skin were 91 ± 77, non-lesional 58 ± 38 and control skin 48 ± 20, the differences being not statistically significant.

4.2.1.6 Immunohistochemistry – increased expression of NKA positive cells and fibres in lesional skin

For NKA positive fibres the immunohistochemical analysis resulted in an increase ($p < 0.01$) in lesional, 2.8 ± 2.4, compared to non-lesional, 1.0 ± 1.3, skin. Control was 1.4 ± 1.4. There was an increase of NKA positive dendritic cells, in the dermis in lesional, 2.1 ± 5.9, compared to both non-lesional, 0.3 ± 0.8 ($p < 0.01$), and control, 0.2 ± 0.6 ($p < 0.05$), skin.

The degranulation of NKA positive cells was increased ($p < 0.05$) in lesional, 1.0 ± 1.1, compared to control, 0.2 ± 0.6, skin, and a tendency ($p = 0.06$) to an increase compared to non-lesional skin, 0.4 ± 0.7.

4.2.1.7 Immunohistochemistry – increased degranulation and epidermal infiltration of NK-1R positive cells in lesional skin

There were no differences for NK-1R positive cells, mostly dendritic between lesional, 57 ± 35, non-lesional, 58 ± 30, and control, 61 ± 18, skin. An increased ($p < 0.05$) degranulation of NK-1R positive cells was observed in lesional, 0.5 ± 0.5, compared to control skin, 0.1 ± 0.3, skin. The non-lesional skin value was 0.3 ± 0.5. There were also epidermal infiltrating mononuclear cells with NK-1R positivity with an increase ($p < 0.001$) in lesional, 0.5 ± 0.8, compared to non-lesional, 0.0 ± 0.0, skin, and a tendency ($p =0.06$) to increase in lesional compared to control skin 0.0 ± 0.0.

4.2.1.8 Correlations – a correlation between NK-1R positive cells and depression

A significant correlation between pruritus and somatic trait anxiety ($r = 0.48$, $p < 0.05$) and also a tendency to a correlation to stress susceptibility ($r = 0.38$, $p = 0.06$) were noted, but not with any of the presently studied histopathological parameters in either lesional nor non-lesional skin.

We could also observe a correlation between NK-1R cells and acanthosis ($r = 0.49$, $p < 0.01$) and inflammation ($r = 0.44$, $p < 0.05$), respectively, in lesional skin.

Futhermore, there were correlations between acanthosis and NKA positive cells ($r = 0.45$, $p < 0.05$) and NKA cell degranulation ($r = 0.43$, $p < 0.05$) in non-lesional skin. In addition, a tendency to correlation ($r = 0.36$, $p = 0.06$) between NKA positive cells and inflammation in non-lesional skin was determined.

Correlations between depression score and NK-1R cells in lesional ($r = 0.43$, $p < 0.05$) and non-lesional skin ($r = 0.54$, $p < 0.01$), were found.

Regarding impulsivity there was a correlation ($r = 0.51$, $p < 0.01$) with NKA positive cells and a tendency to a correlation ($r = 0.37$, $p = 0.06$) with NKA degranulation, both in lesional skin.

No correlation was observed for any of the aggressive personality traits.

4.2.2 Study IV

4.2.2.1 Extent of disease – both groups showed a decrease in SCORAD

In order to investigate the effect of aprepitant on AD, the extent of disease was evaluated by SCORAD. In the aprepitant-treated group, the extent of AD measured by SCORAD decreased ($p < 0.01$) from 40.5 ± 12.0 (mean ± SD) to 32.0 ± 11.2, as well as in the control group, where SCORAD decreased ($p < 0.001$) from 37.0 ± 11.3 to 26.7 ± 14.7. For subjective SCORAD (S-SCORAD) there was a decrease ($p < 0.001$) in the treatment group as well as in the control group, from 49.0 ± 14.1 to 38.1 ± 12.6 and from 47.7 ± 13.7 to 33.0 ± 18.9, respectively. There were no significant differences between the groups.

4.2.2.2 Pruritus – both groups showed a decrease of pruritus

For evaluation of pruritus evaluation with VAS and scratching movements was made. VAS in the treatment group was 5.5 ± 2.1 at the inclusion and decreased ($p < 0.05$) to 3.8 ± 2.2 at the end of treatment visit (Day 7) as well as in the control group, 6.7 ± 2.2 to 4.1 ± 3.0 ($p = 0.001$). In the treatment group there was a decrease ($p < 0.05$) of scratching movements from 77.3 ± 97.9 to 48.3 ± 62.6 and in the control group there was a tendency ($p = 0.07$) to a decrease from 65.0 ± 100.9 to 34.7 ± 45.0. There were no significant differences between the groups at any of the time points, Day 3, Day 5 or Day 7.

4.2.2.3 Total IgE – increases in the group treated with aprepitant

To determine if aprepitant also could affect IgE levels analysis of IgE was made before and after treatment. In the treatment group there was an increase ($p < 0.05$) from 903.7 ± 1391 to 937.9 ± 1403, after treatment. In the control group the mean value before treatment was 876.5 ± 1934 and after treatment 821.1 ± 1754.

4.2.2.4 Depression and anxiety score – decrease of depression in both groups

As aprepitant penetrates the blood-brain barrier and also affects NK-1R in the CNS we therefore considered it of interest to also evaluate depression and anxiety before and after treatment. In both the treatment group and the control group there was a decrease ($p < 0.05$) in MADRS-S score from 5.6 ± 4.9 to 4.0 ± 5.3 and from 9.3 ± 8.9 to 6.5 ± 5.9, respectively. A decrease ($p < 0.01$) of depression score in HAD was also seen in the control group, from 3.8 ± 2.7 to 2.4 ± 2.3. This was not significant in the treatment group, where results were from 1.9 ± 2.1 to 1.8 ± 2.4. Measuring anxiety, there was a decrease in the control group from 6.6 ± 4.8 to 4.8 ± 3.7 ($p < 0.05$), but no difference

was evident in the treatment group, where values were 3.4 ± 4.1 before and 3.5 ± 3.2 after the treatment period.

4.2.2.5 Safety – the treatment was generally well tolerated

The patients were monitored in regard to adverse events (AEs) during the study. Thirteen out of the 21 aprepitant-treated patients reported adverse events (headache, fatigue, dizziness, elevated liver enzymes, palpitations, dyspné, changed ability to react, obstipation, stomach ache, periocular dermatitis, and erectile dysfunction). All AEs were considered mild to moderate. All AEs were transient except for one case of elevated liver enzymes.

5 DISCUSSION

5.1 ANIMAL STUDIES (I-II)

In the first two studies an animal model of mild chronic stress and eczema was used. The chronic mild stress form seems relevant from a patient´s perspective. The minor everyday stressors are often what bother the patients most and many patients experience a worsening of the disease as a physical sign of the stress they experience. Substance P is an important mediator of both stress and inflammation and has a central role in regulating the activity of these pathways and the communication between them. Chronic stress has been shown to result in neurogenic inflammation in the intestines of the rat (66). Another study, in tree shrews, has investigated the effects of chronic stress in the form of daily psychosocial conflict. This resulted in morphological modulations of the hippocampus, which could be prevented by treatment with a substance P antagonist (67). Furthermore, depression has been shown to result in elevation of both central and peripheral levels of substance P (68).

The present study showed that the SE group had lower corticosterone levels compared with the other groups. This could indicate that the SE group had a blunted stress response, a suppression of the HPA axis due to the combination of eczema and chronic stress. A similar condition of blunted HPA response has been reported in AD in humans (35).

5.1.1 Skin (I-II)

In the skin the combination of stress and eczema resulted in a higher degree of inflammation, noted as larger ear diameter in this group compared to the other. This is in concordance with another study reporting increased inflammation as a result of stress (69).

Further investigations of the skin revealed a decrease in innervation (PGP 9.5 positive nerve fibres) in the SE compared to the NSE mice, in both the epidermis and dermis. This might indicate that the stress would have a negative effect on innervation. A decrease in newly formed nerve fibres (GAP-43 positive) was also seen in the SE compared to the SC group in both the epidermis and dermis, indicating that the inflammation *per se* would have the greatest impact on newly formed nerve fibres. Previous studies on AD skin have demonstrated increased innervation (52, 70, 71). Acute stress, e.g. sonic stress for 24 h (72) in mice and acute social stress for 24 h in humans (73) also increases skin innervation. In the literature, there are disparate findings. For example, one human study of AD has, in agreement with our results, shown that the number of nerve fibres was decreased in involved compared to non-involved eczematous skin (74). A possible explanation for the results in study I could be that the stress situation used was a chronic one. Initially acute stress might increase innervation, but it might decrease with time. Another explanation could be that there has been a mechanical injury of the nerve fibres, due to the higher degree of inflammation, and caused by the scratching. The scratching per se might be amplified due to central effects of the chronic stress, lowering the threshold for perception of pruritus, as well as the increased inflammation in the skin.

As mentioned earlier, the degree of inflammation was higher in the SE group compared to the other groups. Alternative mechanisms exists that could affect the innervation and formation of nerve fibres in the skin. One is the possible modulation

of the function of keratinocytes due to the inflammation. Keratinocytes release mediators such as NGF which are reported to have a trophic effect on sensory neurons (75).

The fact that there was no difference in the GAP-43 positive nerve fibre expression between the SE and NSE group indicates that the chronic mild stress does not seem to affect the newly formed nerve fibres. In rat models of chronic stress (chronic psychosocial and chronic restraint stress) it has been shown that stress resulted in a decrease of hippocampal progenitor cells, when analysing the expression of Ki-67 protein (76), and also suppression of neurogenesis in the hippocampus, using a bromodeoxyuridine (BrdU) labelling method (77, 78), both methods measuring cell proliferation. Yet, after using chronic restraint stress, another study reported that GAP-43 expression was only slightly decreased in specific areas of the hippocampus or not decreased when analysing the total hippocampus (79).

Furthermore, when investigating the expression of substance P and NK-1R in skin of the mice, it was found that substance P positive nerve fibres were decreased in the SE compared to the SC group. The values for NSE were also higher, although not significantly. RT-PCR of the skin showed a strong tendency to an increase of NK-1R-mRNA in the SE compared to NSE group. These findings suggest that the effects of inflammation in the skin and stress could be additive, resulting in a decreased distribution of substance P, possibly with a following upregulation of NK-1R in skin. Furthermore, the increase in number of mast cells and the increased mast cell degranulation in the affected skin could possibly be interpreted as a non-neuronal compensatory mechanism to a decreased innervation and a lowered expression of neuronal substance P.

5.1.2 Brain (II)

The study showed a decrease of substance P in the SE compared to the NSE group in the medial hippocampus. In the same area a decrease was also evident in the SC group, even though not statistically significant. This would suggest that the psychological stress, as compared to the physical (in form of skin inflammation) might have the most important effect on the expression of substance P in this region. No significant changes in substance P distribution were observed in amygdala or prefrontal cortex. It seems that there are different responses to psychological stress in the form of chronic mild stress and physiological stress depending on the examined areas of the nervous system. In the skin our results indicate an additive effect of the two different stressors resulting in a slight decrease of substance P and an increase of NK-1R, while results from the brain suggest that the area most sensitive to both stress and eczema is the hippocampus, where chronic stress might result in a decreased expression of substance P. A previous study reported an increased expression of substance P in the central amygdala after exposure to restraint induced stress (80). The findings presented here, showing no significant difference, could be related to the type of stress used, not the acute but the chronic mild stress.

5.2 HUMAN STUDIES (III-IV)

5.2.1 Cross sectional study (III)

The investigation of distribution of tachykinins in skin with correlation to psychological traits showed an increase of substance P positive nerve fibres in lesional compared to non-lesional AD skin as well as a decrease in non-lesional compared to healthy control skin. As previously mentioned, other studies have shown alterations in the number of substance P fibres, and an altered substance P concentration in lesional

AD skin compared to control skin, see (40). Recent studies have also shown an increase in plasma substance P in AD patients compared to healthy individuals (61, 81-84).

A higher number of NKA positive fibres were noted in lesional compared to non-lesional skin and the number of NKA positive cells were increased in lesional compared to both non-lesional and control skin. This finding of NKA positive cells has, to the best of our knowledge, not previously been reported in AD.

The study revealed no difference in numbers of NK-1R positive cells, this being in agreement with earlier studies (85, 86). However, the results reported here indicate a different functional activity with an increased degranulation and epidermal infiltration of NK-1R positive cells in lesional compared to non-lesional and control skin.

Personality is connected to experience of health status and disease. It has been debated if, in addition to abnormal immune response, psychological factors such as stress or personality could play a central role in the pathogenesis of AD (87).

In study III the aim was to investigare the relationship between personality traits and the perceived outcome of AD. The hypothesis was that the study would show a correlation between clinical characteristics, e.g., the extent of disease (SCORAD) and pruritus (VAS), and psychodemographic data, like personality, anxiety or depression, as well as between clinical characteristics and expression of tachykinins in the skin.

The study could not establish any correlation between extent of disease and expression of tachykinins in the skin. In this context, a study by Toyoda *et al.* (84) suggested that substance P could be a useful marker in studying the activity of AD, since they found a correlation to SCORAD, with substance P in plasma. In addition, a correlation between substance P and quality of life has been reported by Hon et al. (81). In contrast, two other studies reported no correlation between AD severity and substance P in plasma (82, 83). Even though the present investigation was not able to

confirm any correlations between tachykinergic markers and clinical characteristics, it should be noted that in our investigation both NKA and NK-1R positive structures were correlated to local inflammatory parameters such as degree of inflammatory infiltrates and acanthosis.

We determined no correlation between pruritus and any of the studied histopathological parameters, but did with somatic trait anxiety and stress susceptibility, respectively. Previous studies in humans and mice have suggested substance P to be an important pruritic mediator. Another study, in psoriasis, has shown that NK-2R positive cells and substance P positive fibres were correlated with the level of pruritus (88). The lack of correlation for pruritus in the present study indicates the complexity of pruritic signalling during skin inflammation. It is also of importance to consider the method by which pruritus is measured when interpreting the data. In this study the level of pruritus was measured at a single time point using a VAS-scale, grading the patient's perceived itch during the last three days. Actual scratching was not determined. Different additional ways to measure pruritus have been used in other studies, for example by recording wrist movements (81). This study of children with AD showed a correlation between pruritus and plasma level of substance P, in contrast to subjective symptoms of pruritus. Another possible option is to use a questionnaire-based pruritus score (83). In that study the authors could show a correlation between plasma levels of substance P and pruritus in AD.

Furthermore, the correlation analysis revealed a correlation between NK-1R positive cells in lesional and non-lesional skin, and depression score. This concords with previously published data regarding psoriasis, where it was reported a correlation between depression score (Beck's Depression Inventory being used) and substance P and NK-1R positive cells (88). This is interesting in the context that NK-1R antagonists have been tested as antidepressive substances (89). The correlation indicates that

tachykinergic signalling pathways are of importance in AD, and that depressive symptoms could have an additional influence on skin immunity.

Finally, regarding the correlation between impulsivity and NKA positive cells in the present study, preclinical data from other species have earlier reported a role of the NK-1R for impulsivity in rodents (90).

5.2.2 Treatment study (IV)

Taken together, the results from the third study, showing increase in substance P and NKA positive nerve fibres/ cells, indicate that neurogenic inflammation and tachykinin mediators are of importance in the atopic skin. To further investigate the functionality of tachykinins in AD, it was of interest to examine if the treatment with an NK-1R antagonist could be effective in AD.

This last study was performed to evaluate potential additive effects of aprepitant to a standard topical treatment. We hypothesized that aprepitant in the dose of 80 mg daily would contribute to a decrease of pruritus and inflammation, and thereby change the course of disease, even for this short time period. Aprepitant is an NK-1R antagonist, available in Sweden and approved for treating nausea during cancer treatment in the dose of 125 mg the first day and 80 mg the two following days. In a study by Ständer *et al*. from 2010 (58), pruritic skin conditions of different types were treated rather successfully with 80 mg daily for 1 week. We therefore chose the dose of 80 mg/day for seven days.

The results showed a significant decrease in extent of disease and pruritus in both the treatment and the control group. However, no additive effect of aprepitant in the dose administered and for the time period studied was evident. This might be due to pharmacokinetics and possible induction of degrading system in the liver as a possible explanation, especially when the interim analysis (after 31 patients) revealed a tendency

of a significantly lowered pruritus on Day 3. The standard dose for the approved indication for chemotherapy-induced nausea (the approved indication in Sweden today), is 150 mg the first day and 80 mg/day on the two following days. In consideration of this, it was of interest to determine whether the effect would be better during other time-points during the week. However, this sub-analysis failed to show any effect and we could not observe any significant changes between the groups on either Day 3 or Day 5 when including all patients (n=39 or 41). Considering the efficient effect of topical treatment alone, it might have been beneficial to even analyse pruritus in more detail, at an earlier time-point, at Day 1 or 2, to lower the impact of the topical treatment.

Regarding the dose of aprepitant used, it should be considered that the dose might have been too low to give an optimal effect. It has been suggested that for treatment of depression near complete receptor occupancy for the NK-1R antagonist is needed, and that an occupancy even up to 90% may be ineffective (91). In another study of co-morbid alcohol dependence and posttraumatic stress disorder a dosage of 125 mg/day for 4 weeks was used based on PET studies reporting >90% central receptor occupancy at this dose (92), although, no effect could be reported. It would therefore have been interesting to evaluate a higher dose of 125 mg/ day. In order to achieve a quick effect and in concert with the protocol recommended when treating nausea, it could also have been of interest to start out the first day with 125 mg followed by 80 mg daily thereafter, in our study.

What could be determined by this study is the very satisfactory effect by topical treatment when being used regularly and correctly, resulting in a highly significant improvement regarding extent and pruritus in both study groups. This was interpreted as due to high treatment compliance during the week of study participation. The topical treatment used was a moderately strong steroid crème as well as a moisturizer, and this

resulted in a significant improvement of both the extent of the disease as well as the pruritus. An earlier meta-analysis of pruritus in AD has shown that topical treatment with steroids would result in a decrease of 34% (93).

Regarding depression and anxiety there was a decrease in MADRS-S in both the treatment and the control group and a decrease in HAD in the control group. We were interested in studying the effect of the treatment on these parameters as well considering that NK-1R antagonists have also been investigated for the treatment of anxiety and depression (94). The effect of other anxiolytic treatments, e.g. tandospirone citrate, a serotonin (5-HT) agonist, have been studied in atopic patients and suggested to be useful (95). Our study was, perhaps, also too short to properly investigate the effects on depression and anxiety. We are, however, now considering analysing a subgroup of patients with high values on MADRS-S to see if they might have had a greater effect of the treatment.

There was a different gender composition in the treatment compared to the control group, with a clearly higher proportion of men than women in the treatment group and *vice versa*. This could also have had affected the results since aprepitant might have different effects dependent on gender. In the study by Ständer *et al.* (58) a higher reduction of pruritus was noted in the male patients, even though no significant gender difference could be found and they suggested that aprepitant could be more effective in male patients. This would have given us enhanced effect in our study considering the gender distribution, with a male predominance in the treatment group, but this was still not observed.

Taken together, the results from these studies indicate that the mechanisms involved in both inflammation and pruritus in AD are complex, and not entirely dependent on substance P as a principle mediator and communicator via NK-1R. As mentioned

before, some human studies have reported no correlation between plasma substance P levels and SCORAD (40, 81, 82), even though this might not reflect tissue levels. Furthermore, animal studies have given contradictory results regarding the pruritic role of substance P and NK-1R. A study of treatment with aprepitant in NC/ Nga mice resulted in decreased IgE levels as well as a decreased density of substance P nerve fibres, although with no effect on clinical signs (96).

One recent study in mice showed no effect by aprepitant on the scratching behaviour (97), whereas an earlier study (55), also in mice, and using a different NK-1R antagonist showed that the scratching behaviour could be inhibited by a NK-1R antagonist.

6 METHODOLOGICAL CONSIDERATIONS

6.1 CHRONIC MILD STRESS STUDIES (I-II)

It should be considered that there might be important differences between species and that data from mouse studies might not, in all aspects, be applicable to human physiology. In addition, the mice being used in study II-III were females in contrast to the human studies III-IV, where we included both genders.

In the animal studies a fourth group, non-stressed control, would have been beneficial to fully been able to evaluate the effects of stress *per se*. However, we primarily aimed to compare stress with inflammation.

6.2 IMAGE ANALYSIS (I-II)

Regarding the method of measuring nerve fibre density in the skin of the mice, using image analysis, it can be discussed if it is the most optimal method. It is a relatively objective method and it limits the risk of bias from the observer, but it might have affected the results of the studies. In the inflamed skin the epidermis is hypertrophic and this might have affected the results, lowering the density of nerve fibres in relation to the area of epidermis. However, if there would be a hypertrophy of the epidermis the nerve fibres would presumably be longer and therefore this would maybe not influence the results to a great deal. Considering this, image analysis may have some limitations when measuring nerve fibres in the skin.

6.3 IMMUNOHISTOCHEMICAL METHODS (II-III)

When staining for the distribution of receptors one has to consider if the distribution of the receptors reflects their physiological activity. With the method used we cannot determine whether the receptors are active on the surface of the cells. This would require other methods. A complementary method to using antibodies would be to use the "binding *in situ*"-method by using a biotinylated peptide to enable investigating binding specificity (85).

In study II we could not detect NK-1R in the investigated areas of the brain. This is in contrast to previously documented expression of NK-1R using different techniques, including immunohistochemistry (94). However, we did find NK-1R-immunoreactive nerve fibres in other parts of the brain, and the PCR results also showed a positive signal. Nakaya *et al.* (98) used an Avidin Biotin Complex staining method, which might have provided higher sensitivity compared to our method. Another possibility is that the stress and/or inflammation might have caused a downregulation of the NK-1R gene, as has been reported previously in rat models of pain and stress (99). This is, however, unlikely.

6.4 TREATMENT STUDY (IV)

The treatment study has given rise to further questions. First of all, it can be discussed if the control group was the most optimal. At first we would have liked to have ensured a more even gender distribution in the two groups, but difficulties recruiting patients made us drop the stratified randomisation. When considering the dose given, a higher dose (125 mg/day) could maybe, as discussed earlier, have given a more optimal effect. To further investigate this it would have been possible to have two different treatment groups with two different doses. However, due to limited resources we chose the dose

of 80 mg/day as was described in the study by Ständer *et al*.(58). For a possible positive treatment effect we would still have had the problem of excluding a placebo effect, since the study was an open randomized trial.

As discussed earlier there are different ways of measuring pruritus. The method we used was an attempt to get an additional tool to evaluate the effect of pruritus. However the method proved to be difficult to standardize and the results showed large variations.

It shall also be noted that the basal treatment that we used in the study might be so potent that the effects of the systemic treatment were not separately detectable. Considering the time it may take to gain the effect of steroids topically it might be of interest to already analyse the effect at Day 1-2.

7 CONCLUSIONS

Chronic mild stress might cause a decrease in preformed cutaneous nerves in eczematous skin in NC/Nga mice. The slowdown of the process of neuronal regeneration in the eczematous skin of atopic mice could be due to the inflammation *per se.*

Furthermore, the combination of chronic mild stress and eczema may result in alterations in substance P expression in the skin and brain. Symptoms of depression and diseases of chronic inflammation in barrier tissues may reflect a common underlying pathophysiology involving substance P as a central mediator. This may be of interest for future pharmacological treatment of AD and the worsening of AD during chronic stress, targeting substance P pathways.

In humans substance P and NKA are likely to have a role in the inflamed skin of AD. In addition, there was a relation between NK-1R positive cells and depression score. This indicates that tachykinergic signalling pathways are of importance in AD, and that depressive symptoms could have an additional influence on skin immunity.

However, when interfering with substance P pathways by blocking the NK-1R with aprepitant, we have not been able to detect an additive effect by this treatment compared to a standardized topical treatment in AD patients. Nevertheless, the clinical trial setting supported the patients to be compliant to all study procedures and resulted in significant relief of the disease symptoms in both study groups.

In conclusion, my studies suggest that tachykinins seem to have a role in pathogenesis of AD, even though the underlying mechanisms are complex and not fully understood.

8 FUTURE PERSPECTIVES

It is still of interest to further investigate the effects of blocking NK-1R with aprepitant. As discussed above, it would be of interest to primarily investigate a higher dose to see if it would be more efficient. It would, even if it probably would result in more difficulties in recruiting patients, be of interest to investigate the treatment with aprepitant alone compared to placebo. This would, however, have to be preceded by a couple of weeks of wash-out from the patients' standard topical treatment, which is in my opinion difficult to motivate the patients to do, and might result in even more recruitment difficulties.

There are other inflammatory skin conditions that would be of interest to treat with aprepitant, for example prurigo nodularis, where the pruritus is even worse and where neuropeptides and neurogenic inflammation are considered to have a role in the pathogenesis. Ständer *et al*. (58) reported promising results treating different inflammatory skin diseases with aprepitant, and it would be interesting to see if the results are reproducible in another setting.

There are other peptides, like CGRP and gastrin releasing peptide (GRP) that have been suggested to be involved in itch in AD, and which are related to inflammation (100) and itch, respectively (101). It would be of interest to further investigate the distribution of these peptides in the skin of AD. There is a new CGRP antagonist developed for the treatment of migraine (102), which would be interesting to examine in relation to AD. There have been a few studies investigating intradermal injection and topical administration of CGRP-antagonists in contact dermatitis, resulting in an inhibition of reaction to nickel in patients allergic to nickel (103) and vasoconstriction

(104), respectively.

Regarding the neurocutaneus perspective it is of interest to further investigare how stress and depression influence different inflammatory skin conditions, and *vice versa.* Since pruritus is a central symptom in many inflammatory skin diseases and is also regulated at a central level this is a symptom that is of high interest. One could for example evaluate the benefit of additional treatment of skin diseases, especially if you can establish stress and depression as a contributing factor for the patient, with anti-depressive agents and/or psychotherapy such as cognitive behavioural therapy (CBT).

It is also of importance to find new forms to collaborate between the university hospitals and private actors. It is, and is going to be an even greater challenge to get the patients in contact with the researchers. We will have to find ways, reach out to the private sector and make it profitable to take part in research and also to create platforms for the patients to more easily get in contact with the researchers.

9 SAMMANFATTNING PÅ SVENSKA

Atopisk dermatit (AD) är en vanlig, till viss del ärftlig, kronisk hudsjukdom vilken ofta uppkommer i tidig barndom (15-20% av barn i Sverige drabbas). Av vuxna har 2-3% kvarstående besvär. Patogenesen involverar bland annat en ändring i distributionen av neuropeptider, däribland substans P. Denna neuropeptid spelar också en viktig roll vid stress och det är välkänt att stress kan ge upphov till försämring av AD.

Målsättningen var att utreda vilken roll substans P och andra tachykininer spelar i utvecklingen av AD, klåda och stressförsämring.

Initialt användes en djurmodell för att studera substans P och dess receptor NK-1R i relation till eksem och stress. Kombinationen av stress och eksem resulterade i en minskning av nervfibrer i huden och själva inflammationen i den eksemdrabbade huden tycks ha haft mest negativ effekt på de nybildade nervfibrerna. Även uttrycket av substans P och NK-1R förändrades i både huden och hjärnan.

Därefter studerades AD hos patienter och då korrelerades uttrycket av substans P och NK-1R i huden till grad av eksemutbredning, klåda samt patienternas oros- och depressionsnivå. Resultaten visade en ökning av substans P-och NKA-positiva nervfibrer samt NKA-positiva celler i eksemdrabbad hud. Vidare kunde det fastställas en korrelation mellan NK-1R-positiva celler i huden och grad av inflammation i huden. NK-1R positiva celler korrelerade även till patienternas grad av depression.

Slutligen genomfördes en öppen randomiserad klinisk prövning där effekten av en NK-1R-antagonist, aprepitant, på utbredningen av eksemet och klådan hos patienter med måttlig/svår AD utvärderades. Ingen additativ effekt av behandlingen med aprepitant jämfört med endast lokalbehandling kunde fastställas. Däremot visade studien på en signifikant förbättring i båda grupperna, något som talar för att god följsamhet ger god behandlingseffekt vid behandling med lokala steroider.

Sammanfattningsvis talar studierna för att tachykininer tycks spela en roll i patogenesen och stressförsämringen av AD. Samspelet är dock komplext och det behövs vidare undersökningar för att vidare utreda de exakta mekanismerna.

10 ACKNOWLEDGEMENTS

The work resulting in this thesis have been made at the Department of Medicine, Solna, Karolinska Institutet, and the Dermatology and Venereology unit, Karolinska University Hospital, Solna. A lot of people have contributed to making this work possible and I would in particular like to thank the following.

First of all would I like to send my greatest appreciations to my supervisor **Klas Nordlind**, for all his support, time and effort during the years. It has been a journey manoeuvring during different situations in life and science. Thank you Klas for your enthusiasm and amazing ability to always find ways. I am grateful for the introduction to research and also to teaching and clinical practice. You have always made time for me and in the end, I greatly appreciated those extra meeting during weekends before deadlines.

My co-supervisor, **Mikael Holst**, for his time, effort and support. I am truly grateful for your encouragement and positive energy as well as for your scientific expertise and many discussions. The promise of you and your band playing at the dissertation party was a great encouraging vision during the last months.

My co-supervisor **Husameldin El-Nour**. I am very grateful for all your time and guidance as well as for all the hours by the microscope and discussions regarding research and life.

My co-supervisor **Sol-Britt Lonne-Rahm** for your encouragement, support and reminding me of what is important in life.

I would like to thank my friend and colleague **Liv Eidsmo** for her support during the last years. For scientific perspective and new energy.

I would also like to send my appreciations to **Mona Ståhle**. I am grateful for your encouragement during different stages of my research and clinical education.

Anna Josephson, my mentor, for advice, energy and support.

A warm hug to all my colleagues at the clinic for your interest and support over the years. Also, for your valuable input to my work. Also, to my chiefs at the clinic, thank you **Toomas Talme** and **Lena Lundeberg** for the clinical resources that made my last study possible.

To all my colleagues at the lab, for great laughs and stimulating conversations.

My greatest appreciations to my colleagues in the private sector and to the nurses at Swedish Psoriasis Association for assistance in recruiting patients.

Nurses **Papely Kassiri** and **Kirsti Ajax** for great patience, assistance and support during my work.

Anna-Lena Kastman for great technical expertise as well as support during my laboratory work.

Gunilla Ekstrand for excellent administrative support and continuous problem solving.

Yvonne Löfgren for laboratory expertise and supervision.

Björn Johansson for his expertise in neuroscience and scientific discussions.

Ronald van Vollenhoven for support and scientific advice.

Laurence Quérat and **Christina Åkerstedt** for monitoring the clinical trial.

Lars Olson for great advice regarding histochemisty and manuscripts.

Robert A Harris for linguistic advice during the writing of my thesis.

Per Näsman for statistical analysis and advice.

Jonathan Grip for great patience and support during my first years of studies, and for great initiatives.

A special thanks to all patients that have participated in the studies.

I would like to thank Karolinska Institutet for giving me the opportunity to do my PhD in parallel to my medical studies, by the MD/PhD scholarship and “forskar AT”. Also I would like to thank Welander/Finsen- Hudfonden for important funding.

Last and most important, I would like to thank my family and friends. For your love and support. A special thanks to my best friend, colleague and husband, **Hassan Killasli**, I couldn´t have done it without you.

11 REFERENCES

1. Odhiambo JA, Williams HC, Clayton TO*, et al.* Global variations in prevalence of eczema symptoms in children from ISAAC Phase Three. J Allergy Clin Immunol. 2009;124(6):1251-8.e23.
2. Vinding GR, Zarchi K, Ibler KS*, et al.* Is adult atopic eczema more common than we think? - A population-based study in Danish adults. Acta Derm Venereol. 2014;94(4):480-2.
3. Harrop J, Chinn S, Verlato G*, et al.* Eczema, atopy and allergen exposure in adults: a population-based study. Clin Exp Allergy. 2007;37(4):526-35.
4. Ronmark EP, Ekerljung L, Lotvall J*, et al.* Eczema among adults: prevalence, risk factors and relation to airway diseases. Results from a large-scale population survey in Sweden. Br J Dermatol. 2012;166(6):1301-8.
5. Hanifin JM, Reed ML. A population-based survey of eczema prevalence in the United States. Dermatitis. 2007;18(2):82-91.
6. Flohr C, Johansson SG, Wahlgren CF*, et al.* How atopic is atopic dermatitis? J Allergy Clin Immunol. 2004;114(1):150-8.
7. Bieber T. Atopic dermatitis. N Engl J Med. 2008;358(14):1483-94.
8. Williams HC, Burney PG, Pembroke AC*, et al.* The U.K. Working Party's Diagnostic Criteria for Atopic Dermatitis. III. Independent hospital validation. Br J Dermatol. 1994;131(3):406-16.
9. Montes-Torres A, Llamas-Velasco M, Perez-Plaza A*, et al.* Biological Treatments in Atopic Dermatitis. Journal of clinical medicine. 2015;4(4):593-613.
10. Hamilton JD, Suarez-Farinas M, Dhingra N*, et al.* Dupilumab improves the molecular signature in skin of patients with moderate-to-severe atopic dermatitis. J Allergy Clin Immunol. 2014;134(6):1293-300.
11. Weidinger S, Rodriguez E, Stahl C*, et al.* Filaggrin mutations strongly predispose to early-onset and extrinsic atopic dermatitis. J Invest Dermatol. 2007;127(3):724-6.
12. Wollenberg A, Seba A, Antal AS. Immunological and molecular targets of atopic dermatitis treatment. Br J Dermatol. 2014;170 Suppl 1:7-11.
13. Goldsby RA, Goldsby RA, Kuby J. Immunology. 5. ed. New York: W.H. Freeman; 2003. xxiii, 551, 15, 14, 16, 25 s. p.
14. Madva EN, Granstein RD. Nerve-derived transmitters including peptides influence cutaneous immunology. Brain Behav Immun. 2013;34:1-10.
15. Burbach GJ, Kim KH, Zivony AS*, et al.* The neurosensory tachykinins substance P and neurokinin A directly induce keratinocyte nerve growth factor. J Invest Dermatol. 2001;117(5):1075-82.
16. Forsythe P, Bienenstock J. The mast cell-nerve functional unit: a key component of physiologic and pathophysiologic responses. Chem Immunol Allergy. 2012;98:196-221.
17. Ding W, Manni M, Stohl LL*, et al.* Pituitary adenylate cyclase-activating peptide and vasoactive intestinal polypeptide bias Langerhans cell Ag presentation toward Th17 cells. Eur J Immunol. 2012;42(4):901-11.
18. Hosoi J, Murphy GF, Egan CL*, et al.* Regulation of Langerhans cell function by nerves containing calcitonin gene-related peptide. Nature. 1993;363(6425):159-63.

19. Huang J, Stohl LL, Zhou X*, et al.* Calcitonin gene-related peptide inhibits chemokine production by human dermal microvascular endothelial cells. Brain Behav Immun. 2011;25(4):787-99.
20. Umeda Y, Takamiya M, Yoshizaki H*, et al.* Inhibition of mitogen-stimulated T lymphocyte proliferation by calcitonin gene-related peptide. Biochem Biophys Res Commun. 1988;154(1):227-35.
21. Lee HR, Ho WZ, Douglas SD. Substance P augments tumor necrosis factor release in human monocyte-derived macrophages. Clin Diagn Lab Immunol. 1994;1(4):419-23.
22. Gordon DJ, Ostlere LS, Holden CA. Neuropeptide modulation of Th1 and Th2 cytokines in peripheral blood mononuclear leucocytes in atopic dermatitis and non-atopic controls. Br J Dermatol. 1997;137(6):921-7.
23. Liezmann C, Klapp B, Peters EM. Stress, atopy and allergy: A re-evaluation from a psychoneuroimmunologic persepective. Dermatoendocrinol. 2011;3(1):37-40.
24. Maier SF. Bi-directional immune-brain communication: Implications for understanding stress, pain, and cognition. Brain Behav Immun. 2003;17(2):69-85.
25. Rosenkranz MA. Substance P at the nexus of mind and body in chronic inflammation and affective disorders. Psychol Bull. 2007;133(6):1007-37.
26. Tancowny BP, Karpov V, Schleimer RP*, et al.* Substance P primes lipoteichoic acid- and Pam3CysSerLys4-mediated activation of human mast cells by up-regulating Toll-like receptor 2. Immunology. 2010;131(2):220-30.
27. Chrousos GP. Stressors, stress, and neuroendocrine integration of the adaptive response. The 1997 Hans Selye Memorial Lecture. Ann N Y Acad Sci. 1998;851:311-35.
28. Lorton D, Lubahn C, Bellinger DL. Potential use of drugs that target neural-immune pathways in the treatment of rheumatoid arthritis and other autoimmune diseases. Curr Drug Targets Inflamm Allergy. 2003;2(1):1-30.
29. Buske-Kirschbaum A, Geiben A, Hollig H*, et al.* Altered responsiveness of the hypothalamus-pituitary-adrenal axis and the sympathetic adrenomedullary system to stress in patients with atopic dermatitis. J Clin Endocrinol Metab. 2002;87(9):4245-51.
30. Harbuz MS, Chover-Gonzalez AJ, Jessop DS. Hypothalamo-pituitary-adrenal axis and chronic immune activation. Ann N Y Acad Sci. 2003;992:99-106.
31. Chowdrey HS, Larsen PJ, Harbuz MS*, et al.* Endogenous substance P inhibits the expression of corticotropin-releasing hormone during a chronic inflammatory stress. Life Sci. 1995;57(22):2021-9.
32. Besedovsky HO, Rey AD. Physiology of psychoneuroimmunology: a personal view. Brain Behav Immun. 2007;21(1):34-44.
33. Kim HS, Yosipovitch G. An aberrant parasympathetic response: a new perspective linking chronic stress and itch. Exp Dermatol. 2013;22(4):239-44.
34. Nussdorfer GG, Malendowicz LK. Role of tachykinins in the regulation of the hypothalamo-pituitary-adrenal axis. Peptides. 1998;19(5):949-68.
35. Buske-Kirschbaum A, Ebrecht M, Hellhammer DH. Blunted HPA axis responsiveness to stress in atopic patients is associated with the acuity and severeness of allergic inflammation. Brain Behav Immun. 2010;24(8):1347-53.
36. Severini C, Improta G, Falconieri-Erspamer G*, et al.* The tachykinin peptide family. Pharmacol Rev. 2002;54(2):285-322.
37. Maggi CA. The mammalian tachykinin receptors. Gen Pharmacol. 1995;26(5):911-44.

38. Singh LK, Pang X, Alexacos N*, et al.* Acute immobilization stress triggers skin mast cell degranulation via corticotropin releasing hormone, neurotensin, and substance P: A link to neurogenic skin disorders. Brain Behav Immun. 1999;13(3):225-39.
39. Pavlovic S, Daniltchenko M, Tobin DJ*, et al.* Further exploring the brain-skin connection: stress worsens dermatitis via substance P-dependent neurogenic inflammation in mice. J Invest Dermatol. 2008;128(2):434-46.
40. Salomon J, Baran E. The role of selected neuropeptides in pathogenesis of atopic dermatitis. J Eur Acad Dermatol Venereol. 2008;22(2):223-8.
41. Toyoda M, Makino T, Kagoura M*, et al.* Immunolocalization of substance P in human skin mast cells. Arch Dermatol Res. 2000;292(8):418-21.
42. Ohmura T, Tsunenari I, Hayashi T*, et al.* Role of substance P in an NC/Nga mouse model of atopic dermatitis-like disease. Int Arch Allergy Immunol. 2004;133(4):389-97.
43. Jarvikallio A, Harvima IT, Naukkarinen A. Mast cells, nerves and neuropeptides in atopic dermatitis and nummular eczema. Arch Dermatol Res. 2003;295(1):2-7.
44. Katayama I, Bae SJ, Hamasaki Y*, et al.* Stress response, tachykinin, and cutaneous inflammation. J Investig Dermatol Symp Proc. 2001;6(1):81-6.
45. Lai JP, Douglas SD, Ho WZ. Human lymphocytes express substance P and its receptor. J Neuroimmunol. 1998;86(1):80-6.
46. Yokote R, Yagi H, Furukawa F*, et al.* Regulation of peripheral blood mononuclear cell responses to Dermatophagoides farinae by substance P in patients with atopic dermatitis. Arch Dermatol Res. 1998;290(4):191-7.
47. Giannetti A, Girolomoni G. Skin reactivity to neuropeptides in atopic dermatitis. Br J Dermatol. 1989;121(6):681-8.
48. Heyer G, Hornstein OP, Handwerker HO. Reactions to intradermally injected substance P and topically applied mustard oil in atopic dermatitis patients. Acta Derm Venereol. 1991;71(4):291-5.
49. Cardona ID, Cho SH, Leung DY. Role of bacterial superantigens in atopic dermatitis : implications for future therapeutic strategies. Am J Clin Dermatol. 2006;7(5):273-9.
50. Kitamura H, Kobayashi M, Wakita D*, et al.* Neuropeptide signaling activates dendritic cell-mediated type 1 immune responses through neurokinin-2 receptor. J Immunol. 2012;188(9):4200-8.
51. Mollanazar NK, Smith PK, Yosipovitch G. Mediators of Chronic Pruritus in Atopic Dermatitis: Getting the Itch Out? Clin Rev Allergy Immunol. 2015.
52. Tominaga M, Takamori K. Itch and nerve fibers with special reference to atopic dermatitis: therapeutic implications. J Dermatol. 2014;41(3):205-12.
53. Yosipovitch G, Papoiu AD. What causes itch in atopic dermatitis? Curr Allergy Asthma Rep. 2008;8(4):306-11.
54. Hagermark O, Hokfelt T, Pernow B. Flare and itch induced by substance P in human skin. J Invest Dermatol. 1978;71(4):233-5.
55. Ohmura T, Hayashi T, Satoh Y*, et al.* Involvement of substance P in scratching behaviour in an atopic dermatitis model. Eur J Pharmacol. 2004;491(2-3):191-4.
56. Carstens EE, Carstens MI, Simons CT*, et al.* Dorsal horn neurons expressing NK-1 receptors mediate scratching in rats. Neuroreport. 2010;21(4):303-8.
57. Wilson SR, Nelson AM, Batia L*, et al.* The ion channel TRPA1 is required for chronic itch. J Neurosci. 2013;33(22):9283-94.
58. Stander S, Siepmann D, Herrgott I*, et al.* Targeting the neurokinin receptor 1 with aprepitant: a novel antipruritic strategy. PLoS One. 2010;5(6):e10968.

59. Tanaka A, Matsuda H. Evaluation of itch by using NC/NgaTnd mice: a model of human atopic dermatitis. J Biomed Biotechnol. 2011;2011:790436.
60. Shiohara T, Hayakawa J, Mizukawa Y. Animal models for atopic dermatitis: are they relevant to human disease? J Dermatol Sci. 2004;36(1):1-9.
61. Lanfumey L, Pardon MC, Laaris N*, et al.* 5-HT1A autoreceptor desensitization by chronic ultramild stress in mice. Neuroreport. 1999;10(16):3369-74.
62. Schram ME, Spuls PI, Leeflang MM*, et al.* EASI, (objective) SCORAD and POEM for atopic eczema: responsiveness and minimal clinically important difference. Allergy. 2012;67(1):99-106.
63. Gustavsson JP, Bergman H, Edman G*, et al.* Swedish universities Scales of Personality (SSP): construction, internal consistency and normative data. Acta Psychiatr Scand. 2000;102(3):217-25.
64. Svanborg P, Asberg M. A comparison between the Beck Depression Inventory (BDI) and the self-rating version of the Montgomery Asberg Depression Rating Scale (MADRS). J Affect Disord. 2001;64(2-3):203-16.
65. Stein DJ, Lopez AG. Effects of escitalopram on sleep problems in patients with major depression or generalized anxiety disorder. Adv Ther. 2011;28(11):1021-37.
66. Chen JH, Wei SZ, Chen J*, et al.* Sensory denervation reduces visceral hypersensitivity in adult rats exposed to chronic unpredictable stress: evidences of neurogenic inflammation. Dig Dis Sci. 2009;54(9):1884-91.
67. Czeh B, Simon M, van der Hart MG*, et al.* Chronic stress decreases the number of parvalbumin-immunoreactive interneurons in the hippocampus: prevention by treatment with a substance P receptor (NK1) antagonist. Neuropsychopharmacology. 2005;30(1):67-79.
68. Bondy B, Baghai TC, Minov C*, et al.* Substance P serum levels are increased in major depression: preliminary results. Biol Psychiatry. 2003;53(6):538-42.
69. Amano H, Negishi I, Akiyama H*, et al.* Psychological stress can trigger atopic dermatitis in NC/Nga mice: an inhibitory effect of corticotropin-releasing factor. Neuropsychopharmacology. 2008;33(3):566-73.
70. Urashima R, Mihara M. Cutaneous nerves in atopic dermatitis. A histological, immunohistochemical and electron microscopic study. Virchows Arch. 1998;432(4):363-70.
71. Tobin D, Nabarro G, Baart de la Faille H*, et al.* Increased number of immunoreactive nerve fibers in atopic dermatitis. J Allergy Clin Immunol. 1992;90(4 Pt 1):613-22.
72. Peters EM, Kuhlmei A, Tobin DJ*, et al.* Stress exposure modulates peptidergic innervation and degranulates mast cells in murine skin. Brain Behav Immun. 2005;19(3):252-62.
73. Kleyn CE, Schneider L, Saraceno R*, et al.* The effects of acute social stress on epidermal Langerhans' cell frequency and expression of cutaneous neuropeptides. J Invest Dermatol. 2008;128(5):1273-9.
74. Lonne-Rahm SB, Rickberg H, El-Nour H*, et al.* Neuroimmune mechanisms in patients with atopic dermatitis during chronic stress. J Eur Acad Dermatol Venereol. 2008;22(1):11-8.
75. Ulmann L, Rodeau JL, Danoux L*, et al.* Trophic effects of keratinocytes on the axonal development of sensory neurons in a coculture model. Eur J Neurosci. 2007;26(1):113-25.
76. Rosenbrock H, Koros E, Bloching A*, et al.* 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.

77. Pham K, Nacher J, Hof PR*, et al.* Repeated restraint stress suppresses neurogenesis and induces biphasic PSA-NCAM expression in the adult rat dentate gyrus. Eur J Neurosci. 2003;17(4):879-86.
78. Czeh B, Welt T, Fischer AK*, et al.* Chronic psychosocial stress and concomitant repetitive transcranial magnetic stimulation: effects on stress hormone levels and adult hippocampal neurogenesis. Biol Psychiatry. 2002;52(11):1057-65.
79. 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. Brain Res Mol Brain Res. 1998;59(1):35-9.
80. Hwang BH, Katner J, Iyengar S. Corticotropin-releasing factor mRNA and substance P receptor binding in the paraventricular hypothalamic nucleus, central nucleus of the amygdala, and locus coeruleus of Sprague-Dawley rats following restraint-induced stress. J Mol Neurosci. 2005;25(3):239-50.
81. Hon KL, Lam MC, Wong KY*, et al.* Pathophysiology of nocturnal scratching in childhood atopic dermatitis: the role of brain-derived neurotrophic factor and substance P. Br J Dermatol. 2007;157(5):922-5.
82. Hosokawa C, Takeuchi S, Furue M. Severity scores, itch scores and plasma substance P levels in atopic dermatitis treated with standard topical therapy with oral olopatadine hydrochloride. J Dermatol. 2009;36(4):185-90.
83. Panahi Y, Taherzadeh ES, Davoudi SM*, et al.* Investigation of serum substance P status in patients with chronic pruritic skin lesions due to sulfur mustard: a cross-sectional study. Cutan Ocul Toxicol. 2013;32(1):4-8.
84. Toyoda M, Nakamura M, Makino T*, et al.* Nerve growth factor and substance P are useful plasma markers of disease activity in atopic dermatitis. Br J Dermatol. 2002;147(1):71-9.
85. Misery L, Bourchanny D, Kanitakis J*, et al.* Modulation of substance P and somatostatin receptors in cutaneous lymphocytic inflammatory and tumoral infiltrates. J Eur Acad Dermatol Venereol. 2001;15(3):238-41.
86. Staniek V, Liebich C, Vocks E*, et al.* Modulation of cutaneous SP receptors in atopic dermatitis after UVA irradiation. Acta Derm Venereol. 1998;78(2):92-4.
87. Buske-Kirschbaum A, Ebrecht M, Kern S*, et al.* Personality characteristics in chronic and non-chronic allergic conditions. Brain Behav Immun. 2008;22(5):762-8.
88. Amatya B, El-Nour H, Holst M*, et al.* Expression of tachykinins and their receptors in plaque psoriasis with pruritus. Br J Dermatol. 2011;164(5):1023-9.
89. Hokfelt T, Pernow B, Wahren J. Substance P: a pioneer amongst neuropeptides. J Intern Med. 2001;249(1):27-40.
90. Porter AJ, Pillidge K, Grabowska EM*, et al.* The angiotensin converting enzyme inhibitor, captopril, prevents the hyperactivity and impulsivity of neurokinin-1 receptor gene 'knockout' mice: sex differences and implications for the treatment of attention deficit hyperactivity disorder. Eur Neuropsychopharmacol. 2015;25(4):512-21.
91. Schank JR. The neurokinin-1 receptor in addictive processes. J Pharmacol Exp Ther. 2014;351(1):2-8.
92. Kwako LE, George DT, Schwandt ML*, et al.* The neurokinin-1 receptor antagonist aprepitant in co-morbid alcohol dependence and posttraumatic stress disorder: a human experimental study. Psychopharmacology (Berl). 2015;232(1):295-304.
93. Sher LG, Chang J, Patel IB*, et al.* Relieving the pruritus of atopic dermatitis: a meta-analysis. Acta Derm Venereol. 2012;92(5):455-61.

94. Ebner K, Singewald N. The role of substance P in stress and anxiety responses. Amino Acids. 2006;31(3):251-72.
95. Kawana S, Kato Y, Omi T. Efficacy of a 5-HT1a receptor agonist in atopic dermatitis. Clin Exp Dermatol. 2010;35(8):835-40.
96. Lee JH, Cho SH. Korean red ginseng extract ameliorates skin lesions in NC/Nga mice: an atopic dermatitis model. J Ethnopharmacol. 2011;133(2):810-7.
97. Nakasone T, Sato T, Matsushima Y*, et al.* Characteristics of scratching behavior in ADJM mice (atopic dermatitis from Japanese mice). Immunopharmacol Immunotoxicol. 2015;37(2):202-6.
98. Nakaya Y, Kaneko T, Shigemoto R*, et al.* Immunohistochemical localization of substance P receptor in the central nervous system of the adult rat. J Comp Neurol. 1994;347(2):249-74.
99. Duric V, McCarson KE. Hippocampal neurokinin-1 receptor and brain-derived neurotrophic factor gene expression is decreased in rat models of pain and stress. Neuroscience. 2005;133(4):999-1006.
100. Pincelli C, Steinhoff M. Recapitulating atopic dermatitis in three dimensions: cross talk between keratinocytes and nerve fibers. J Invest Dermatol. 2013;133(6):1465-7.
101. Tirado-Sanchez A, Bonifaz A, Ponce-Olivera RM. Serum gastrin-releasing peptide levels correlate with disease severity and pruritus in patients with atopic dermatitis. Br J Dermatol. 2015;173(1):298-300.
102. Edvinsson L, Linde M. New drugs in migraine treatment and prophylaxis: telcagepant and topiramate. Lancet. 2010;376(9741):645-55.
103. Wallengren J. Dual effects of CGRP-antagonist on allergic contact dermatitis in human skin. Contact Dermatitis. 2000;43(3):137-43.
104. Wallengren J, Edvinsson L. Topical non-peptide antagonists of sensory neurotransmitters substance P and CGRP do not modify patch test and prick test reactions: a vehicle-controlled, double-blind pilot study. Arch Dermatol Res. 2014;306(5):505-9.