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SEROTONERGIC MECHANISMS IN ATOPIC DERMATITIS

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ABSTRACT

Atopic dermatitis (AD) may be worsened by stress and anxiety. Serotonin (5-hydroxytryptamine; 5-HT) is an important mediator in stress and anxiety.

In the present thesis serotonergic mechanisms were studied in atopic dermatitis (AD).

In an atopic-like mouse model, NC/Nga, that was subjected to chronic mild stress, we studied expression of serotonergic markers 5-HT1A and 5-HT2A receptors (R) and serotonin transporter protein (SERT) in skin, cerebrum and cerebellum. There was an upregulation of 5-HT1AR in the skin, cerebrum and cerebellum, during inflammation, irrespective of stress, while the 5-HT2AR was upregulated in the cerebrum, hippocampal CA1 area, and in the cerebellum, Purkinje cell layer, while being downregulated in the skin, during chronic mild stress.

In human AD patients serotonergic markers in relation to extent of the disease, pruritus, chronic stress and psychodemographic data with focus on trait anxiety and depression, were studied. We found a correlation between the extent of the disease and dermal 5-HT1AR-positive dermal inflammatory cells in the lesional skin and 5-HT2AR-positive vessels in the non-lesional skin, respectively. There was a correlation between depression with the epidermal positive 5-HT1AR fraction, while a reverse correlation with the number of 5-HT2AR expressing vessels, both in the lesional skin. In the lesional skin there was a reverse correlation for the basal SERT immunoreactivity with stress susceptibility.

Moreover, the effect of intradermal injection of 5-HT was studied in patients with AD and in healthy controls, on vascular response and pruritus, estimated by a computerized VAS recorder. No difference was seen regarding pruritus, while the vascular response to 5-HT was reduced in the AD patients, compared to the healthy controls.

5-HT seems to have a role in AD.

LIST OF PUBLICATIONS

I. **Aram Rasul**, Husameldin El-Nour, Randy D. Blakely, Sol-Britt Lonne-Rahm, Johan Forsberg, Björn Johansson, Elvar Theodorsson, Klas Nordlind. Effects of chronic mild stress on the expression of serotonergic markers in the skin and brain of the atopic-like mouse strain. Arch Dermatol Res 2011; 303:625–633.

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CONTENTS

LIST OF ABBREVIATIONS

5-HT	5-hydroxytryptamine, serotonin
5-HT1AR	5-hydroxytryptamine 1A receptor
5-HT2AR	5-hydroxytryptamine 2A receptor
ACTH	Adreno-corticotropic hormone
AD	Atopic dermatitis
AUC	Area under the curve
BSA	Bovine serum albumin
CNS	Central nervous system
FLG	Filaggrin
HPA	Hypothalamic-pituitary-adrenal
ID	Itch duration
Ig	Immunoglobulin
IL	Interleukin
IM	Itch max
IR	Immunoreactivity
KLH	Keyhole limpet hemocyanin
L	Lesional
MADRS-S	Montgomery-Åsberg Depression Rating Scale-Self assessment N
N-L	Non-lesional
NSE	Non-stressed eczematous
PBS	Phosphate buffered saline
POEM	Patient´s oriented eczema measurement
PsTA	Psychic trait anxiety
RIA	Radioimmunoassay
R	Receptor
SCORAD	SCORing of Atopic Dermatitis
SERT	Serotonin transporter protein
STA	Somatic trait anxiety
S. aureus	Staphylococcus aureus
SC	Stressed control
SE	Stressed eczematous
SS	Stress susceptibility
SSP	Swedish Universities Scales of Personality
Th	T helper
VAS	Visual analogue scale

1 INTRODUCTION

1.1 ATOPIC DERMATITIS

Atopic dermatitis (AD) is a chronic inflammatory highly pruritic disease, with a dry skin. AD usually occurs in people who have an atopic constitution. This means that they may develop any or all of three closely linked conditions; AD, hay fever (allergic rhinitis) and asthma.

1.1.1 Epidemiology

AD affects up to 15% of the Swedish population among children and around 2-3% of adults. AD most often begins in childhood before the age of five and may persist into adulthood. For some, it flares periodically and then subsides for a time, even up to several years.[1]

A female preponderance for AD has been reported,[1] also dependent on age.[2] The prevalence of the AD is increasing continuously. AD is considered as a major health problem worldwide, with a high prevalence in children in the USA, the Northern and Western Europe, urban Africa, Japan, Australia, and industrialized countries. Interestingly, the prevalence of AD is lower in agricultural countries such as China and in Eastern Europe, rural Africa, and central Asia.[1]

1.1.2 Clinical features

The diagnosis of AD may be made by using the Williams criteria.[3] The clinical signs and symptoms of AD include itching and scratching, which may be severe, especially at night. The disease is also characterized by erythematous papules with excoriations and serous exudates in the acute phase and, in addition, lichenification in the chronic phase, and with a generally dry skin. Typical distribution in small children includes

extensor side and face, while there is a predilection for flexures in older children and adults. In children and adults the disease may also affect the skin around the eyes, including the eyelids. In the adult phase there may also be a cranial localization as well as a localization to the hand area.

1.1.3 Worsening factors

AD can be worsened by several different factors such as climate, environmental factors, microbial agents and stress. AD is usually aggravated by a low humidity climate, high ambient temperatures and during dry cold weather. Among environmental factors, soaps, antiseptics, chlorine in swimming pools may worsen the disease. Bacterial infection like staphylococci and streptococci and/or yeasts like malassezia and candida, may play an important role in triggering AD.[1]

Finally adults and children with AD are usually experiencing an exacerbation in AD secondary to mental or social stress. Thus, it has been earlier reported that AD is often worsened due to stress and anxiety [4,5] and a special personality of these patients, being more prone to anxiety, has been described.[6] Patients with AD have been reported to have higher values for state and trait anxiety compared to healthy controls.[7] While the extent of AD did not show a correlation with psychological parameters, pruritus correlated with state and trait anxiety.

1.1.4 Pathogenesis

The pathogenesis of AD is complicated.[8] It is necessary to study the skin barrier dysfunction, itch sensation, abnormal immune responses, and incessant infection of Staphylococcus (S.) aureus to develop an effective treatment; however, the mechanisms have not yet been fully revealed.

AD is a highly complex genetic disease with phenotype-specific genes likely playing a significant role along with atopy genes. Filaggrin (FLG) mutations have an important role for the skin barrier. FLGs are filament-associated proteins which bind to keratin fibres in the epidermal cells. Skin barrier defect is considered as one of the basic pathophysiologic abnormality of AD. Moreover, a dysfunctional skin barrier precedes the inflammation process, as well as the epidermal barrier defects initiate the development of AD. An intact skin barrier is an important first line of defense against various factors such as irritants, allergens and microbes.

It is well known that itch is the major symptom of AD, which impacts most considerably on the quality of life of patients. The limited effect of non-sedating anti-histamine indicates that there are other mediators than histamine which might be involved, such as neuropeptides and cytokines.

There are several well-characterized cutaneous immune and systemic abnormalities in AD, including raised T helper (Th) 2-type cytokine expression in acute lesions, elevated serum immunoglobulin (Ig) E and sensitization to allergens, increased numbers of T cells expressing cutaneous lymphocyte-associated antigen (CLA), as well as decreased expression of anti-microbial peptides. In chronic lesions there is a Th1-type cytokine expression besides the Th2-type. Inappropriate adaptive immune response, with elevated IgE levels has been correlated with the severity of disease and infectious complications. Abnormalities in the body innate immune system, including diminished recruitment of cells such as neutrophils to the skin, reduction of the anti-microbial peptides, and epidermal barrier defects may play an essential role in infection or microbial colonization in the skin of AD patients.

Patients with AD have a unique susceptibility to be infected or colonized by a number of microbial organisms, mainly S. aureus, which can be cultured from 90% of skin lesions and significantly can colonize normal appearing skin in AD.[8]

1.1.5 The atopic mouse

There are limitations on studies using human AD subjects, and application of the experimental approach to patients is difficult. Therefore, analysis using laboratory animals is essential. There are several mouse models to study AD. [9-10]

The probably most used animal model is the NC/Nga mouse, originated from Japan as a fancy mouse and established as an inbred strain.[11] The NC/Nga spontaneously develop dermatitis, when kept in a non-sterile pathogen-free environment, but there is also a substrain in which dermatitis is induced by topical application of a mite antigen[12] or picryl chloride.[13]

NC/Nga mouse model manifests clinical and immunological aspects similar to AD patients-like skin manifestations, suggesting that this NC/Nga mouse model is an appropriate model for exploring the pathogenesis and treatment of human AD. The skin lesions of inbred NC/Nga mice are clinically and histologically very similar to human AD. Scratching behavior, the first sign of the skin changes, occurs at 6-8 weeks, and is followed by rapidly developing erythematous, erosive lesions with edema, and hemorrhages on the face, ears, neck, and back. The histological examination shows dermal infiltration with eosinophils and mononuclear cells before the appearance of clinical skin manifestations. Hyperparakeratosis, hyperplasia, and spongiosis are observed in the skin lesions at the age of 17 weeks.[10,14]

1.2 NEUROCUTANEOUS INTERACTION

There is a bilateral contact between the neuroendocrine system and the immune system, including the skin, to which the skin belongs.[5,15] (Fig.1).

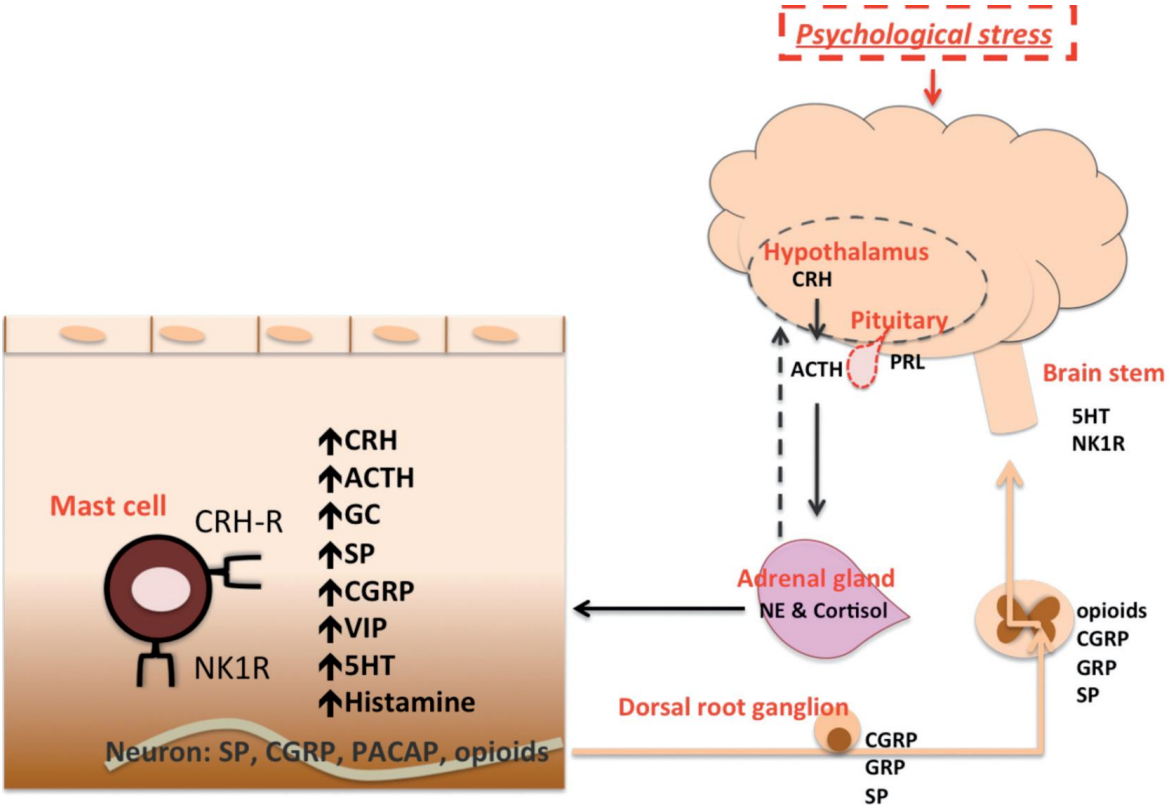

Fig. 1. Neuroendocrine system and skin response to psychological stress (Suarez et al.[16] , with permission from the authors).

That means that we may find ligands and ligand-receptors for neuromediators at central and peripheral locations. Stress involves an activation of the hypothalamic-pituitary-adrenal (HPA) axis, autonomic nervous system, and also neuropeptides.

Different mediators are responsible for this contact between the neuroendocrine and skin, being activated during stress.[16] One important mediator is 5-hydroxytryptamine (5-HT; serotonin). It has earlier been reported that acute stress is generally associated with an increase in the turnover of 5-HT, whereas chronic stress is usually associated with a sustained increase in the plasma cortisol, and causes a reduction in 5-HT turnover and release.[17]

There are important brain areas associated with stress, such as the prefrontal cortex, hippocampus and amygdale. In addition, profound changes in neuromediator expression, including serotonergic changes, in the cerebellum due to chronic stress, have been reported.[18] Recent studies [19-22] have indicated a connection between chronic

inflammation and the cerebellum, adding to the complex interaction between the neuro-endocrine and the immune systems.

1.2.1 Neurocutaneous interaction in atopic dermatitis

In AD lesions, there are numerous specific alterations in skin neurophysiology as well as skin neurobiology. It has been stated that the sensory functions of the skin are modified in AD patients.[20] There are various neurotransmitters and neurohormones that are involved in AD pathophysiology. Approximately 30 of them have been described in human skin, e.g., hormones, neuropeptides and classical neurotrans-mitters such as monoamines, acetylcholine, adrenocorticotropic hormone (ACTH), 5-HT, calcitonin gene-related peptide, angiotensin, corticotropin-releasing hormone (CRH), endorphins, enkephalins, galanin, histamine, gastrin-releasing hormone, melanocyte stimulating hormone (MSH), nitric oxide (NO), neurokinin A and B, neuropeptide Y, neurotensin, peptide histidine isoleucine (PHI), peptide histidine methionine (PHM), prolactin, parathyroid hormone, somatostatin, substance P, and vasoactive intestinal polypeptide (VIP).[20]

Itch is an important hallmark of symptoms of the AD disease. There is a variety of peripheral and central mediators suggested to play a role in the pathophysiology of this itch. The damaged barrier function is also connected with the itch-scratch cycle and further augments this vicious cycle.[21] There are several new mediators that have been suggested to be involved in AD- induced itch, such as interleukin(IL)-31, serine proteases, and nerve growth factor (NGF). This indicates that there are peripheral and central mechanisms and mediators implicated in the pathogenesis of itch in patients with AD.[21]

As stated above AD is a disease known to be worsened by psychological stress [5,16] and itch may be triggered or enhanced by acute or chronic stress. Interestingly, the

NC/Nga mouse has been used in stress experiments, and it was shown that water avoidance stress could lead to eczema even if the mice were kept in a sterile environment.[22]

1.3 SEROTONIN

5-HT is a monoamine. It is synthesized from tryptophan, which contains an indole ring and a carboxyl-amide side-chain. The rate limiting agent regarding the synthesis of 5-HT is tryptophan hydroxylase.

5-HT is a signalling molecule distributed broadly throughout the body, acting in the central and peripheral nervous system as a neurotransmitter. There are widespread serotonergic projections arising from the raphe nuclei in the brainstem, and 5-HT is one of the most broadly distributed neurotransmitters within the brain.[23] In man it is mainly found in the periphery, in platelets, while in lower animals also being found in mast cells.[24] The major source of 5-HT is the gut (enterochromaffin cells). 5-HT has profound effects both at the central and peripheral levels of the neuroendocrine system. It is playing an important role in regulating appetite, learning, behaviour, sexual desire, sleep, memory, and stress response.[25] It is also found in high concentrations at sites of inflammation. Platelets will release 5-HT upon aggregation after tissue injury. It is also assumed that 5-HT at the cellular or molecular level might influence differentiation, life span and dendricity of different cells. [25-26]

In the skin, 5-HT is expressed by melanocytes, Merkel cells and inflammatory cells, with a difference depending on the species.[27,28]

1.3.1 Serotonin receptors

5-HT, like a few other neurotransmitters, such as acetylcholine, glutamate, and γ-aminobutyric acid (GABA), acts via two categories of receptors: ionotropic and metabotropic receptors. Ionotropic receptors (channel receptors) have a low affinity for their neurotransmitter ligand but a rapid activation constant (a few milliseconds). In contrast, metabotropic receptors (receptors acting through G protein activation and second messenger production) exhibit both a high affinity for their neurotransmitter and a slow activation constant (in seconds or longer).[29]

The effects of 5-HT are thus mediated by cell surface membrane-bound receptors (R) of at least 21 different subtypes. The most well-characterized are the 5-HT1A and 5-HT2A receptors.[25]

5-HT1AR is a "transiently expressed" intronless receptor, that is, at specific times in development of/or during stress, quickly expressed at very high amounts. Decrease in 5-HT1AR number is probably due to increased 5-HT brain levels, since the 5-HT1AR expression is sensitive to autoinhibition.

The 5-HT1AR and 5-HT2AR have opposing functions in a variety of cellular and behavioral processes. The 5-HT1AR develops early in the central nervous system (CNS) and is associated with reduction of c-AMP levels in neurons. These actions provide intracellular stability for the cytoskeleton and result in cell differentiation and cessation of proliferation. Clinically, 5-HT1AR drugs decrease brain activity and act as anxiolytics. The 5-HT2AR develops more slowly and is associated with increased Ca^{++} availability in neurons. These actions destabilize the internal cytoskeleton and result in cell proliferation, synaptogenesis, and apoptosis. In humans, 5-HT2AR drugs produce hallucinations.[25]

Both these 5-HT receptors are involved in anxiety and stress.[30,31]

1.3.2 Serotonin transporter protein

The serotonin transporter protein (SERT; 5-HTT) belongs to the family of monoamine transporters and serves the high affinity reuptake of 5-HT into presynaptic terminals. SERT thus reuptakes, but may also release, 5-HT and therefore determines the magnitude and duration of the serotonergic response. Serotonin reuptake inhibitors (SSRIs), used for treating mood disorders, are acting by inhibiting the reuptake of 5-HT by SERT. Therefore, SERT has effectively been established as the initial target for clinically useful anxiolytic and antidepressant drugs.[23]

Genetic variations in promoter and intron regions of the SERT-gene are seen in individuals more prone to stress and depression.[32,33] An increase of epidermal and dermal cells expressing SERT, being dendritic or round mononuclear, in involved compared to non-involved inflammatory skin, have been observed in previous studies.[34]

1.3.3 Serotonin and inflammation

During inflammation the 5-HT expression in the skin is limited to platelets. 5-HT during inflammation activates receptors, such as 5-HT1AR and 5-HT2AR. Regarding 5-HT1AR, 5-HT endorses inflammation by way of increasing the number of mast cells at the site of the injury via this receptor. The human mast cells contain and releases 5-HT, which has been demonstrated to contribute to allergic inflammation in mice.[35]

5-HT in the skin has proinflammatory, vasodilatory and pruritogenic effects.[36, 37]

Agonists to 5-HT1AR, buspirone, and spiperone [37] were able to suppress allergic contact eczema,[38] while an antagonist to a 5-HT2AR, ketanserine, also suppressed allergic contact eczema.[39] SERT is involved in inflammation via different neuronal and non-neuronal pathways.[40]

1.3.4 Serotonin in atopic dermatitis

It has been reported that 5-HT plasma levels were elevated in AD patients compared with a healthy control group, and this elevation was correlated with the extent of the disease.[41] In addition, a 5-HT1A agonist, tandospirone, has been shown to improve the clinical features of AD.[42,43]

2 AIMS

To investigate the role of 5-HT in a mouse model of atopic eczema, which is subjected to chronic mild stress, via studying the expression of serotonergic markers, 5-HT, 5-HT1A and 5-HT2A receptors and SERT, in the skin, cerebrum and cerebellum (I and II).

To study the expression of these serotonergic markers in the skin of human AD patients and to correlate clinical, laboratory and psychodemographic data, focusing at chronic stress, anxiety and depression (III).

To investigate 5-HT as a pruritogenic and vascular affecting substance in the skin of AD patients compared to healthy controls (IV).

3 MATERIALS AND METHODS

3.1 ANIMAL STUDIES (I AND II)

In total, 24 6-week-old female NC/Nga mice (Charles River Laboratories, Germany) were used. The experiments were approved by the local Animal Ethics Committee. Animals were left to acclimatize for 1 week prior to the experiments after delivery from the vendor.

Chronic stress and immunization

The chronic mild stress procedure used has been described by Lanfumey et al.[44] and was used in a previous study.[45]

Briefly, mice were kept in a Scantainer box type 50-SCNT-Z11 (Scanbur AS, Köge, Denmark), in a conventional facility, and fed with pellets (R70, Granngården, Malmö, Sweden) and tap water. Different stressors were used on weekly basis, such as reversed light/dark cycle, one period of confinement to small cages for 12 h, two periods of placement with foreign mice for 2 h, one period of continuous overnight illumination, one overnight period of wet soil, one period of cage-tilting (30°) for 12 h and one period, 3 h, of food and water deprivation. Mice were divided into three groups (eight mice per group). One group was stressed and sensitized (stressed eczematous, SE). The mice in this group were subjected to chronic mild stress for 12 weeks, and eczema was induced from week 9, by painting their ears with a mite antigen, Dermatophagoides pteronyssinus (Allergon, Ängelholm, Sweden), at a concentration of 10 mg/ml, which had been dissolved in phosphate-buffered saline (PBS) and 0.5% Tween 20. A non-sensitized control group was similarly stressed (stressed control, SC), and had their ears painted using the solvent. A second sensitized group was relieved from stress (non-stressed eczematous, NSE), being kept in a regular cage and the mice in this group were also painted on their ears with the mite antigen from week 9. These mice were

maintained on a 12 h light/dark cycle under controlled temperature between 18 and 22°C and a humidity of 40-60%.

Processing of samples

After 12 weeks, the animals were killed by cervical dislocation and the ear thickness was immediately measured using a calliper (Kroeplin, Schluchtern, Germany), before being preserved together with the cerebri and cerebelli, for further analysis. Plasma corticosterone was measured using a corticosterone RIA kit (RS 490 11) from IBL (Hamburg, Germany) following the instructions of the manufacturer.

Tissue samples from the ears, cerebrums and cerebelli were subjected to immunohistochemistry in order to analyze changes in the serotonergic markers. For this purpose, the samples were fixed in Lana´s fixative (phosphate buffered 4% formaldehyde containing 0.2% picric acid) for 2 h at 4°C. They were then rinsed with 0.1 mol/L phosphate buffer containing 10% sucrose for at least 24 h. Tissues were then embedded in Tissue-Tek OCT Compound (Sakura Finetek, Zoeterwoude, The Netherlands) and sectioned (14 μm thick) using a Microm cryostat (Heidelberg, Germany). Cryosections were then mounted on Super Frost Plus glass slides (Menzel-Gläser, Freiburg, Germany) and stored at -70°C until being used for immunohistochemistry employing an immunofluorescence technique.

Immunohistochemistry

A biotinylated-streptavidine technique was used to detect all antibody-labelled molecules. The primary antibodies used overnight at 4°C, were rabbit polyclonal rabbit antibodies against 5-HT (20080; dilution 1:10,000; DiaSorin, Stillwater, MN, USA), 5-HT2AR (24288; 1:300; ImmunoStar, Hudson, WI, USA) and SERT (SERT#48; 1:2,000),[46,47] as well as a polyclonal guinea pig antibody against 5-HT1AR (AB5406;

1:7,500; Chemicon, Temecula, CA, USA). Slides were then incubated with secondary biotinylated anti-rabbit (BA-1000) or anti-guinea pig (BA- 7000) antibodies (1:2,000; Vector, Burlingame, CA, USA) for 40 min at room temperature, and finally the fluorochrome Cy2-labelled streptavidin (PA42001; 1:2,000; Amersham Pharmacia Biotech, Uppsala, Sweden) was added for 40 min at room temperature for visualization of antibody target labelling. When staining for 5-HT, adjacent sections were also stained with a rabbit polyclonal antibody against tryptase (1:20,000), a kind gift from prof. I. Harvima, Kuopio, Finland. We also performed double staining in order to confirm the neuronal characteristics of the 5-HT2AR- and SERT-positive nerve-like bundles. For this purpose we used a guinea pig polyclonal antibody against protein gene product 9.5 (PGP 9.5) (GP14104), at a dilution of 1:1,000 (Neuromics, Minneapolis, MN, USA), the secondary biotinylated anti-guinea pig antibody (from above) and then streptavidine-conjugated Texas Red (SA-5006; 1:2,000; Vector). All antibody solutions were diluted in PBS containing 1% bovine serum albumin (BSA) (A9418, Sigma-Aldrich, Stockholm, Sweden) prior to use.

As controls, the primary antibodies were omitted or normal rabbit serum IgG (X 936, Dako, Glostrup, Denmark), or guinea pig serum IgG (006-000-003; Jackson Immuno-Research, West Grove, PA, USA) were used at the same dilution as the primary antibodies. In addition, we performed preadsorption for 5-HT (serotonin creatine sulfate monohydrate, 85030; Sigma-Aldrich, Stockholm, Sweden), 5-HT1AR (AG349, Chemicon), 5-HT2AR (24333, Immunostar) and SERT (K596 peptide- the target for the SERT antisera). In the case of 5-HT, we utilized serotonin creatine sulfate at a concentration of 10^{-3} mol/L, the 5-HT1AR and 5-HT2AR peptides were used at a concentration of 5 μg/ml, respectively, whereas the SERT peptide was used at 1 μM. All the above mentioned controls resulted in substantially decreased or abolished

signals. Finally, the sections were mounted with Kaiser's glycerol gelatine (Merck, Darmstadt, Germany) before being covered with glass slips.

Microscopy

Labelled skin sections were analyzed using a fluorescence Zeiss Axioskop 2 MOT microscope (Carl Zeiss, Stockholm, Sweden). The numbers of 5-HT-positive mononuclear cells were counted in four representative wells per ear section of the skin and the mean was calculated. Epidermal area of expression was estimated as: 0–25% (1), 26–50% (2), and higher than 50% (3). Fluorescence intensity was scored as: none = 0, low = 1, moderate = 2 and high = 3. The number of immunoreactive nerve bundles were calculated per section, the absolute number being given, and the intensity of staining being semiquantitatively evaluated as none = 0, low= 1, moderate = 2 and high = 3.

For the cerebrum, a semiquantitative technique was used. The cells in the prefrontal cortex immunoreactive for the different markers were evaluated as: none = 0, low = 1, moderate = 2 and high = 3, and their intensity as above. In the hippocampus, we focused on CA1 and CA3 regions and used 1 = 1–3, 2 = 4–6 and 3 = 7 or more positive cells in the respective area. The intensity was scored as above.

In the cerebellar study, for the 5-HT1AR, an image analysis technique was used with an appropriate software (Easy Image Analysis, Bergström Instruments, Solna, Sweden). Labelled sections were examined using the fluorescence microscope. Images of 4-6 sections were captured by a digital camera connected to a PC before being analyzed using the software. The area fraction (ratio of specifically immunoreactive/ nonimmunoreactive areas) was calculated.

For the 5-HT2AR a semiquantitative technique was used, focusing at the density of-the Purkinje cells, being absent=0, low=1, medium=2 and high=3, and the intensity of the staining signal, none=0, low=1, moderate =2 and high =3. Slides were coded prior

to analysis to permit blind evaluation. Also here four to six microscopic fields per section were selected and used for quantification.

3.2 HUMAN STUDIES

3.2.1 Immunohistochemical-correlative study (III)

Patients

Twenty eight patients, 18 females and 10 males, with a mean age of 29.5 years (range 19-48 years) were recruited. They had ongoing AD, according to the criteria of Williams et al.[3] and itching (in the past 3 days), and had been referred by their family doctors to The Department of Dermatology. The patients should not have had systemic therapy (including phototherapy and antihistamines) within one month before the study.

Ten healthy control individuals, mainly staff and students, 5 males and 5 females (mean age 37.7 years (range 20–61 years)), with no history of atopic manifestations for themselves, previous and present hay fever, asthma nor AD, were also recruited.

Ethical permission was obtained from the local ethical board.

SCORAD

Extent of the disease was determined using SCORing of Atopic Dermatitis (SCORAD).[48] Both objective and subjective SCORAD were determined.

Clinical pruritus

The degree of clinical pruritus was determined using a visual analogue scale (VAS).

Chronic stress

Degree of chronic stress was measured by the salivary cortisol test.[49] Samples were obtained from 20 patients. Salivary cortisol was measured in the morning of three consecutive days. In the evening of day 3, 0.5 mg dexametasone was administered orally, thereby blocking input from hypothalamus on adrenal cortex, followed by a new salivary cortisol test on the fourth day. A ratio between the mean value of the first three days and day 4 was calculated, where a ratio below 2 is an indicator of chronic stress. Immunoassay was done at the Sahlgrenska University Hospital, Gothenburg, using a radioimmunoassay (RIA) method.

Psychodemographic data

The patients personality traits were evaluated by using the SSP (Swedish Universities Scales of Personality),[50] a 91 item questionnaire, filled by the patients and analyzed regarding somatic trait anxiety (STA), psychic trait anxiety (PsTA), and stress susceptibility (SS). Instructions on how to fill in the forms were given by the physician. To assess depression MADRS-S (Montgomery-Åsberg Depression Rating Scale-Self assessment) [51] was used.

Processing of biopsy specimens

Biopsies, 3 mm, were taken from L (lesional) skin (with dryness, paples, often lichenified) of the elbow, and N-L (non-lesional) skin from the lower back region. No topical steroids had been used on any of the areas for at least 14 days. Lana´s fixative was used for fixation of the biopsies for 2 h at 4^{o}C. After fixation they were rinsed in 0.1 M Sörensen's phosphate buffer supplemented with 10 % sucrose for at least 24 h and then rapidly frozen and stored at -70°C until further processing. While still frozen, the biopsy material was placed in a tissue holder, embedded in Tissue-Tek OCT

Compound. A Microm HM 500 cryostat was used for cutting the biopsies into 14-µm thick sections and mounted onto Super Frost glass slides for immunohistochemical staining using a biotinylated-streptavidine procedure.

Immunohistochemistry

Sections prepared as described above were incubated first with 10% normal goat serum (S 100, Vector, Burlinghame, CA, USA) for 40 min and were then incubated overnight at 4^{o}C with rabbit polyclonal antibodies against 5-HT (20080, dilution 1:10,000; Diasorin, Millwater, USA) or 5-HT1AR (SlA-170; used at a dilution of 1:1,000). The 5-HT1AR antibody is directed against amino acids 170–186 in the second extracellular loop.[52] Alternatively, the sections were incubated with 10% normal horse serum (S-2000, Vector) followed by mouse monoclonal antibodies toward SERT(ST51-5, diluted 1:10,000, MabTechnologies, Stone Mountain, GA, USA) directed against amino acid residues 51-66 coupled to keyhole limpet hemocyanin (KLH) through an additional N-terminal cysteine residue or 5-HT2AR (sc-166775, dilution 1:400; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Thereafter, incubation with streptavidine-biotin-labeled anti-rabbit (BA-1000) or anti-mouse (BA-2000) IgG (both in dilution 1:200; Vector) as the secondary antibodies was performed for 40 min at room temperature, followed by treatment with the fluorochrome Cy2-labelled streptavidine (PA42001, 1:2,000; Amersham Biosciences) for 40 min at room temperature.

Control staining with unspecific mouse IgG of the same isotype (X0931, Dako, Glostrup, Denmark) and in the same dilution as the monoclonal antibody against SERT or 5-HT2AR, was negative. In the case of polyclonal antibody toward 5-HT, preadsorption with this compound (85030, Fluka, Sigma-Aldrich), at a concentration of 10^{-6} mol/L, eliminated the immunostaining. Moreover, when the primary antibodies were omitted, a substantial reduction or no staining occurred.

Microscopy

Of general histopathological changes, hyperkeratosis, acanthosis and degree of infiltration by inflammatory cells in the dermis were graded semiquantitatively, 0-3 (0= normal appearance, 1= mild, 2= moderate and 3= severe).

The degree of 5-HT-IR was determined in the epidermis and inflammatory infiltrates, graded 0-3, and the absolute number of 5-HT-positive platelets were determined.

For 5-HT1AR the fraction of positive staining in the epidermis was evaluated, 0=0%, 1=25%, 2=50% and 3=75%. The absolute number of positive 5-HT1AR inflammatory cells in the papillary dermis was determined.

For 5-HT2AR the epidermal fraction expressing 5-HT2AR of the total thickness of epidermis was, similarly as the 5-HT1A epidermal fraction, graded 0-3. The basal membrane intensity was graded 0 for minimal staining, and 1-3 for increased staining intensity. The number of vessels expressing 5-HT2AR in papillary dermis was graded 0-3 (0<40, 1=40-60, 2=60-80 and 3=80 vessels per section).

The number of SERT-positive mononuclear cells was counted in the epidermis and papillary dermis, respectively. In addition, the immunoreactivity was determined in the basal epidermal layer and the signal intensity assessed 0-3, as absent, weak, moderate or strong.

3.2.2 Intradermal injection study (IV)

Subjects

Twenty five patients, 14 females and 11 males, with a mean age of 31.1 ± 7.8 years (range 19-46 years), with ongoing AD and itching (in the past 3 days and with a wide individual itch range), were recruited. They had been referred by their family doctors to The Department of Dermatology.

The patients were diagnosed as having AD using the criteria of Williams et al.[3] The scoring of AD (objective SCORAD) was 32.8 ± 12.3, the clinical itch for the previous three days according to VAS was 3.9 ± 2.1cm, and a Patient-Oriented Eczema Measure (POEM) [48] was 12.1 ± 6.3.

The patients should not have had systemic therapy (including phototherapy and antihistamines) within one month before the study. They were allowed to use topical therapy (glucocorticoids, calcineurin inhibitors) on L skin of the arm to be injected, but not on N-L skin (the site of injection).

Twenty five healthy control individuals, mainly staff and students, 17 females and 8 males, mean age 30.4 ± 6.2 years (range 22-41 years), with no history of previous or present hay fever, asthma or AD/eczema, were also recruited.

The experiment was run during the time period of March until May 2011. Ethical permission was obtained from the local ethical board.

Substances

All substances were dissolved in sterile, pyrogen-free, physiological saline containing 10% (v/v) Sörensen phosphate buffer ($Na_2 HPO_4 + KH_2 PO_4$ 67 mmol/L; pH 7.4). 5-HT was used at a concentration of 2.5 mg/ml, chosen after a pilot study. Histamine dihydrochloride 10 µg /ml (Sigma-Aldrich, Sweden) was used as a positive control and Sörensen's buffered saline as a negative control. The histamine dosage is one of the standard dosages that we, as well as others, have used in studies of experimental itch with histamine, see, e.g.,[53-66]. The substances were injected intradermally and separately in random order in volumes of 20 µl. Injections were given into N-L areas, with no visible or palpable lesion within a 5 cm area, on the lateral part of the upper arms of the subjects. No injections were given into L areas, because of difficulties to standardize such lesions and to measure vascular effects.

The substances had been coded in vials with different colours by a lab technician. Decoding was done after the completion of the whole study. Thus, the experiment was carried out under double-blind conditions. To maintain experimental consistency, the same individual performed the experiment, including the injections and supervision of the recordings in all subjects.

Recordings of experimental pruritus

Both the duration and intensity of pruritus were recorded simultaneously with the VAS attached to a computer (Somedic, Hörby, Sweden), whereas the areas of the flare and wheal reactions were measured with a ruler after each injection. The subjects were asked to rate their itch by moving a knob on a 100-mm VAS, which was graded from 'no itch' (0 mm) to 'maximum itch' (100 mm). All subjects were instructed to continuously adjust the position of the knob so that the position should always reflect the present itch. The investigator also regularly reminded the subjects about this during the experiment. The subjects always used their dominant hand to ensure the best motoric precision of the rating. In addition, the localization of the different injection sites was randomized to avoid a systematic error depending on localization.

The time intervals between each injection and the initiation and termination of pruritus were recorded. Thus, calculations of itch latency (sec), itch duration (sec), maximum itch intensity (mm) and area under the curve (AUC) (mm x sec) were automatically downloaded into the computer. Such methods for measuring experimental pruritus have been used extensively and were validated in previous studies.[54-56] The areas of flare and wheal reactions were recorded after 5 min and outlined on a transparent plastic film. We considered 5 min as most appropriate based on earlier studies [57,58,69] and, in addition, a pilot study in the present investigation showed rather constant values for the wheal, while the flare was slightly increasing

with time (0-15 min). The maximum perpendicular diameters for the flare were measured in mm and the surface calculated in square mm,[59] while in case for the wheal the maximum diameter in mm was recorded. The wheal was recorded by measuring only one diameter, because pilot tests with the dosages we used showed that the reactions were always round or oval and symmetrical with no pseudo-pods, making it necessary to measure a perpendicular diameter. The reactions to all injected substances were followed for 15 min regardless of having itch or not.

3.3 STATISTICAL ANALYSIS

Study I

For comparison of the number of 5-HT-positive cells between the groups, Student's t-test was used. For the semiquantitative data, the chi-square test and/or Fisher's exact test were used. A p value of <0.05 was regarded as significant (also being the case for the following studies).

Study II

Multiple comparisons of continuous data were performed by analysis of variance, ANOVA. In the case of a statistically significant result in the ANOVA, statistical comparisons between two arbitrary groups were made by use of the post-hoc test proposed by Fisher to control for multiplicity.[19,60]

Study III

Processing of the absolute numerical data was done to find out if there was any difference between L and N-L skin, and normal healthy skin (non-parametric test). For

the semiquantitative data, the chi-square test and/or Fisher's exact test were used in non-dependant samples, and Student´s t-test or non-parametric test, if dependent samples. Correlation between the different parameters was measured using Spearman´s or Pearson's tests.

Study IV

Student´s t-test, and the Wilcoxon signed-rank test for dependant samples and the Mann-Whitney test for independent samples were used. The Spearman´s rank-order test was used to determine the correlation between the variables.

4 RESULTS

4.1 ANIMAL STUDIES (I AND II)

General findings

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

Corticosterone levels

The SE group showed lower ($p < 0.05$) corticosterone values 496.4 ± 221.5 ng/ml, compared to the other groups, 738.6 ± 232.1 in the NSE and 805.2 ± 144.4 ng/ml in the SC.

Skin

There was an increase ($p < 0.01$) in the number of 5-HT-containing dermal mono-nuclear cells in the mice with induced eczema (SE 295 ± 46 cells/section and NSE 259 ± 58) compared with the control group (SC 164 ± 23). There was a trend ($p = 0.09$) toward more 5-HT-containing cells in the SE group, compared with the NSE. In the SC group, the 5-HT-positive cells were smaller and less granulated, whereas in the SE group, they were larger, often degranulating, widely distributed and located closer to the epithelium compared to both the NSE and SC groups. When staining adjacent sections for tryptase a similar expression was obtained indicating that these 5-HT-positive cells are mast cells.

5-HT1AR-IR was seen in the skin sections, with an increase in the eczematous groups ($p < 0.01$ for SE and $p < 0.05$ for NSE) compared to the control, and with no difference between the SE and NSE groups.

5-HT2AR-IR was found in nerves, Langerhans cell-like cells and in the apical epithelium. In both eczema groups, the dendrites of the Langerhans cell-like cells were longer than in the SC group. The 5-HT2AR epidermal area immunoreactivity was most evident in the SE (2.4 ± 0.5) and NSE (2.1 ± 0.4) groups compared to the SC (1.3 ± 0.5) group, with a difference ($p < 0.01$) between the SE and NSE groups, and between the SE and SC ($p < 0.001$) groups, whereas the intensity was highest in the SE (2.8 ± 0.4) and NSE (2.6 ± 0.6) groups compared to the SC (1.4 ± 0.5) group. A difference in intensity levels was detected ($p < 0.05$) between the SE and NSE, and also between SE and SC ($p < 0.001$). 5-HT2AR expression was also seen on nerve bundles, which was confirmed by double staining with PGP 9.5.

The number of 5-HT2AR-positive nerve bundles was decreased ($p < 0.01$) in the SE (6.3 ± 3.1 bundles per section) compared to the NSE (11.3 ± 3.6), and SC (8.8 ± 3.4) groups. In addition, the fluorescence intensity of the nerve bundles for 5-HT2AR was lower ($p < 0.01$) in the SE (1.8 ± 0.5) as compared with the NSE (2.6 ± 0.7) and SC (2.3 ± 0.4) groups.

SERT-IR, in addition to the epidermis, was also found in nerve bundles with a decreased number in the SE (5.9 ± 2.4) compared to the NSE (11.9 ± 3.1; $p < 0.001$) and SC (9.5 ± 3.2; $p < 0.05$) groups. There was no difference in bundle intensity between the NSE (2.4 ± 0.6), SE (2.1 ± 0.4) and the SC (2.2 ± 0.6) groups.

Cerebrum

Prefrontal cortex

No significant changes between the mouse groups were found. The expression of all the serotonergic markers was limited in this area.

Hippocampus

There was no difference in neither 5-HT- nor SERT-IR in CA1 and CA3 areas between the groups.

There was a hippocampal (CA1 and CA3) expression of 5-HT1AR. There was an increase ($p < 0.05$) in the SE compared to the SC group, however, with no difference between the SE and NSE, regarding fluorescence intensity in CA1. In the CA3, a higher ($p < 0.05$) immunoreactive cell number in the eczematous groups compared to SC, and an increase ($p < 0.05$) in fluorescence intensity in the NSE in contrast to SC, were obtained.

In the CA1 area, we noted an increase in the quantity of cells immunoreactive for 5-HT2AR in the SE (1.5 ± 0.5) compared to the NSE (1.0 ± 0.0; $p < 0.05$), and SC (1.0 ± 0.0; $p < 0.05$) groups, whereas the fluorescence intensity was 1.6 ± 0.5 in SE, 1.1 ± 0.4 in NSE and 1.3 ± 0.5 in SC, with a tendency ($p = 0.07$) toward an increase in SE compared to NSE. In the CA3 area, no differences could be found between the SE and NSE.

Cerebellum

Expression of 5-HT1AR was seen on the Purkinje cells and also some small interneuronal cells in the molecular layer. There was a difference ($p < 0.001$) in the 5-HT1AR area fraction between the SE, 0.77 ± 0.24 (mean ± SD) and NSE, 1.56 ± 0.42, groups, thus, with a lower value in the SE group. The lowest value was found in the SC group, 0.18 ± 0.08, also significantly different ($p < 0.001$) from the other groups.
The 5-HT2AR was expressed in the Purkinje cells and sometimes in nerve fibres extending far out into the molecular layer. The cell density of the immunoreactive cells was highest in the SE, 2.2 ± 0.3, compared to the other groups, NSE, 1.7 ± 0.2 ($p =$

0.001), and SC, 1.3 ± 0.3 ($p < 0.001$), and there was also a difference ($p = 0.05$) between NSE and SC.

The intensity of the fluorescence signal was also highest in the SE group, 1.9 ± 0.2, compared to the NSE, 1.6 ± 0.3 ($p < 0.05$), and SC, 1.5 ± 0.4 ($p < 0.01$).

4.2 HUMAN STUDIES

4.2.1 Immunohistochemical-correlative study (III)

Clinical data

The objective SCORAD was 42.3 ±11.5 (mean ± SD) (range 21.2 to 65.5) and subjective SCORAD 51.6 ± 13.4 (range 26.2 to 73.5). The pruritus, using the VAS scale, was 5.2 ± 2.4 (range 0-10).

Cortisol ratio

The cortisol ratio was 2.6 ± 3.0 (range 0.3-14.1).

Psychodemographic data

STA was 15.1 ± 4.3 (range 8-22), PsTA was 15.2 ± 3.8 (range 9-25), and SS 16.3 ± 4.1 (range 7-24). The score for MADRS-S was 8.0 ± 6.5 (range 0-24).

General histopathological findings

The degree of hyperkeratosis was higher in L, 1.9 ± 0.7 compared to N-L, 1.2 ± 0.4 skin and normal healthy skin, 0.6 ± 0.5, a statistical difference ($p < 0.001$) between L, and NL and control skin, respectively, and also ($p = 0.001$) between N-L and control skin. The degree of acanthosis was 2.1 ± 0.8 in L skin, 0.6 ± 0.8 in N-L and 0.5 ± 0.5 in normal healthy skin, with a significant difference ($p < 0.001$) between L, and N-L skin

as well as the control skin. The degree of inflammation was 2.4 ± 0.7 in L skin, while it was 1.1 ± 0.8 in N-L skin and 0.3 ± 0.5 in normal healthy skin. This difference was significant ($p < 0.001$) between L, and N-L as well as control skin, and there was a strong tendency ($p = 0.06$) for a difference between N-L and control skin.

5-HT

The epidermal immunoreactivity for 5-HT was similar, 2.1 ± 0.4 in L and 2.0 ± 0.3 in N-L skin, while 2.5 ± 0.5 in normal healthy skin. There was a statistical difference between N-L ($p < 0.01$) compared to the healthy control skin, and a tendency ($p = 0.07$) to a difference between L and control skin

The 5-HT staining of the inflammatory infiltrate was 2.0 ± 0.6 in L and 1.8 ± 0.5 in N-L skin, while being 2.5 ± 0.5 in normal healthy skin. There was a statistical difference between L ($p < 0.05$) and N-L ($p = 0.001$) skin, compared to the healthy control skin.

The highest number of 5-HT-positive platelets, 5.0 ± 2.3, was found in L skin, being 2.0 ± 0.8 in N-L skin and 1.0 ± 0.7 in normal healthy skin. The difference between L and N-L skin was statistically significant ($p < 0.001$), so was the difference between healthy control skin, and L ($p < 0.001$) and N-L ($p < 0.01$) skin, respectively.

5-HT1AR

A 5-HT1AR-positive epidermal fraction was found in the apical part of the epidermis, more evident ($p < 0.001$) in the L, 1.1 ± 0.7, compared to N-L skin, 0.3 ± 0.5, and also with a higher signal intensity. The fraction in normal healthy skin was 0.3 ± 0.5, which means that there is a difference ($p < 0.01$) against L skin.

There were also dermal inflammatory, mast cell-like, cells that expressed the 5-HT1AR, their number being higher ($p < 0.001$) in L, 31.9 ± 10.6, compared to N-L skin, 17.5 ± 6.0, also the value in the L skin being higher ($p < 0.001$) compared to normal healthy control skin, 14.4 ± 3.6.

5-HT2AR

There was a significant difference ($p < 0.05$) in epidermal fraction expressing 5-HT2AR in L compared to N-L skin, reaching 1.8 ± 0.7 in L and 1.3 ± 1.1 in N-L skin. In the normal healthy skin the fraction was 0.6 ± 0.5, which is different ($p < 0.001$) from L skin. There was no difference in the epidermal 5-HT2AR intensity.

There was a basement membrane signal being more evident in N-L, 2.3 ± 0.7, and healthy control skin, 2.4 ± 0.4, compared to L skin, 1.6 ± 0.6. The difference between L skin and N-L skin was significant ($p < 0.001$) and also significant ($p < 0.001$) between L and normal healthy skin.

The number of 5-HT2AR immunoreactive vessels in papillary dermis was increased ($p < 0.001$) from normal control skin, 0.8 ± 0.6, and N-L, 0.8 ± 0.7, respectively, to L skin, 2.3 ± 0.8.

SERT

There was a higher number of the epidermal SERT-positive cells, 13.8 ± 5.7 and 20.4 ± 12.0 in N-L and L skin, respectively, normal control skin showing 11.5 ± 2.1. There was a statistical difference ($p < 0.05$) between L skin, and N-L skin and healthy control skin, respectively. The dermal SERT-positive cells were 20.3 ± 4.5 and 39.9 ± 9.2 in N-L and L, respectively, while in normal healthy skin being 16.5 ± 3.2. There was a difference ($p < 0.001$) between L and N-L skin as well as healthy control skin and also a difference ($p < 0.05$) between N-L skin and healthy control skin, indicating a role of SERT for antigen presentation. There was a higher immunoreactivity in the basal layer of the L, 1.6 ± 0.6, compared to N-L, 0.5 ± 0.5, skin, while in normal healthy skin, 0.4 ± 0.5, which indicates that keratinocyte proliferation might be affected by modulating

this protein. There was a difference ($p < 0.001$) between L and N-L skin, as well as between L and normal skin.

Correlations

The degree of subjective itch correlated with STA (r = 0.50; $p = 0.01$) and SS (r = 0.44; $p < 0.05$).

In the L skin there was a correlation (r = 0.38; $p < 0.05$) between the number of 5-HT1AR-positive inflammatory dermal cells and objective SCORAD. Moreover, a correlation (r = 0.39; $p = 0.05$) between the epidermal 5-HT1AR fraction with the MADRS-S score and also a reverse correlation (r = -0.48; $p < 0.05$) between the 5-HT2AR-positive vessels and the MADRS-S score, was found. There was a reverse correlation (r = -0.42; $p < 0.05$) for the basal epidermal SERT-IR with the SS and a tendency (r = 0.36; $p = 0.07$) to a reverse correlation with PsTA.

In N-L skin the objective SCORAD correlated (r = 0.56; $p < 0.01$) with the degree of acanthosis. There was a correlation (r = 0.53; $p < 0.05$) between the epidermal fraction of 5-HT2AR and the cortisol ratio. In addition, a correlation was found between the number of 5-HT2AR-positive vessels in the N-L skin and the objective (r = 0.38; $p = 0.05$) and subjective (r = 0.39; $p < 0.05$) SCORAD, respectively.

4.2.2 Intradermal injection study (IV)

The quality of sensation of the experimentally induced pruritus varied among participants. Some described pruritus as burning, others as painful.

However, there was no difference between the qualitative descriptions reported by patients with AD and by healthy controls, regardless of the substance injected.

5-HT, histamine and buffer induced itch in 23, 22 and 14 patients with AD and 21, 23 and 9 healthy controls, respectively. For the different itch variables there was no significant difference between patients with AD and healthy controls. However, itch latency differed significantly between the substances, being shorter for 5-HT compared with histamine, both in patients with AD ($p = 0.001$) and in healthy controls ($p < 0.05$).

5-HT, histamine and buffer induced flare and wheal in all 25 patients with AD and in all 25 healthy controls, respectively. However, flare and wheal differed between patients with AD and controls, with lower values in patients with AD for 5-HT ($p < 0.01$ and $p < 0.05$, respectively) as well as for histamine ($p < 0.001$ and $p < 0.01$, respectively).

There was no difference between the sexes for itch, flare or wheal results.

There was a correlation between the objective SCORAD and the clinical SCORAD pruritus ($p < 0.001$) and POEM ($p < 0.01$), respectively. There were no correlations between the clinical findings (i.e. clinical SCORAD pruritus, POEM), and the recorded experimental itch, nor flare or wheal responses for 5-HT in patients with AD. There was, however, a negative correlation ($p < 0.05$) for histamine, between clinical SCORAD pruritus and wheal, as well as between POEM and wheal, in patients with AD, i.e. the higher the SCORAD pruritus or POEM score, the smaller the wheal.

5 DISCUSSION

5.1 ANIMAL STUDIES (I AND II)

The highest degree of eczema in these studies was found in the mouse group exposed to chronic mild stress. This finding is consistent with other studies showing that chronic stress may increase an eczematous reaction.[22,61 ,62]

It is interesting that the SE group had the lowest level of corticosterone, while both the NSE and SC groups had higher levels. The low level in the SE group indicates a chronic HPA axis suppression, when both stress and inflammation are combined. In AD patients, a blunt HPA axis responsiveness to stress has been described, resulting in a failure to mount a sufficient cortisol response.[63]

We observed a decreased neuronal expression of 5-HT2AR and SERT in the skin of the atopic-like mouse strain exposed to chronic mild stress. At the same time, we detected an increased hippocampal expression of 5-HT2AR, in the CA1 area of the cerebrum. These findings are interesting since the CA1 area has been associated with chronic stress.[64,65] The discrepancy with an increased hippocampal signal for 5-HT2AR but a decreased skin nerve signal for this marker, in the SE group, might be due to a downregulation of this receptor in the peripheral neurons and a central upregulation in the hippocampus. In addition, the increased 5-HT2AR expression may suggest a lower level of the ligand influenced by both stress and eczema. This may in turn be reflected on the activity of the HPA system and the subsequent lower levels of corticosterone in the SE mice.

It has been recently reported that 5-HT1/5-HT2 receptors may be involved in scratching in mice.[66] Prior electron microscopy studies demonstrated that 5-HT2AR receptors are expressed on peripheral sensory axons in the rat skin.[67] We confirmed by immunohistochemistry that the 5-HT2AR also exists on sensory nerve fibres extending far out into the mouse epidermis. The 5-HT2AR has also been reported to

contribute to mechanical hyperalgesia in a rat model of neuropathic pain.[68] Both nociceptive and A-δ nerve cell bodies are also known to express 5-HT2AR in rat dorsal root ganglia.[69]

We also detected an increase of 5-HT2AR-IR in the ear epithelium of the SE compared to the NSE and SC mice. The chronic mild stress appears to have resulted in a worsening of the inflammatory status of the skin exposed to the mite antigen, and also induced a stronger scratching behavior. The explanation could either be that the stress reduced the degree to which the mice were able to cope with the itch stimuli, causing a greater scratching behavior. Alternatively, stress responsive pathways in the brain may indirectly, via neural or humoral pathways, increase the inflammatory response through an impact on inflammatory cells, such as keratinocytes, which have been shown to express 5-HT2AR and 5-HT1AR.[70,71]

SERT was shown to be present in nerve bundles in the mouse ears, where we observed a downregulation in the eczema group exposed to stress compared to non-stressed eczema group. This is interesting, since SERT modulating effects have been suggested to be mediated through the 5-HT2AR.[72]

It has been suggested that a 5-HT1AR agonist may be of use in the clinical management of stress-associated aggravation of AD in humans.[43] However, we could not find any difference in 5-HT1AR expression between the eczematous groups, SE and NSE, neither in the skin nor brain. It was more an upregulation dependent on the inflammation, which could be seen in the skin and in the brain.

5-HT-containing mast cell-like cells were found to be more numerous in the skin of the SE and NSE groups compared to the SC, suggesting that a good part of this effect being caused by the eczema *per se*. However, there was also a tendency for more 5-HT-positive cells in the SE than in the NSE group suggesting a possible added effect of stress. The cells were also larger, more often degranulating and located closer to

the basement membrane, consistent with a more vigorous inflammation. In this context we note a lack of 5-HT1A receptors on the mast cells, in contrast to findings in humans and dogs.[24,70] The increase in the number of mast cell-like cells and their degranulation with secretion of 5-HT in the skin might contribute to a decreased peripheral neuronal 5-HT2AR expression.

In the **c**erebellum, chronic mild stress gave a reduced immunoreactivity for the 5-HT1AR in SE compared to NSE mice, however, the lowest values were found in the SC group. This indicates a contribution of the inflammation *per se* to the increased immunoreactivity of this receptor, but at the same time that the stress *per se* gives a decreased immunoreactivity. This reduced immunoreactivity might be due to a decreased synthesis of 5-HT1AR, but there are also other possibilities such as internalization of the receptor.

The 5-HT1AR may be involved in the serotonergic control of growth of 5-HT fibers and/or the modulation of Purkinje cell activity [73,74] Functionally 5-HT has been shown to be able to modify the firing of these cells in the short term by changing the firing pattern from regular to burst firing.[74] This might have an impact on the motor pattern, including the scratching behaviour. In the long term 5-HT may regulate dendrite formation in these cells via the 5-HT1AR and 5-HT2AR.[75]

Regarding the 5-HT2AR, the highest expression was found in the SE group, while the lowest was found in the SC group, indicating a role for the receptor both for the inflammation and the chronic stress. These results for the 5-HT2AR are also in line with our results from the hippocampus, CA1 area.[76] Additionally, our results were consistent with a recent report about an increase in 5-HT2AR in the cerebellum in a model of chronic stress in gold-fish.[77]

The functional impact of the modulation of the cerebellar serotonergic receptors during chronic mild stress remains to be shown. The Purkinje cells are critical to the

output of cerebellum to other brain areas.[60] In the Purkinje cell layer an expression of heat shock protein 70 was shown in an animal model of sustained muscular contraction,[78] suggesting an involvement of these Purkinje cells in muscle tension. AD patients may show increased muscular tension,[79] which may vary depending on the degree of anxiety/chronic stress, and here we might be dealing with a possible role for the cerebellum and its Purkinje cells.

Chronic skin inflammation, as in atopic eczema, may thus affect the function of the cerebellum through the serotonergic system. At least two mechanisms might be possible- a chemical effect of blood carried inflammatory mediators on the cerebellum- or an effect of a changed movement pattern. Johansson et al.[80] showed that skin or brain inflammation may have an impact on the cerebellum, confirmed by the increased fusion between Purkinje cells in the cerebellum and transplanted bone marrow derived stem cells.

Since female mice were used in our animal studies we can not rule out the possibility of a hormonal impact on the results during the estrous cycle.

5.2 HUMAN STUDIES

5.2.1 Immunohistochemical study (III)

We found a correlation between itch, which is a primary and critical symptom in AD, and SS and PsTA. However, we did not find a correlation between the eczema severity (SCORAD) and any psychodemographic data. In addition, it was a rather wide deviation regarding the psychodemographic scores. Previously, Oh et al.[7] reported an association of stress with symptoms of AD. Pruritus was correlated with state anxiety and trait anxiety, while the SCORAD did not show a correlation with psychological parameters. This is to be compared with earlier reports of a special personality in AD

being prone to an increased SS.[63] This also indicates the complexity of this field which includes several confounding factors being involved in AD and its worsening.

The patients in the study by Oh et al.[7] included both males and females as was the case in our study. This may have an impact on the results. There are gender differences regarding anxiety and SS, but we in the present study were primarily interested to study serotonergic mechanisms in the different individuals. When extracting the female patients we found a correlation between SS and objective SCORAD, which is interesting in this respect. Further extended studies, which incorporate gender aspects would be of value.

Moreover, we did not see a correlation between the cortisol ratios and the severity of the disease. The mean cortisol ratio values were in the range of what we define as chronic stress. Furuichui et al.[90] reported increased salivary cortisol levels in AD patients in comparison to healthy subjects and that these levels correlated with SCORAD values.

Furthermore, we did not find a correlation between the extent of the eczema (SCORAD) and the pruritus, which contradicts with our earlier findings.[91] Here we might be dealing with confounding factors, such as the climate.

There was a difference between N-L and normal healthy control skin regarding hyperkeratosis and also a tendency to a difference regarding general inflammation. Moreover, there was a correlation between objective SCORAD and the degree of acanthosis in N-L skin. These findings are in line with the notion that N-L skin is not to be regarded as normal skin. As ´L´ skin we always chose skin from the elbow. This skin area is prone to be subjected to scratching. On the other hand most of the lesions of AD are dynamic, in contrast to inflammatory lesions of psoriasis. This also supports the value of studying N-L skin in AD.

A 5-HT1AR- positive signal was found in the apical part of the epidermis, more evident in the L compared to N-L skin, which indicates that this receptor has a role for keratinocyte differentitation. There were also inflammatory, mast-cell-like cells, which expressed the 5-HT1AR, their number being higher in L compared to N-L skin, and which may have an impact on the inflammatory process. There was a correlation between the number of 5-HT1AR-positive inflammatory dermal cells in the L skin and objective SCORAD. In earlier studies on contact eczema [92] and psoriasis [93] we found a lower number of 5-HT1AR-positive dermal cells in L skin. The reason for this discrepancy with our findings is not evident, but these inflammatory conditions, with the stable psoriasis, and the well defined contact eczematous reaction, are quite different from the often scratched AD.

Regarding 5-HT2AR, there was a higher epidermal fraction expressing this receptor in L compared to N-L skin. In N-L skin there was also a correlation between epidermal fraction of 5-HT2AR and the cortisol ratio. This indicates a role for this receptor for the keratinocyte proliferation/differentiation and also the importance of chronic stress.

The number of 5-HT2AR-positive vessels in the papillary dermis was increased from N-L to L skin. The finding of positive vessels might be due to a general importance of vessels for the inflammation, but a more specific role of the 5-HT2AR expressed on the vessels can not be excluded. There was a correlation between the number of 5-HT2AR-positive vessels in the NL skin and the objective and subjective SCORAD, respectively.

In L skin there was a reverse correlation for the 5-HT2AR immunoreactive vessels with the depression score. This might indicate a protective role for this receptor regarding depression, which is a somewhat an unexpected finding. However, an anti-inflammatory effect by this receptor has earlier been reported in rheumatoid arthritis,[94] pointing at a complex situation regarding the 5-HT2AR.

There were a higher number of the epidermal and dermal SERT-positive cells in the L skin compared to N-L skin, indicating a role for SERT in antigen presentation. There was also a difference between N-L and control skin regarding the number of dermal SERT-positive cells.

There was also a higher SERT-IR in the basal epidermal layer of the L compared to N-L skin, which indicates that keratinocyte proliferation might be affected by modulating this protein. In the L skin there was a reverse correlation for this expression with the SS and a tendency to a reverse correlation with PsTA. At the same time a correlation was found with the degree of acanthosis and a tendency to a correlation with inflammation. This highlights the importance of the SERT both for the inflammatory process and possibly a protective effect against SS and PsTA, and suggests that this protein might be a target in the future in stress-worsened AD.

5.2.2 Intradermal injection study (Study IV)

There was a similar itch response to 5-HT and to histamine, at the group level, in N-L skin of patients with AD and healthy controls. On the other hand, 5-HT, analogous to histamine, gave smaller flare and wheal responses in patients with AD compared with healthy controls.

It is well known that patients with AD have an abnormal vascular response with a tendency to vasoconstriction.[95,96] This may explain the different vascular effects induced by 5-HT and histamine in our patients with AD and healthy controls.

Hosogi et al.,[58] using iontophoresis, found a lower itch response to 5-HT in N-L compared with L skin of patients with AD, whereas the itch response of healthy controls was in the range of the L skin.

The means of administration of substances and the locally reached tissue concentration might be responsible for different results at the group level in our study

and earlier studies. We chose the injection technique rather than iontophoresis, as it has the advantage that the dosage can be calculated from the injection volume and substance concentration. A concentration of 2.5 mg/ml was used because it gave a reliable itch reaction in our pilot study. This corresponds to an absolute dosage of 50 μg, which is similar to 5-HT doses used by Fjellner and Hägermark.[81] We have previously found the concentration of 5-HT to be 9.85 ng/g in healthy skin and 24.25 ng/g in eczematous skin.[97] Our dosage might not be physiological; however, during inflammation high concentrations in local tissue may be achieved.

It is not surprising that iontophoretically applied pruritogens induce a more pronounced itch response in L skin, with its damaged skin barrier, than in N-L skin. The question is whether this reflects only the delivery of a higher dosage through the damaged skin or increased sensitivity to these pruritogens in the L skin.

There was a difference in sex proportion between patients with AD and healthy controls, yet we could not observe a difference between the sexes either for the itch or the flare or wheal results. This is in accordance with no difference between men and women for itch responses elicited experimentally with histamine, compound 40/80 or wool.[55]

5-HT also induced pruritus in normal individuals. This is in line with previous studies.[81,82] The question is how 5-HT causes a pruritic response. A possible mechanism is via activation of keratinocytes, e.g., transient receptor potential vanilloid (TRPV) receptors,[98] which, in turn, might activate sensory nerves. Sensory nerve fibres may also be triggered, either directly, via 5-HT receptors[36] or indirectly via effect on inflammatory cells, which like keratinocytes, have receptors for 5-HT.[70] Another possible mechanism is via an effect on the vessel wall. The fact that the pruritic effect did not differ significantly between patients with AD and controls, while at the same time, the vascular response did, indicates that the latter is not the

primary cause of pruritus. This is supported by a study in which vasoregulation at the site of 5-HT injection occurred in the absence of scratching reflexes.[99] This difference between pruritic and vascular effects of 5-HT was studied earlier by Yamaguchi et al.[36] using rodents. In this case it was also stated that the vascular response was of less importance in relation to pruritus due to 5-HT. Moreover, the lower itch latency for 5-HT compared with histamine might support a direct effect of 5-HT on itch receptors on sensory nerves.

Warmer skin temperatures may evoke itch due to a decrease in threshold for 5-HT-evoked itch signalling. TRPV4, a warmth-sensitive cation-channel expressed in skin cells and sensory neurones, plays an important role in the enhancement of 5-HT-evoked itch by skin warming.[100] This may be of particular importance in patients with AD, who are sensitive to heat. The room temperature was kept constant for our patients with AD and healthy controls, when performing the intradermal injection study.

6 CONCLUSIONS

AD may be worsened by stress and anxiety. 5-HT is an important mediator in stress and anxiety. In the present study serotonergic mechanisms were studied in AD.

In an atopic-like mouse model, NC/Nga, that was subjected to chronic mild stress we studied expression of serotonergic markers 5-HT, 5-HT1AR, 5-HT2AR and SERT in cerebrum, cerebellum and skin. There was an upregulation of 5-HT1AR in the skin, cerebrum and cerebellum, during inflammation, irrespective of stress. The 5-HT2AR was upregulated in the cerebrum, hippocampal CA1 area, and in the cerebellum, Purkinje cell layer, while being downregulated in the skin, during chronic mild stress.

In human AD patients serotonergic markers in relation to extent of the disease, pruritus, and psychodemographic data with focus on anxiety traits and depression, moreover, levels of chronic stress, were studied. We found a correlation between the extent of the disease and dermal 5-HT1AR-positive dermal inflammatory cells in the L skin and 5-HT2AR-positive vessels in the N-L skin. There was a correlation between depression with the epidermal positive 5-HT1AR fraction, while a reverse correlation with the number of 5-HT2AR expressing vessels, both in the L skin. In the L skin there was a reverse correlation for the basal epidermal SERT-IR with the SS.

Moreover, the effect of intradermal injection of 5-HT was studied in patients with AD and in healthy controls, on vascular response and pruritus, estimated by a computerized VAS recorder. No difference was seen regarding pruritus, while the vascular response, was reduced in the AD patients compared to the healthy controls. This study confirms a pruritogenic role of 5-HT, both in patients with AD and in healthy controls, and shows a lower vascular response of 5-HT in patients with AD compared with healthy controls. In addition, the short itch latency time might indicate a direct effect of 5-HT on itch receptors.

5-HT seems to have a role in AD.

7 FUTURE PERSPECTIVES

In the present thesis we have studied the expression of serotonergic markers in atopic eczema/AD by immunohistochemistry. It is difficult to tell exactly how this expression mirrors the synthesis or internalization of, e.g., serotonergic receptors.

It would in this respect be interesting to use confocal microscopy to be able to study the localization of the serotonergic markers more in detail.

There is a lack of methods in order to study the functionality of the receptors using methods such as autoradiography, mainly due to the probably low numbers of receptors in the skin and lack of suitable tools such as labelled ligands. We need techniques, where we could study both ligands and receptors in the same tissue sections.

We need to use more NC/Nga mice in order to study a non-stressed control group. Also use male NC/Nga mice to study possible gender differences. The latter is also the case for human study, where the patient material should be increased.

We have been focusing at trait anxiety in the present thesis. There was a correlation between our anxiety parameters and depression. Still it may be of interest to use tools to measure the ongoing (state) anxiety in patients with AD, such as Hamilton Anxiety Rating Scale (HAM-A) and Hospital Anxiety and Depression Scale (HAD).

It should be of interest to extract human AD patients with increased SS and investigate such patients with fMRI in order to be able to investigate brain tracts for possible future pharmacological treatment of stress worsened AD. In that respect we should focus on the amygdale.

We need to perform a clinical treatment study of patients with AD using a serotonergic compound, such as an SSRI. We will monitor the extent of the disease, the degree of pruritus, chronic stress and psychodemographic data, and quality of life in these patients

8 SAMMANFATTNING PÅ SVENSKA

Atopisk dermatit (AD) kan förvärras av stress och ångest. Serotonin (5-hydroxitryptamin; 5-HT) är en viktig mediator i stress och ångest. I denna avhandling har serotonerga mekanismer studerats vid atopisk dermatit.

I en atopisk-liknande musmodell, NC/Nga, som utsatts för kronisk mild stress, studerade vi uttryck av serotonerga markörer, serotonin, 5-HT1A och 5-HT2A receptorer (R) och serotonintransportör protein (SERT) i hud, cerebrum och cerebellum. Det fanns en uppreglering av uttrycket av 5-HT1AR i huden, cerebrum och cerebellum, under inflammation, oberoende av stress. Samtidigt uppreglerades 5-HT2AR uttryck i cerebrum, hippocampala CA1 området, cerebellum, i Purkinje cellskiktet, medan nedreglerades i huden, under kronisk mild stress.

Hos AD patienter studerades dessa serotonerga markörer i förhållande till omfattningen av sjukdomen, klåda, kronisk stress och psykodemografisk data med fokus på ångestdrag och depression. Vi fann ett samband mellan utbredningen av sjukdomen och 5-HT1AR positiva dermala inflammatoriska celler i lesionell hud respektive 5-HT2AR positiva blodkärl i icke lesionell hud. Det fanns en korrelation mellan depression och den epidermala 5-HT1AR positiva fraktionen, medan en omvänd korrelation med antalet 5-HT2AR uttryckande kärl, i båda fall i den lesionella huden. I lesionell hud fanns en omvänd korrelation mellan den basala epidermala SERT immunreaktiviteten och stresskänslighet.

Dessutom har effekten av intradermal injektion av 5-HT studerats hos patienter med AD och friska kontroller, beträffande vaskulärt svar och klåda, med hjälp av en datoriserad klådmätningsmetod. Ingen skillnad sågs avseende klåda jämfört med friska kontroller, medan det vaskulära svaret på 5-HT var minskat hos AD patienter.

Serotonin verkar ha en roll i AD.

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