Study of Serotonin, Innervation and Sensory Neuropeptides in Allergic Contact Dermatitis

Husameldin El-Nour
MD

Stockholm 2005

From the Unit of Dermatology and Venereology,
Department of Medicine, Karolinska University Hospital,
Solna, Karolinska Institutet, Stockholm, Sweden

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Unit of Dermatology and Venereology
Department of Medicine
Karolinska University Hospital, Solna
SE- 171 76 Stockholm
Sweden

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To my mother, father and Ibtisam

To my sisters, brothers and their families

ABSTRACT

In a murine model of contact allergy, 24 h after challenge with antigen, increased axonal growth using an antibody to growth associated protein-43 (GAP-43) was seen in the epidermis and dermis of eczematous skin. Protein gene product 9.5 (PGP 9.5) positive nerve fibres tended to increase in the eczematous skin. There was also a higher number of galanin and a tendency to a higher number of calcitonin gene-related peptide (CGRP) and substance P positive fibres. In contrast, the concentrations of galanin and CGRP were lower and substance P concentration tended to decrease. Serotonin positive platelets were also increased in the eczematous skin.

Expression of 5-HT2C receptor (R) was seen on murine epidermal dendritic, I-A positive cells, being more numerous in the eczematous than in the control skin. With the 5-HT2CR agonist (RO 60-0175/007), at high concentrations, and the murine XS52 Langerhans cell-like line, there was an increased interleukin (IL)-1β production, by using ELISA.

In human positive epicutaneous patch test reactions, at 72 h, an increased number of GAP-43 immunoreactive nerve fibres were found in the eczematous compared to control skin. Semiquantification showed an increase in dermal substance P and its neurokinin 1 receptor positive inflammatory mononuclear cells. Serotonin positive platelets were increased in the eczematous skin. A decrease in the number of 5-HT1AR positive cells, the majority being mast cells, was seen in the eczematous compared to control skin. Contrariwise, both 5-HT2AR, being CD3, CD4 or CD8 positive cells, and serotonin transporter protein (SERT) positive cells, being CD1a, CD4, CD8 positive or $CD56^{+}CD3^{-}$ cells, were increased. 5-HT3R immunoreactivity was seen in the basal epidermis of the inflamed and control skin. A high concentration of the 5-HT2AR agonist DOI inhibited the proliferation and tended to inhibit the IL-2 production by nickel-stimulated peripheral blood mononuclear cells from nickel-allergic patients. A tendency to an inhibition of IL-1β production from the XS52 cell line was seen by the SERT inhibitors fluoxetine and citalopram at 10^{-5}-10^{-6} mol/L, respectively.

In conclusion, the serotonergic system, nerve supply and sensory neuropeptides may all be of importance in the pathophysiology of allergic contact dermatitis.

Key words: Allergic contact dermatitis, serotonin, nerve density, sensory neuropeptides

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LIST OF ORIGINAL PAPERS

This thesis is based on the following studies, which will be referred to by their Roman numerals:

I. El-Nour H, Lundeberg L, Boman A, Beck O, Harvima IT, Theodorsson E, Nordlind K. (2005). Study of Innervation, Sensory Neuropeptides, and Serotonin in Murine Contact Allergic Skin. Immunopharmacol Immunotoxicol 27: 67-76.

II. El-Nour H, Lundeberg L, Boman A, Theodorsson E, Hokfelt T, Nordlind K. (2004). Galanin Expression in a Murine Model of Allergic Contact Dermatitis. Acta Derm Venereol (Stockh) 84: 428-432.

III. El-Nour H, Lundeberg L, Boman A, Abramowski D, Holst M, Nordlind K. Expression and function of serotonin 2C receptors on murine Langerhans cell-like cells and murine contact allergy. Submitted.

IV. Lundeberg L, El-Nour H, Mohabbati S, Morales M, Azmitia E, Nordlind K. (2002). Expression of serotonin receptors in allergic contact eczematous human skin. Arch Dermatol Res 294: 393-398.

V. El-Nour H, Lundeberg L, Al-Tawil R, Granlund A, Abdel-Magid N, Lonne-Rahm S-B, Azmitia EC, Nordlind K. Modulation of Axonal growth, Substance P and Serotonin in the human contact allergic reaction. Submitted.

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LIST OF ABBREVIATIONS

5-HT	5-hydroxytryptamine
ACD	Allergic contact dermatitis
ACTH	Adrenocorticotropic hormone
APCs	Antigen presenting cells
CGRP	Calcitonin gene-related peptide
CHS	Contact hypersensitivity
CNS	Central nervous system
DLNs	Draining lymph nodes
DRG	Dorsal root ganglion
ELISA	Enzyme-linked immunosorbent assay
FITC	Fluorescein isothiocyanate
GAP-43	Growth associated protein-43
GC-MS	Gas chromatography-mass spectrometry
GM-CSF	Granulocyte macrophage-colony stimulating factor
HPA	Hypothalamic-pituitary-adrenal
IFN-γ	Interferon-gamma
IL	Interleukin
LCs	Langerhans cells
LPS	Lipopolysaccaride
NK1	Neurokinin 1
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PGP 9.5	Protein gene product 9.5
RIA	Radioimmunoassay
SERT	Serotonin transporter protein
SSRIs	Selective serotonin reuptake inhibitors
TNF	Tumor necrosis factor

1. INTRODUCTION

1.1 Allergic contact dermatitis

Allergic contact dermatitis (ACD) is a common inflammatory skin disorder which is responsible for a considerable rate of morbidity in industrialized societies. It has an estimated prevalence of 6.2 – 10.6% over a period of 1-3 years (Traidl *et al*., 2000). ACD is synonymous with contact hypersensitivity (CHS) induced in laboratory animals through the application of skin sensitizers.

The cellular mechanisms in ACD are separated into two phases, a sensitization (induction) phase and a challenge (elicitation) phase (Saint-Mezard *et al*., 2004b) (Fig. 1). During sensitization the skin is exposed to an allergen (antigen), a low (<1000 Dalton) molecular weight chemical (hapten) (Basketter *et al*., 1995), which is *per se* unable to induce skin sensitization and therefore after penetration forms a hapten-protein complex (antigen) (Kimbe *et al*., 2002). The antigen is then captured by predominantly, epidermal Langerhans cells (LCs), which are a type of antigen presenting cells (APCs) in the skin derived from bone marrow that comprise 2-5% of the total number epidermal cell population (Teunissen, 1992). There are approximately 800 LCs per mm^2 in human skin with dendritic processes that fan out in epidermal sheets to form a network of reticuloepithelial traps for contact allergens.

LCs are considered to be sentinels for the immune system, surveying and monitoring the changes in the microenvironment with a high potency to capture, process and present antigens to naïve T-cells in the draining lymph nodes (DLNs) (Grabbe and Schwarz, 1998; Kimber and Dearman, 2002). LCs mature during their course towards the DLNs, implying the loss of their potent ability to internalize antigens and the adaptation of the characteristic features of immunostimulatory APCs (Kitajima *et al*., 1995) including the surface expression of major histocompatibility molecules (MHC) I and MHC II as well as costimulatory molecules (e.g. B7-1), and also the production of proinflammatory cytokines such as interleukin (IL)-1β. This cytokine is important for maturation and the induction of primary immune responses in the skin (Enk *et al*., 1993). Antigen presentation occurs in the paracortical regions of the DLNs where an antigen is presented via MHC I or II to $CD8^+$ and $CD4^+$ cells, respectively. At this stage the cells become sensitized and their frequency increases dramatically (Saint-Mezard *et*

al., 2003), these primed/memory T cells then being acquired into the circulation. In humans, the sensitization phase takes between 10 to 15 days, whereas in mice it takes 5-7 days (Saint-Mezard *et al*., 2004b). Upon reexposure the same antigen is presented by LCs and other cutaneous cells, as well as infiltrating APCs, to memory T lymphocytes at the site. These effector cells are activated to release a cascade of inflammatory cytokines (e.g. IL-2 and IFN-γ) and chemokines (Saint-Mezard *et al*., 2004a). As a result, the inflammatory process is amplified and further attracts leukocytes, including mononuclear cells, macrophages, NK cells and neutrophils to the site of assault, giving rise to epidermal spongiosis (intercellular edema). These cellular events, together with the formation of edema, are manifest as a characteristic contact eczematous reaction. The challenge phase of ACD takes about 48-72 h in humans and 24-48 h in mice (Saint-Mezard *et al*., 2004a).

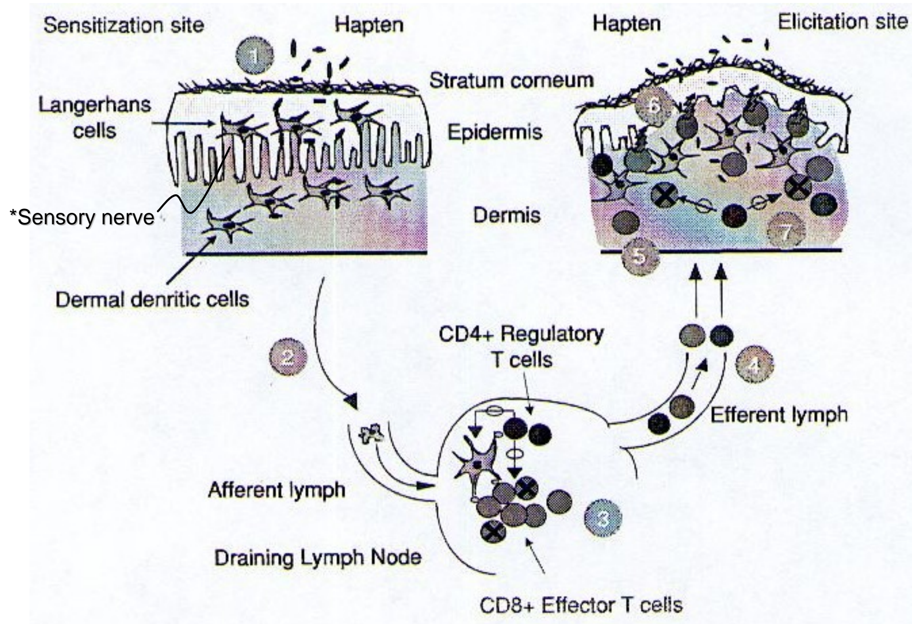

Figure 1. Pathophysiology of ACD: (1) Hapten penetrates the skin and is then engulfed by LCs. (2) Migration of APCs to draining lymph nodes. (3) Presentation of haptenated peptides to T cells. (4) Sensitized T cells diffuse to blood circulation via the efferent lymphatics. (5) Sensitized T cells trafficking into the dermis. (6) Activation of memory T cells upon reexposure to the same hapten and the release of inflammatory mediators. (7) Recruitment of leukocytes leading to the development of characteristic ACD reaction. *Sensory nerves release proinflammatory neuropeptides upon stimulation. Modified from Saint-Mezard *et al*. 2004b.

1.2 The skin

The skin is the largest organ of the body. It is a major sense organ that weighs an average of 4 kg and covers an area of 2 m^2. It is a barrier between the external and internal environments, regulates heat and water loss, protects the body from harsh external stimuli and prevents the loss of important body constituents. The skin utilizes a highly developed architecture and well-established intercommunication with the nervous and immune systems to protect our bodies from assaulting agents. Internal and external factors that affect skin condition include: sunshine, heat and cold, chemicals, infectious agents, trauma, psychological factors, drugs, internal diseases and genetic factors.

The structure of the skin is composed of three layers: the epidermis (outer layer), dermis (middle) and subcutis/hypodermis (inner). The epidermis adheres to the dermis partly by interlocking of downward projections (epidermal ridges) with upward projections of the dermis (dermal papillae). It varies in thickness from less than 0.1 mm to approximately 1 mm depending on the anatomical distribution. The epidermis lacks a blood supply but obtains nutrition from the underlying dermis by diffusion of the nutrients through the basement membrane. Keratinocytes comprise the majority of the cellular component of the epidermis (around 85%), other cell types residing in the epidermis including melanocytes, LCs and Merkel cells. Most of these epidermal cells are involved in immunoregulation in various skin conditions. Leukocytes infiltrate the epidermis during inflammation.

The dermis is a connective tissue layer between the epidermis and hypodermis, which provides structural and nutritional support to the epidermis. It is composed of cells, connective tissue fibres, ground substance, blood vessels, lymphatics, muscles and nerves. The cells of the dermis are fibroblasts, mononuclear phagocytes, lymphocytes, Langerhans cells and mast cells. Polymorphonuclear cells also occur during inflammation. Fibroblasts are responsible for the synthesis of connective tissue fibres such as collagen and elastin which are important for the structural support and elasticity of the skin. The cells of the dermis are involved in innate and adaptive skin immunity.

1.3 Skin innervation

The skin has a rich nerve supply (approximately one million nerve fibres) and its innervation can be classified into autonomic and sensory. The autonomic (adrenergic and cholinergic) nerves are responsible for innervating eccrine sweat glands, apocrine glands, arrector pili muscles and blood vessels. This process is mediated by autonomic neuropeptides such as neuropeptide Y (NPY), vasoactive intestinal peptide (VIP), and the classical neurotransmitters noradrenaline (NA) and acetylcholine (ACh).

Skin sensory innervation is transmitted by nerves that are myelinated Aδ-fibres (thinnest among A-fibres) or nonmyelinated C-fibres. The velocity of conduction depends on the thickness of a nerve, with A-fibres being the thickest and fastest and C-fibres being the slowest. These cutaneous nerves may be free nerve endings or specialized end organs in the dermis (i.e. Meissner's corpuscle and Pacini's corpuscle). Cell bodies of the cutaneous sensory nerve fibres are found in the dorsal root ganglia (DRG) of the spinal nerves and some cranial nerves.

A general neuronal marker that detects cutaneous nerves is protein gene product 9.5 (PGP 9.5), newly generated nerves being detected by an axonal growth marker (growth associated protein-43, GAP-43). Immunoreactivity for both PGP 9.5 and GAP-43 has been reported in human skin, for refs see Verze *et al*. (2003).

1.4 Neuroimmune interactions

Interaction between the nervous, endocrine and immune systems has been well documented. There is crosstalk between the immune system and neuroendocrine systems that is mediated by a number of biochemical substances including classical neurotransmitters (e.g. serotonin (5-hydroxytryptamine, 5-HT)), neuropeptides (e.g. calcitonin gene-related peptide, CGRP and neuropeptide Y, NPY), hormones such as adrencorticotropic hormone (ACTH), and cytokines (e.g. IL-1 and tumor necrosis factor, TNF). These substances act in concert with target receptors in the immune system, central nervous system (CNS), peripheral nervous system (PNS) and endocrine system, see e.g. Blalock (2005).

During the last decades, it has been accepted that cells of the immune system produce neuropeptides and neurotransmitters; for example, lymphocytes are known to produce several

peptide hormones and neurotransmitters including ACh, 5-HT, ACTH and endorphins (Tayebati *et al*., 2002). Immune cells were also shown to express receptors for these neuropeptides/neurotransmitters (Carr *et al*., 1989). Cytokines, a family of glycoproteins that include members that mediate inflammation, are produced by neurons in the nervous system as well as cells of the immune system. For example, IL-1 is expressed in human hypothalamus (Breder *et al*., 1988), and the administration of this cytokine and others such as IL-6 activates the hypothalamic-pituitary-adrenal (HPA) axis and results in the release of neuromediators at targeted lymph organs (Straub, 2004) (Fig. 2). In this context the immune system was suggested to function as a sensory organ that is responsible for sensation and reporting of noncognitive stimuli (e.g bacteria and viruses) that are not perceived by our common sense organs to the CNS via signaling molecules. These signaling molecules (both in CNS and immune cells) then mediate the CNS responses to the incoming information, and thus a bidirectional transmission is established that will eventually result in a physiological response at the site of trauma. An example is the activation of the vagus nerve by proinflammatory mediators (TNF, IL-6 and IL-16) released from macrophages during an infection that will lead to an increase in ACh secretion, which in turn will act on its designated receptors on macrophages, resulting in suppression of mediator production and thus blocking the inflammation (Borovikova *et al*., 2000).

In the skin, sensory and autonomic cutaneous nerve fibres as well as epidermal and dermal immune cells are capable of producing neuromediators that can activate specific receptors on target cells and thus modulate physiological and pathophysiological effects and contribute to cutaneous neurogenic inflammation, see Luger and Lotti (1998) and Luger (2002). Neurogenic inflammation in the skin implies that a signal being transmitted through afferent fibres to the DRG and the spinal cord (orthodromic signal) stimulates the release of a second signal (antidromic). This travels in the opposite direction to the source of the stimulus, and subsequently neuropeptides are released that will in turn act on blood vessels, resident cells and transient immunocompetent cells expressing the relevant receptors (Holzer, 1988; Rees *et al*., 1996).

Neuropeptides have been found to activate a number of cutaneous cells via the activation of an intracellular G-protein signaling cascade. The sensory neuropeptides CGRP and

substance P are released in the skin during inflammation and are known to exert an effect on blood vessels, causing an alteration of the expression of adhesion molecules and leading to vasodilation and plasma extravasation as well as chemotaxis of leukocytes to the site of inflammation (Goebeler *et al*., 1994). Other neuropeptides expressed in skin nerve fibres include the autonomic neuropeptides NPY (sympathetic) and VIP (parasympathetic) (Lundberg *et al*., 1980; Lundberg and Hokfelt, 1986) and proopiomelanocortin (POMC)-derived peptides such as alpha melanocyte stimulating hormone (α-MSH) and β–endorphins.

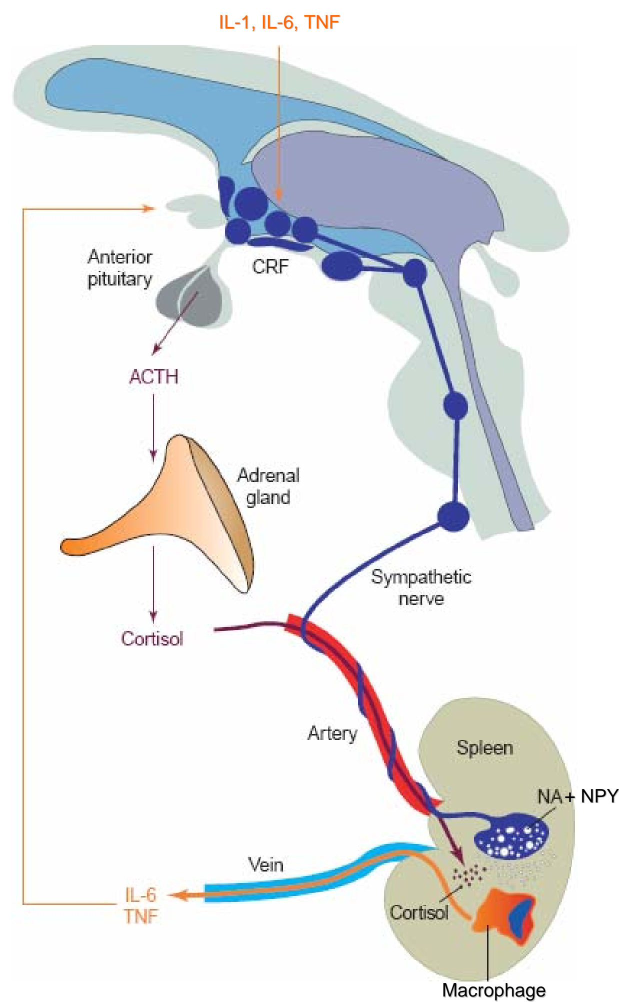

Figure 2. Activation of the sympathetic nervous system and the hypothalamic-pituitary-adrenal (HPA) axis by proinflammatory cytokines such as interleukin (IL)-1, IL-6 and tumor necrosis factor (TNF). Modified from Straub 2004.

1.5 The hypothalamic-pituitary-adrenal axis

The hypothalamic-pituitary-adrenal axis is inevitably activated by immune stimuli as well as stress. It is responsible for the production of stress hormones (glucocorticoids), regulation of energy metabolism and restoration of tissue homeostasis (Bhatia and Tandon, 2005). In the case of stressors, corticotropin-releasing factor (CRF) released by the hypothalamus increases the pituitary production of ACTH, which is then released into the circulation, from where it is transported to the adrenal glands. The hormone then stimulates the adrenal cortex to produce and secrete glucocorticoids, including the main stress hormone cortisol.

Psychopathological conditions have been reported to be associated with several inflammatory skin diseases such as psoriasis, atopic dermatitis and urticaria and stress was suggested to exacerbate these diseases (Gupta *et al*., 1994). There is increasing evidence of a stress element contribution to ACD (Nakano, 2004) whereby acute stress was shown to suppress murine ACD whereas chronic stress enhanced it. In the later context, a role for LCs has been proposed and was attributed to an increased number of hapten-induced cells in DLNs and an increased antigen presenting capacity. Moreover, levels of substance P were increased in chronically stressed mice, accompanied by a raised expression of neurokinin 1 receptor (NK1R) mRNA in the epidermal cells (Nakano, 2004).

1.6 Serotonin (5-HT)

In 1947, the neuromediator 5-HT released from blood platelets during clotting was isolated from serum by Rapport *et al.* (for review see Grimaldi and Fillion (2000)). This vasotonic monoamine exerted a tensing effect on smooth muscles and hence got its name *sero-tonin*. It is also abundantly present in the gastrointestinal tract where it is described as an enteramine.

Since its discovery, 5-HT has received considerable focus for investigation by several scientists and was found to have a widespread distribution in the CNS and periphery. It is synthesized from the essential amino acid tryptophan (L-Trp). L-Trp (supplied from diet) availability is an important factor that determines the levels of 5-HT in the brain, in addition to the activity of the rate-limiting enzyme tryptophan hydroxylase. Intracellular 5-HT, which is not protected by vesicular storage, is degraded by monoamine oxidase to the main metabolite 5-hydroxyindoleacetic acid (5-HIAA) that is eliminated via urine (Lambert *et al*., 1995).

Central 5-HT is produced by raphe nuclei near the midline of the brain stem (Jacobs and Azmitia, 1992). It is then transported to almost all parts of the brain via a highly sophisticated network of axonal arborization with a very high number of varicosities (Audet *et al*., 1989). Thus 5-HT has an enormous ability to interact with other neural networks and neurotransmitters in the brain and, accordingly, it is involved in the regulation of a number of physiological functions such as behaviour, temperature, sleep, appetite, learning and memory, mood and sexual activity. Dysfunction of the serotonergic system can lead to several pathological conditions including anxiety, depression, schizophrenia, sleep disturbances, sexual dysfunctions, aggression and obsessive compulsive disorders. There are also several reports in the literature that indicate a stress modulation of the serotonergic activity and 5-HT regulation of the HPA axis (for example see Fuller (1992) and Shimizu *et al*. (1992)).

Brain-synthesized 5-HT comprises a minor proportion (approximately 2% of total body content) compared to enterochromaffin cell-derived 5-HT, which comprises over 95%. 5-HT is also present in other organs of the body such as the heart, kidneys, spleen and thyroid gland. Enterochromaffin cell-derived 5-HT is stored in blood platelets whereas in rodents it is also found in mast cells and basophils. Physiological and pathological stimuli that cause platelet aggregation and mast cell degranulation lead to the release of 5-HT into the surrounding tissue, where it can impose a wide range of action via several receptor subtypes.

1.7 5-HT receptors

5-HT exerts an effect via a number of receptor subtypes (mostly heteroreceptors) distributed throughout the body. They are classified into at least 21 structurally and pharmacologically distinguished receptors that belong to a superfamily of seven members: 5-HTR1 (A, B, D, E, F), 5-HTR2 (A, B, C), 5-HTR3 (A, B), 5-HTR4 (A, B, C, D), 5-HTR5 (A, B), 5-HTR6 and 5-HTR7 (A, B, C, D) (Meneses, 1999; Hoyer *et al*., 2002; Kroeze *et al*., 2002). All members of the 5-HT receptor family are transmembrane spanning G-coupled receptors, except 5-HT3R which is an ion-gated channel. 5-HT1R and 5-HT5 act by inhibiting the activity of adenylate cyclase and thus decrease the production of cyclic adenosine monophosphate (cAMP), whereas 5-HT4R, 5-HT6R and 5-HT7R activate adenylate cyclase. 5-HTR2 is a phospholipase C activator. These receptor subtypes are responsible for the diversity of signaling that conducts

different effects on target or producer cells. 5-HT1A receptor is an autoreceptor which upon stimulation decreases 5-HT frequency of discharge (Sprouse and Aghajanian, 1987). Conversely, the other autoreceptor (5-HT1B) controls the amount of released 5-HT. The 5-HT2C receptor has not been reported in the periphery to date (Abramowski *et al*., 1995). Of the above-mentioned serotonergic receptors, 5-HTR1 and 5-HTR2 are the most well-characterized.

1.8 5-HT and neuroimmune interactions

5-HT is a major neurotransmitter in the CNS and the periphery, and among other neurotransmitters is involved in neuroimmune interactions in both the brain and peripheral tissue (for review see Mossner and Lesch (1998)). 5-HT is also implicated in different stages of the cell cycle with an impact on proliferation, differentiation and apoptosis (Azmitia, 2001). It is known that infections can produce sickness behaviours that can be manifest as anxiety and alteration of mood and depression. These effects are thought to be the result of an interaction between proinflammatory cytokines (e.g. IL-1 and TNF-α) and different neural networks in the brain, including the hypothalamus, with a subsequent activation of the HPA axis leading to the release of stress hormones and activation of neurotransmitter receptors such as 5-HT receptors. In the brain, it was demonstrated that an increase in 5-HT levels could result from the administration of IL-1 (Gemma *et al*., 1991; 1994). Depression and stress are associated with deficits in 5-HT as well as a suppressed immune system (Chaouloff, 1993).

There is increasing evidence in the literature which indicates the presence of 5-HT in immune cells such as T lymphocytes, monocytes, macrophages and granulocytes (Csaba *et al*., 2005a; b). In addition, expression of serotonergic receptors on immune cells has been reported (Ameisen *et al*., 1989; Aune *et al*., 1993; Fiebich *et al*., 2004). Depending on the concentration, 5-HT can cause stimulation or inhibition of the immunocompetent cells. Low levels of 5-HT increased mitogen-induced lymphocyte proliferation and IL-2R expression *in vitro* (Kut *et al*., 1992; Young *et al*., 1993). Contrariwise, high concentrations of 5-HT gave an inhibitory effect. Furthermore, 5-HT and IFN-γ had a bidirectional influence on their production by stimulated lymphocytes, monocytes and NK cells (Finocchiaro *et al*., 1988; Hellstrand *et al*., 1993).

An increased concentration of 5-HT was determined in positive epicutaneous patch test reactions in humans in comparison with control skin (Lundeberg *et al*., 1999). A role for 5-HT in murine contact allergy was demonstrated via the modulation of 5-HT1A and 5-HT2A receptor activities, in which the 5-HT1A receptor agonist buspirone inhibited murine contact allergy (McAloon *et al*., 1995). The same inhibitory effect also being obtained by antagonizing the 5-HT2A receptor (Ameisen *et al*., 1989).

The serotonin transporter protein (SERT) determines the magnitude and duration of the serotonergic response. There is significant data in the literature regarding the expression of SERT on human lymphocytes (Faraj *et al*., 1994) as well as macrophages (Rudd *et al*., 2005). Moreover, the selective serotonin reuptake inhibitors (SSRIs) fluoxetine and citalopram were reported to modulate cells of the immune system (Frank *et al*., 1999; Pellegrino and Bayer, 2000). Fluoxetine has been found to cause a decrease in lymphocyte proliferation mediated by an activation of central 5-HT2AR (Pellegrino and Bayer, 2002).

1.9 Sensory neuropeptides (CGRP, substance P and galanin)

CGRP is a 37 amino acid peptide that belongs to a family of calcitonin peptides consisting of calcitonin, amylin and adrenomodulin. It coexists with 5-HT and other neuromediators in the CNS (see Hokfelt *et al*. (2000a)).

The effects of CGRP are mediated via a calcitonin-like receptor (CRLR) encoded by the expression of receptor activity modifying protein 1 (RAMP1) (see Luger (2002)). CRLR is expressed on vascular endothelial cells, immunocompetent cells as well as resident epidermal cells. Upon stimulation, CGRP is released from sensory C-fibres in the skin and initiates several inflammatory responses such as vasodilation, plasma extravasation, cellular infiltration and cytokine production.

There is a close anatomical association between the key player in ACD induction phase, LCs, and CGRP containing nerves in the skin (Hosoi *et al*., 1993). Once released, CGRP plays a role in suppressing CHS (Asahina *et al*., 1995) mediated by a downregulatory effect on LCs (Hosoi *et al*., 1993; Torii *et al*., 1997a) altering their antigen presenting capability as well as the expression of cytokines and costimulatory molecules (Torii *et al*., 1997b). CGRP is also known to influence effector T cells during CHS (Wang *et al*., 1992), and to cause mast cell

degranulation leading to the release of immunoregulatory cytokines such as TNF-α and IL-10 (Streilein *et al*., 1999).

Substance P is an 11 amino acid peptide discovered in 1931 by Von Euler and Gaddum. It belongs to the tachykinin family along with neurokinin A, neurokinin B, neuropeptide K and neuropeptide γ. Substance P coexists in the CNS with other classical neurotransmitters such as 5-HT (see Hokfelt *et al*. (2000a)). Substance P is also known to coexist with other neuropeptides such as CGRP, galanin and somatostatin. Furthermore, cutaneous sensory nerve fibres were demonstrated to coexpress both substance P and CGRP (Brain and Williams, 1988), and the release of these peptides in a rat skin was stimulated by inflammatory mediators such as 5-HT and bradykinin (Averbeck and Reeh, 2001). A nonneuronal source for substance P in the skin is mast cells, see Toyoda *et al*. (2000) and lymphocytes, see Lambrecht (2001). Like CGRP, substance P is a proinflammatory peptide that exerts a number of effects on the microenvironment including vasodilation, plasma extravasation, leukocyte infiltration and mast cell degranulation (see Goebeler *et al*., 1994). In addition, substance P has been reported to have a trophic effect on nerves, for review see Hokfelt *et al*. (2000b). The actions of substance P are predominately mediated by one of three tachykinin receptors, NK1R (see Ruocco *et al*. (2001)). NK2R and NK3R have a lower binding affinity for substance P but a higher binding affinity for NKA and NKB, respectively (Regoli *et al*., 1997). Substance P conversely to CGRP, was shown to enhance ACD (Niizeki *et al*., 1999). This enhancing effect of substance P is mediated via the action of NK1R (Scholzen *et al*., 2004) and is inhibited by the action of degrading enzymes such as neutral endopeptidases (Scholzen *et al*., 2001).

Galanin is a widely distributed 29/30 amino acid peptide discovered in 1983 in extracts from the porcine jejunum (Tatemoto *et al*., 1983). It coexists in the central and peripheral nervous systems with the sensory neuropeptides CGRP and substance P (Hokfelt *et al*., 1993), and classical neurotransmitters such as 5-HT and ACh (Melander *et al*., 1986). Galanin has been implicated in inflammation (Heppelmann *et al*., 2000). It is upregulated in dorsal horn neurons following peripheral inflammation (Zhang *et al*., 1998), and is involved in the development of inflammatory arthritis in the rat (Calza *et al*., 1998; Qinyang *et al*., 2004). In addition, the concentration of galanin has been found to be increased in chemically induced ileitis (Pidsudko *et al*., 2003). Carrageenan-induced inflammation in rat skin caused an

increased galanin mRNA signal in epidermal cells as well as in the dermis, but no immunoreactive nerve fibres were apparent (Ji *et al*., 1995). Contrariwise, galanin immunoreactive nerve fibres were evident in the lamina propria of rat molar gingiva (Baumann *et al*., 2003). As galanin was suggested to counteract the effects of CGRP and substance P and to suppress plasma extravasation (Xu *et al*., 1991) it is thus considered to be an inhibitory peptide regarding inflammation (Heppelmann *et al*., 2000).

2. AIMS OF THE STUDY

To study neuronal contribution to the allergic contact reaction in mice and humans by investigating:

- Serotonin and its receptors 5-HT1A, 5-HT2A,C and 5-HT3, as well as the serotonin transporter protein (SERT) (I, III, IV, V).

- The effect of serotonin receptors and SERT inhibitors on inflammatory cells *in vitro* (III, V).

- Innervation (I, V).

- Sensory neuropeptides and substance P receptor NK1 (I, II, V).

3. MATERIALS AND METHODS

3.1 Animals (I-III)

In total 48 female Balb/c mice (Charles River, Uppsala), age 8-10 weeks, were used (32 in study I, and 16 in studies II and III). The animals were housed four per cage (37 x 21.5 x 15 cm) in the animal research facility at Karolinska University Hospital, Solna. The animal room was maintained with a 12 h light-dark cycle. The animals were given free access to food and water. Approval was obtained from the local animal ethical committee.

For induction of contact allergy a modification of the procedure by Goebeler *et al*. (1994) was performed, in which the mice were sensitized on two consecutive days by applying 100 µl 3% oxazolone (4-ethoxymethylene-2-phenyl-2-oxazolin-5-one; Sigma-Aldrich, Stockholm, Sweden) dissolved in olive oil/acetone (1:4) on the shaved back. They were challenged 6 days later by painting 50 µl 0.1% oxazolone dissolved in arachis oil/acetone (1:4) on the dorsal surface of both ears. The increase in ear thickness (degree of edema) was used as a sign of inflammation and was measured using a spring-loaded micrometer (Kroeplin, Schluchtern, Germany) before and 24 h after challenge with the antigen. Control mice were painted with the vehicle and kept in separate cages.

Mice were sacrificed 24 h after challenge, and one ear was fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) pH 7.0 (studies I, II, III) for 4 h at 4°C, or in 10% formalin with 0.2% picric acid (study I) for 2 h at 4°C, then rinsed with 0.1 mol/L phosphate buffer containing 10% sucrose for at least 24 h. Tissues were then embedded in Tissue-Tek (Zoeterwoude, The Netherlands) and sectioned (14 µm thick) using a Dittes cryostat (Heidelberg, Germany). In order to include areas with different degrees of inflammation sections were cut at approximately 100 µm intervals and mounted on SuperFrost/Plus glass slides (Menzel-Glaser, Freiburg, Germany), which were then stored at –70°C until the application of immunohistochemistry. The other ear was directly frozen on dry ice and stored at –70°C for radioimmunoassay (RIA) (I and II), gas chromatography-mass spectrometry (GC-MS) (I) or *in situ* hybridization (II).

3.2 *Patients (IV, V)*

In total 35 female patients with patch test verified positive reactions (grade +++ according to the International Contact Dermatitis Research Group (ICDRG)) to 5% nickel sulphate (n=32), 0.5% cobalt chloride (n=2) and 25% Balsam of Peru (n=1), participated in the study. The patch tests, using Finn Chambers (Epitest, Helsinki, Finland) and polypropylene-coated aluminium discs on Scanpor tape (Norgesplaster A/S, Oslo, Norway), had been applied to normal skin on the back for 48 h and were read after an additional 24 h. Biopsies were taken from eczematous (n=18) and control (n=17) skin of the back of the patients after local anaesthesia and immediately prepared for immunohistochemistry by immersion in 10% formalin with 0.2% picric acid for one to two hours, followed by rinsing in 10% sucrose solution for at least 24 h. The biopsies were then frozen and kept at –70°C before being sectioned for immunohistochemistry.

Peripheral blood was obtained from 7 of these patients with a positive patch test reaction to nickel as well as a positive history of nickel allergy.

Ethical approval was obtained from the local Ethics Committee.

3.3 *Substances*

Nickel sulphate was obtained from Sigma-Aldrich, St Louis, USA. Oxazolone, the 5-HT2AR agonist DOI (2,5-dimethoxy-4-iodoamphetamine) and the SSRIs (fluoxetine and citaloprame) were purchased from Sigma-Aldrich (Stockholm). The 5-HT2C receptor agonist RO60-0175/007 ((S)-2-(6-chloro-5-fluoro-indol-1-yl)-1-methyl-ethyl-amine hydrochloride) was a generous gift from Roche (Basel, Switzerland), and the antagonist mesulergine was from ICN Biomedical (Aurora, Ohio, USA).

3.4 *Immunohistochemistry (I-V)*

Polyclonal antibodies (I-V)

A panel of polyclonal antibodies were used in all studies presented in this thesis, containing rabbit antisera directed against PGP 9.5 (dilutions 1:5,000 (study I) & 1:10,000 (study V); UltraClone, Cambridge, England), GAP-43 (1:3,000 (I) & 1:1,500 (V); Chemicon, Temecula,

CA, USA), CGRP (1:8,000 (I) & 1:800/1:10,000 (V)), substance P (1:20,000 (I) & 1:800/1:10,000 (V)) and galanin (1:10,000 (II), all from Bachem, St. Helens, England), NK1R (1:5,000 (V); Abcam, Cambridgeshire, UK), 5-HT (1:10,000 (I & V); DiaSorin, Stillwater, MN, USA), two 5-HT1AR (S1A-170 and S1A-258, the latter only being used in study IV; dilutions 1:1,000 (IV) & 1:5,000 (V)), antibodies. S1A-170 is directed against amino acids 170-186 in the second extracellular loop of rat (Azmitia *et al*., 1992). This amino acid sequence shows complete homology between the human and rat sequences, unlike S1A-258 that recognizes amino acids 258-274 in the third intracellular loop. The staining was positive with the antibody against the extracellular loop, which labels a major band of 64,000 Daltons in immunoblots of solubilized protein from neonatal rat brains, and in different recombinant systems specifically labels cells transfected with constructs containing the 5-HT1A receptor coding sequence (DeFelipe *et al*., 2001). A rabbit anti-rat 5-HT2CR (study III) antibody (522) against a fusion protein of the two major cytoplasmic domains of the 5-HT2C receptor, anti-betaGAL-2CP4, directed against the C-terminal, was used in addition to an antibody, 526, against the third cytoplamic loop (Abramowski *et al*., 1995). These antibodies do not react with the 5-HT2A receptor. There have been similar sites defined for the staining with the 522 antibody as with autoradiographic labelling using ^{3}H-mesulergine, in rat brain slices (Abramowski *et al*., 1995). Of the two 5-HT2CR antibodies, staining with the antibody 522 was positive. An anti-human 5-HT3R antibody (dilution, 1:2,000, Morales *et al*., 1996) was used in study IV. A mast cell marker, anti-tryptase antibody (human) (dilution 1:20,000, Harvima *et al*., 1988), was also used, this antibody also having been shown to stain cultured murine mast cells (personal communication with Dr Monika Welle, Institute of Animal Pathology, University of Berne, Switzerland).

A guinea pig anti-human PGP 9.5 antibody (1:2,000; Neuromics, Minneapolis, MN, USA) was used for double staining experiments in study II.

Monoclonal antibodies (IV & V)

In studies IV and V a number of monoclonal antibodies were used including a 5-HT2AR (G186-1117) antibody (dilutions 1:150 (IV) & 1:500 (V); a generous gift from Pharmingen, San Diego, CA, USA, this antibody now being commercially available) that is directed against

amino acid residues 1-76 in the N-terminal extracellular domain of the human 5-HT2A receptor (Wu *et al*., 1998). A mouse monoclonal anti-SERT antibody (dilution 1:20,000 (V); Mab Technologies, Stone Mountain, GA, USA), being raised against a peptide corresponding to amino acids 51-66 coupled to KLH by the addition of an N-terminal cysteine, was also used. This antibody has a high degree of specificity (Freedman and Maddox, 2001; Salli *et al*., 2004). For double staining experiments a panel of mouse antibodies was utilized including melanoma-associated antigen NKI-beteb (1:80 (IV); Sanbio BY, Am Uden, The Netherlands), the LCs marker CD1a (dilutions 1:50 (IV) & 1:320 (V)), the lymphocyte markers CD3, CD4 and CD8 (all diluted 1:40 in (IV) & 1:80 in (V)), fluorescein isothiocyanate (FITC)-conjugated CD3 (1:20; (V)), and the macrophage marker HLA-DR (1:50; (IV)), all these antibodies were purchased from Becton-Dickinson (San Jose, CA, USA). A mouse anti-GAP-43 (1:10,000 (V); Sigma) and an anti-tryptase antibody (dilution 1:5,000 (V); Chemicon), were used. A rat anti-mouse FITC-conjugated I-A antibody (1: 100; Becton Dickinson) that detects LCs was also used for double staining (III).

Indirect fluorescence method (IV)

After blocking the non-specific binding sites with 10% normal sera, sections were incubated with optimal concentrations of the primary antibodies diluted in 1% BSA/PBS overnight at 4°C in humid chambers. The following day, sections were rinsed in PBS for 10 min with three buffer changes and were then incubated with a rhodamine-conjugated swine anti-rabbit antibody (1:40; Euro-Diagnostica, Malmo, Sweden), a rhodamine-conjugated rabbit anti-mouse (1:20; DakoCytomation, Glostrup, Denmark), or (in double staining experiments) a FITC-conjugated swine anti-rabbit antibody (1:30; Dako) for 30 min at 37°C. Finally, the sections were mounted with glycerol/PBS (10:1) containing 0.1% para-phenylenediamine, and covered with glass slips.

Secondary biotinylated antibodies (I, II, III, V)

All secondary biotinylated antibodies were obtained from Vector, Burlingame, CA, USA, and diluted 1:200. They included goat anti-rabbit (studies I, II, III, V), goat anti-guinea pig (II) and horse anti-mouse (V) antibody.

Biotinylated streptavidine method (I, II, III, V)

After blocking of non-specific binding sites with normal sera, sections were incubated with the primary antibodies overnight at 4°C. The following day sections were incubated with a biotinylated secondary antibody for 40 min at room temperature. The primary antibody was visualized by incubating the sections with fluorochromes Cy2 (dilution 1:2,000; Amersham Pharmacia Biotech, Uppsala, Sweden) or Texas Red (1:2,000, Vector Laboratories).

For controls, the primary antibodies were omitted (I-V), preimmune serum was incubated (III) or the antibodies were preabsorbed with their corresponding peptides (I, II, IV and V).

Double staining method (V)

Sections were incubated with polyclonal or monoclonal primary antibodies followed by biotinylated goat anti-rabbit or horse anti-mouse secondary antibodies (1:200, Vector), and by Texas Red. Staining was continued with the addition of polyclonal or monoclonal antibodies, followed by the biotinylated goat anti-rabbit and Cy2 (1:2,000), FITC-conjugated swine anti-rabbit antibody (1:40, Dako) or donkey anti-mouse FITC-conjugated antibody (1:150; Jackson ImmunoResearch Lab, Pennsylvania, USA).

Microscopy

Sections were examined using a Nikon Eclipse E 800 (Yokohama, Japan) microscope equipped for epifluorescence. Cy2 and FITC fluorescent elements were visible with a B-1E filter cube (excitation at 465-495 nm) and the Texas Red fluorescence with a G-1B filter cube (excitation at 540-580 nm). Black and white photographs were taken on Kodak TMax 400 pro films. Colored images were generated using a digital video camera system (Nikon DXM 1200, Yokohama, Japan) attached to the fluorescence microscope and connected to a PC.

All the immunoreactive nerve fibres were analysed and counted manually by one observer (H.E.) using a coded method except for study I, in which murine PGP 9.5 and GAP-43 positive nerve fibres were analysed using a software program. Nerve fibres which crossed the dermoepidermal junction were counted within the layer that contained the largest part of

the nerve. Four sections (three in murine samples) were counted and the mean values were calculated.

The number of positive cells was counted using the above-mentioned method, whereas in study IV the percentages of 5-HT1A positive cells in 200 papillary dermal mononuclear cells in the eczematous and control skins were calculated. For 5-HT positive platelets, substance P and NK1R positive cells, semiquantification was performed, the results being expressed as low (+), medium (++) or high (+++) number.

Image analysis (I)

The object area in mm for both murine PGP 9.5 nerve fibres and GAP-43 nerve fibres was analysed using the software Easy Image Analysis (Bergström Instruments, Solna, Sweden).

3.5 Radioimmunoassay (I, II)

Frozen tissues were cut into small pieces and boiled for 10 min in 10 volumes of 11 mol/L acetic acid. After homogenization using a polytron and a Vortex mixer the samples were centrifuged at 2,500 x g for 10 min. Supernatants were lyophilized and kept at –20ºC. Just before analysis the precipitates were dissolved in 0.05 mol/L phosphate buffer pH 7.4 (Theodorsson and Rugarn, 2000).

CGRP concentration was analysed using rabbit antiserum CGRPR8 against conjugated rat CGRP. High performance liquid chromatography (HPLC)-purified [^{125}I]-Histidyl rat CGRP was used as radioligand and rat CGRP as standard. The detection limit of the assay for rat CGRP is 9 pmol/L and the cross-reactivity of the assay to bombesin, calcitonin, gastrin, neurotensin, neurokinin A, neurokinin B, neuropeptide K, neuropeptide Y, and substance P was less than 0.01%. Cross-reactivity to human CGRP α and β was 93% and 24%, respectively, and to rat CGRP α and β, 100% and 120% respectively. Intra- and inter-assay coefficients of variation were 8% and 14%, respectively.

Substance P (SP-LI) was analysed using antiserum SP2 (2), which reacts with substance P and substance P sulfoxide but not with other tachykinins. The detection limit was 10 pmol/L. Intra- and inter-assay coefficients of variation were 7% and 11%, respectively (Brodin *et al.*, 1986).

The *galanin* concentration was determined using antiserum RatGala4 raised against conjugated synthetic rat galanin (Theodorsson and Rugarn, 2000). This antiserum does not crossreact with substance P, neurokinin A, neurokinin B, neuropeptide K, neuropeptide Y, gastrin, pancreatic polypeptide, glucagon or neurotensin. HPLC-purified [^{125}I]- rat galanin was used as radioligand and rat galanin as standard. The detection limit of the assay was 5 pmol/L. Intra- and interassay coefficients of variation were 6 and 10%, respectively.

3.6 Gas chromatography-mass spectrometry (I)

The procedure was a modification of a method described for plasma determination of 5-HT (Beck *et al*., 1993). Mouse ears were homogenized in 2.0 ml saline containing internal standard (tetradeuterated serotonin, 2 ng) using an Ultra-Turrax homogenizer. The homogenate was mixed for 10 min with 2.0 ml methanol and 3.0 ml chloroform. The upper methanolic phase was transferred to a new test-tube and evaporated to dryness. The residue was dissolved in 1.0 ml water and treated as described above, omitting the first solid-phase extraction.

3.7 In situ hybridization (II)

Treated and control ears were rapidly frozen and cut using a Dittes cryostat (as above) at 14 µm thickness. An oligonucleotide probe complementary to nucleotides 324-371 of rat galanin mRNA (Vrontakis *et al*., 1987) (SGS/AB, Köping, Sweden) was purified through QIA quick spin columns (QIAGEN/Gmbh, Hilden, Germany). DTT was added to a final concentration of 10 mM. The specific activities obtained ranged between 0.6 and 1.8×10^9 cpm/µg oligonucleotide.

Tissues were hybridized according to published procedures (Dagerlind *et al*., 1992). Briefly, the sections were covered with a hybridization buffer, placed in a humidified chamber and incubated for 16-18 h at 42°C. After being rinsed and air-dried, the sections were dipped in NTB2 nuclear track emulsion (Kodak), exposed, developed, fixed and mounted in glycerol-phosphate buffer. Some sections were subsequently stained with cresyl violet and mounted. Sections were analyzed with a microscope equipped for bright-field and dark-field illumination (Microphot FX, Nikon, Japan).

3.8 In vitro experiments (III, V)

XS 52 cells (III, V)

The XS52 Langerhans cell-like line was generated from the epidermis of newborn BALB/c mice (Xu *et al*., 1995a). This dendritic cell line resembles LCs by their ability to present antigen, growth factor requirement and characteristic surface phenotypes (Xu *et al*., 1995b). The cells were maintained in complete (c)-RPMI medium (Invitrogen) supplemented with recombinant murine granulocyte macrophage-colony stimulating factor (GM-CSF, 2ng/ml, Sigma-Aldrich) and supernatant from the NS47 cell line (10%, see ref. Schuhmachers *et al*. (1995)). Cells floating over confluent cultures were collected from the medium and used in the experiments.

Peripheral blood mononuclear cells (PBMC) (V)

Peripheral blood lymphocytes were isolated from venous blood of nickel-allergic subjects using Ficoll-Paque (Amersham). Cells were incubated at a concentration of $2x10^6$ cells/ml in 96-well microplates, in a 100 µl volume, and in macrocultures in a 500 µl volume (both from Becton Dickinson). After preincubation for 30 min, nickel sulphate in saline, 20 µl per well in the microplates and 100 µl for the macrocultures was added, giving a final concentration of $3.8x10^{-5}$ mol/L. After an additional 30 min the serotonergic modulators, DOI (which was dissolved in deionized water and diluted in culture medium to give a final concentration of $5x10^{-5}$-10^{-10} mol/L), fluoxetine or citalopram (diluted at 10^{-5}-10^{-6} mol/L), were added in volumes of 20 µl and 100 µl, respectively.

Proliferation assay (III, V)

In study III, 10^4 XS52 cells in 100 µl cell-culture medium per well were cultured for 5 days in a 96 well-flat bottomed plate at 37°C in a humidified atmosphere of 5% CO_2 in air. After preincubation for 4-5 h, 20 µl of the moderately selective 5-HT2C receptor agonist RO60-0175/007 dissolved in deionized water and diluted in culture medium to give a final concentration of $5x10^{-5}$-10^{-10} mol/L, was added to the wells. Saline (20µl/well) was added to control wells in the same plate. To analyse DNA synthesis 0.5 µCi of [methyl-^{3}H] thymidine (5 Ci/mmol, Amersham) in 10 µl saline was added to each well 24 h before the end of

incubation. The cells were then collected on glass fibre filters using a Skatron cell harvester (Molecular devices, Sunnyvale, CA, USA). Radioactivity incorporated in DNA was determined by assay in a Packard liquid scintillation spectrometer (Packard BioScience, Meriden, CT, USA). Results were expressed as counts per minutes (cpm) of triplicate cultures.

In study V, PBMC were incubated for 5 days, and 6 h before interrupting the incubation 0.5 μCi of [methyl-^{3}H] thymidine with a specific activity of 5 Ci/mmol was added in 10 μl of saline. The cells were then harvested on glass fibre filters using an automatic cell harvester, then dried for 30 min and covered with melt-on scintillation sheets (all from Wallac, Upplands Väsby, Sweden). DNA incorporation was measured on a liquid scintillation & luminescence counter (Wallac) and the results were expressed as counts per minutes (cpm).

IL-1β ELISA (III, V)

To quantify the IL-1-β production by XS52 cells in response to the different concentrations of RO60-0175/007, a mouse IL-1β ELISA kit from R&D systems (Abingdon, UK), was used according to the manufacturer's protocol. Cells were cultured at 5x10^{4} cells/well in a 96 well-flat bottomed plate for 48 h in cRPMI medium without NS47 supernatant, GM-CSF or lipopolysaccaride (LPS, Sigma-Aldrich). The cell culture medium was then aspirated and 100 μl cRPMI medium containing GM-CSF (2ng/ml) and LPS (1μg/ml) was added, followed by the addition of 20 μl of RO60-0175/007, fluoxetine, citalopram or 20 μl diluent. After 12 h culture supernatants were collected. In blocking experiments, mesulergine (20 μl) was added 20 min before adding RO60-0175/007.

IL-2 ELISA (V)

For IL-2 measurements the cells were incubated for 48 h, and after centrifugation, the supernatants were collected and used. A DuoSet ELISA development kit (R&D systems) that measures natural and recombinant IL-2 in cell culture supernatants was used according to the manufacturer's protocol. Briefly, 96 well-microplates were coated with capture antibody diluted in PBS 100 μl per well and incubated overnight at room temperature. Plates were blocked by adding 300 μl/well of blocking buffer (1% bovine serum albumin (BSA), 5%

sucrose in PBS with 0.05% NaN3) for 1 h. Diluted standards and samples (100 µl/well) were then added and incubated for 2 h. Then 100 µl of detection antibody was added to each well and incubated for 2 h, followed by 100 µl/well of streptavidin-horseradish-peroxidase detection reagent for 20 min. One hundred microlitres per well of substrate solution was added for 20 min. The plates were read at 450 nm within 30 min. Repeated washings were performed throughout the procedure. All the incubations were carried out at room temperature.

Polymerase chain reaction, PCR (III)

Total RNA was extracted from the XS52 Langerhans-like cells after 12 h incubation using an RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

The isolated RNA was dissolved in RNase-free water and stored at –70°C. mRNA was further processed using Superscript cDNA kit (Life Technologies, Gaithersburg, MD, USA) to synthesize cDNA using Oligo (dT) Primer (Invitrogen, Paisley, UK) according to the manufacturer's protocol.

Two 22/19 nucleotide primers were synthesized using the mouse IL-1-β sequence published by (Torii *et al.*, 1997b) as a template. The upstream primer (5'-AAAAGATGAAGGGCTGCTTCCA-3') corresponded to positions 154-175 in the published sequence and the downstream primer (5'-TGGAGAATATCACTTGTTG-3') was complementary to bases at positions 549-531. The primers were deliberately chosen so that they would encompass two introns to avoid confusion with any amplified genomic IL-1-β. Expression of the housekeeping gene beta-actin was analyzed for reference of basic mRNA synthesis in the cell samples.

PCR was performed using a Perkin-Elmer GeneAmp PCR System 2700 in a reaction volume of 50 µl in the presence of 0.5 µM primers, 0.2 mM dNTP, 1.5 mM $MgCl_2$ and 1.75 U Expand High Fidelity (Roche Diagnostics Scandinavia, Bromma, Sweden). The steps in the PCR reaction included time delay (96°C for 5 min), step cycle (96°C for 30 sec, 52°C for 1 min and 72°C for 2 min) altogether 25-40 cycles, after that a 5-min time delay at 72°C, and finally a soak at 4°C for at least 5 min. Aliquots (10 µl) of all samples were separated in 2%

agarose gels containing ethidium bromide (50 µg to 100 ml of agarose gel). The gels were run for 1.5 h at 75 V and photographed under UV light.

3.9 Statistical analysis

For statistical calculation the Student's *t*-test, Mann-Whitney *U* test or Equality of Variances F test were used. A p value of <0.05 was regarded as being significant.

4. RESULTS

4.1 Animal studies (I-III)

Serotonin and serotonin receptor 2C

Immunohistochemistry

5-HT immunoreactive mast-like cells as well as positive platelets were detected in the dermis of both eczematous and control skin. In parallel sections, tryptase positive cells (mast cells) shared similar distribution and features with 5-HT positive cells. The number of these mast-like cells did not differ between the two groups (eczematous at 144±14 (mean±STD) and control at 139±31 per section) but degranulation of some inflamed cells was seen. On the other hand, semi-quantification of 5-HT positive platelets demonstrated an increase in the eczematous (++) compared to control ears (+). Preincubating the antibody with serotonin at 10^{-3} mol/L abolished cellular immunoreactivity.

Expression of 5-HT2CR was recorded on epidermal dendritic cells. The number of these cells was increased ($p<0.01$) in the eczematous skin, at 209±59 cells/section, in contrast to control skin, at 122±63. In addition, dendritic cells in the eczematous skin were enlarged and exhibited longer dendrites than their normal counterparts. Double staining experiments, using the Langerhans cell marker I-A antigen, revealed coexpression of both receptor and antigen on the same cell.

GC-MS

The concentration of 5-HT was almost similar in both the eczematous and control groups, the levels being 16.2±7.3 ng/ear and 17.9±7.6 ng/ear, respectively.

In vitro study of XS52 cell line

Since our immunohistochemical findings showed the expression of 5-HT2CR on epidermal Langerhans cells, we tested the effect of 5-HT2CR agonist RO60-0175/007 on an XS52 Langerhans cell-like line, regarding their proliferation, cytokine IL-1β synthesis and production. XS52 cellular proliferation was decreased ($p<0.05$) by the lowest tested dose (10^{-10} mol/L) of RO60-0175/007. IL-1β mRNA synthesis was clearly detected by PCR in all the

samples but there was no difference between the different concentrations of the agonist and the control. Conversely, ELISA revealed an increased production of IL-1β with the highest concentrations $5x10^{-5}$ ($p<0.01$) and 10^{-5} mol/L ($p<0.05$) as well as a strong tendency to an increase with $5x10^{-6}$ mol/L ($p=0.055$), and a tendency (p=0.2) to a decreased production with 10^{-10} mol/L. Adding equal concentrations of the 5-HT2CR antagonist mesulergine blocked the effects produced by the highest doses of RO60-0175/007.

Innervation (PGP 9.5 and GAP-43)

Immunohistochemistry

Epidermal PGP 9.5 positive nerve fibre density tended to increase from 1.3±0.9 mm per section in the control skin to 1.6±0.7 mm per section in eczematous skin. The same trend was also evident with dermal fibres, being 4.5±2.9 in control skin and 4.8±1.2 in the eczematous skin. GAP-43 immunoreactive nerve fibres in the epidermis were increased ($p<0.01$) at 2.7±1.0 mm per section in the eczematous versus control skin, at 1.3±0.8. Moreover, dermal GAP-43 fibres were increased ($p<0.01$) in eczematous skin (9.7±3.2) in contrast to control skin (5.5±2.5).

Sensory neuropeptides (CGRP, substance P and galanin)

Immunohistochemistry

The total number of CGRP and substance P positive nerve fibres in the epidermis and dermis tended to increase in the eczematous skin, 56.0±29.3 fibres/section and 23.9±13.6 fibres/section, respectively, compared with control skin at 51.0±21.4 and 16.2±4.9 fibres per section. This trend was also apparent in the epidermis and dermis, separately. The number of galanin positive fibres was increased ($p<0.01$) in the eczematous skin (median 8.0, quartile deviation 4.5, fibres per section) in contrast to control skin (median 2.5, quartile difference 2.5, fibres per section). Nerve bundles expressing galanin were also detected in the dermis and double staining revealed the coexpression of PGP 9.5 with galanin-like immunoreactivity in small nerve bundles.

CGRP, substance P and galanin immunoreactivity were abolished by preabsorbing the antisera with their respective peptides at 10^{-5} and 10^{-6} mol/L.

RIA

RIA measurements for the concentration of CGRP showed a decreased ($p<0.05$) concentration in the eczematous ears at 11.5±3.8 pmol/g compared to control ears at 16.0±2.1 pmol/g. Substance P concentration tended to decrease in the eczematous ears, 2.6±0.5 pmol/g in contrast to control ears, 3.0±0.4. The concentration of galanin peptide was lower ($p<0.04$) in the eczematous ears (median 1.7, quartile deviation 1.0 pmol/g) than in control ears (2.4, quartile deviation 1.3 pmol/g).

4.2 Human studies (IV, V)

Serotonin, serotonin receptors 1A, 2A, 3 and SERT

Immunohistochemistry

Serotonin expression was observed on immunoreactive platelets, which were more numerous in eczematous (++) than in control skin (+).

Immunoreactivity for 5-HT1AR was recorded on basal epidermal cells, which were more elongated and possessed longer dendrites in the contact eczematous compared to control skin. Double staining demonstrated the colocalization of both the melanocyte cell-marker NKI-beteb and 5-HT1AR on these epidermal cells. Using indirect immunohistochemical staining, intense epidermal immunoreactivity for 5-HT1AR was also observed on the upper quarter of the epidermis with no difference between the eczematous and control skin. Employing the biotinylated technique, this epidermal immunoreactivity was stronger in eczematous than in control skin.

5-HT1AR expression was also evident on vessels wall and on papillary dermal mononuclear cells, which had a decreased ($p<0.001$) percentage (33.3±6.5%) in eczematous skin, in contrast to control skin (63.7±11.3% (study IV)), and 64±18 cells per section, in contrast to 97±32 ($p<0.01$) in study V. The majority of these cells were found to be tryptase positive and showed more degranulation in the eczematous skin.

Immunoreactivity for 5-HT2AR was recorded with the same intensity throughout the epidermis of control skin, but was more confined to the upper epidermis in the eczematous skin. 5-HT2AR immunoreactivity was also apparent in the vessel wall, smooth muscles and dermal mononuclear cells. The number of the immunoreactive dermal mononuclear cells was increased ($p<0.001$) from 2±2 per section in control skin to 100±50 in the eczematous skin. In the latter, 5-HT2AR positive cells were seen to transmigrate vessel walls, infiltrate the epidermis and lay within vesicles. 5-HT2AR positive cells were determined to be CD3, CD4 or CD8 positive.

Basal epidermal cells expressed 5-HT3R, but the expression was more evident in the acrosyringium and in the epithelium of hair follicles without any difference between the eczematous and control skin. Immunoreactivity for 5-HT3R was also seen on smooth muscles.

SERT positive dermal mononuclear cells infiltrated the epidermis in the contact eczematous skin, more cells ($p<0.001$) being present in the eczematous skin (96±19 cells per section) than in the control skin (2±2). Double staining showed the colocalization of SERT with CD1a, CD4 or CD8 positive or $CD56^{+}CD3^{-}$ cells.

In vitro study XS52 and PBMC

The SERT inhibitors fluoxetine and citalopram (dose range 10^{-5}-10^{-6} mol/L) had a tendency to decrease IL-1β production by XS52 cells. This tendency was more evident with citalopram at 10^{-6} mol/L ($p=0.07$).

PBMC were stimulated with nickel sulphate, which gave a 31-fold stimulation of proliferation compared to nonstimulated cells (649±857 cpm) and (192±268 pg/ml) for IL-2 production. IL-2 for saline-treated cultures could not be detected in most of the experiments, but was 0.7 pg/ml in one experiment.

DOI at $5x10^{-5}$ mol/L decreased ($p<0.001$) the proliferation of these nickel-stimulated cells, and also tended ($p=0.2$) to inhibit their IL-2 production. Adding ketanserin (5-HT2AR antagonist) at $5x10^{-7}$ mol/L moderately blocked the inhibitory effect of DOI on nickel-stimulated cells. Application of the SERT inhibitor fluoxetine at 10^{-5} mol/L resulted in a 47% decrease of nickel-stimulated PBMC proliferation, although not significantly ($p=0.17$). Citalopram did not affect cellular proliferation.

Innervation (PGP 9.5 and GAP-43)

Immunohistochemistry

The total number (epidermis and dermis) of PGP 9.5 immunoreactive nerve fibres did not differ between the eczematous (165.3±42.1) and control skin (165.6±70.4), whereas an increase ($p<0.01$) in total GAP-43 positive fibres was evident. In the epidermis the number of these fibres was 28.4±10 fibres/section in the eczematous skin in contrast to 16.6±6.8 in control skin ($p<0.01$). The number of dermal GAP-43 immunoreactive fibres almost doubled in the eczematous skin, 16.6± 6.0, compared to the control skin, 9.4±5.4 ($p<0.01$).

Sensory neuropeptides and NK1 receptor

Immunohistochemistry

The number of CGRP immunoreactive nerve fibres in the papillary dermis showed a slight decrease in the eczematous, 5.5±2.9 fibres/section, compared to control skin, 7.1±4.0, whereas no difference between the number of substance P positive fibres in the eczematous (4.2±1.7) and control skin (4.4±2.1) was detected. Unlike substance P fibres, CGRP immunoreactive fibres were positive for GAP-43.

Semiquantification of substance P and NK1R positive papillary dermal mononuclear cells demonstrated an increase in the eczematous (+++) in contrast to control (+) skin. Moreover, substance P positive cells intruded into the epidermis. NK1R immunoreactivity in basal keratinocytes was higher in the eczematous than in control skin. The intensity of this immunoreactivity also appeared to be increased with more intense inflammation.

Double staining revealed the coexpression of substance P and tryptase, CD3, CD4 or CD8 on mononuclear cells, whereas NK1R cells were found to be tryptase or CD3 positive.

5. DISCUSSION

5.1 General discussion

The aim of our study was to investigate the contribution of the nervous system to allergic contact dermatitis. ACD is generally a good model to study neuroimmune interaction in the skin because of its acute nature and defined time points at which the allergic reaction is initiated. We studied such neurogenic pathophysiological mechanisms in ACD at the peak of inflammation in humans, three days after challenge with the antigen. In mice, after performing pilot studies to test several time points, 24 h postchallenge was selected as the optimal time point, when a prominent inflammation was established.

Our scope was to focus on the contribution of the serotonergic system, nerve fibres and sensory neuropeptides to ACD in humans as well as in the mouse.

5.2 Serotonin, serotonin receptors 1A, 2A, 2C and SERT

Through semiquantification, we determined an increase in 5-HT immunoreactive platelets in both murine and human inflamed tissues. However, in both murine eczematous and control skin there was no difference in the number of 5-HT positive mast cells or 5-HT concentration. The similar concentrations of 5-HT in both groups, despite an increased platelets number, might be due to the smaller contribution of platelets to the total 5-HT quantity in murine skin, since mast cells are the main source of 5-HT in rodents (Van Loveren and Askenase, 1984). Regarding the concentration of 5-HT in human ACD, 5-HT was reported by Lundeberg *et al*. (1999) to be increased in eczematous skin.

Expression of the serotonergic receptors 1A, 2A as well as SERT in human and 5-HT2C in murine ACD may indicate a role for these receptors in the pathophysiological mechanisms in ACD. There was a decrease in 5-HT1AR immunoreactive mononuclear cells in the dermis of inflammatory skin. The expression of this receptor on dermal mast cells supports the notion of the importance of 5-HT in initiating ACD. This finding is consistent with the report by McAloon *et al*. (1995) in which buspiron (5-HT1AR agonist) inhibited murine allergic contact dermatitis. Moreover, the epidermal immunoreactivity was higher in the eczematous skin, suggesting that keratinocytes may express and synthesize this receptor. This may also add to

the importance of 5-HT1AR since keratinocytes may enhance the process of ACD by initiating the production of IL-1β, which will participate in amplification of the inflammatory response.

5-HT2AR expression was evident on dermal mononuclear cells that were seen to infiltrate the epidermis in the contact eczematous skin (study V). In study IV, epidermal immunoreactivity for 5-HT2AR was recorded with the same intensity in control skin and was rather confined to the upper part of the epidermis in the eczematous skin. Contrariwise, the immunoreactivity in study V was essentially negative in the epidermis and revealed more immunoreactive immune cells intruding into the epidermis. In addition, the abundant expression of this receptor on inflammatory cells may serve as a marker for skin inflammation. Ameisen *et al*. (1989) demonstrated a role for 5-HT2R in murine contact allergy, using ketanserin, which is a 5HT2A/C receptors antagonist. Hence 5-HT2A/C receptor positive cells might be targets for therapeutic drugs.

Nonetheless, our *in vitro* findings with 5-HT2AR agonist (DOI) treated nickel-stimulated PBMC showed a decreased cellular proliferation and a tendency to a decreased IL-2 production at a high agonist concentration. During certain inflammatory conditions, the tissue concentrations of 5-HT were reported to be in the range of 10^{-4} mol/L (Benedict *et al*., 1986). 5-HT in the range of 10^{-4}–10^{-5} mol/L was also discerned to increase the viability and prevent apoptosis in mitogen-stimulated lymphocytes (Abdouh *et al*., 2004).

Regarding the role of the serotonin transporter protein, SERT, dermal immunoreactive cells infiltrated the epidermis and were significantly increased in the eczematous skin. Both SERT and 5-HT2AR expression was observed on CD3 positive lymphocytes, implying that they can be coexpressed on the same cell in the skin, and infiltrating into the epidermis. In addition, the distribution of these immunoreactive cells was rather similar in both cases. A close association of SERT and 5-HT2R has been earlier described, where a fluoxetine-induced decrease in lymphocyte proliferation to mitogens was suggested to be mediated by an activation of central 5-HT2R (Pellegrino and Bayer, 2002).

The SERT inhibitor fluoxetine in the present study tended to decrease nickel-stimulated PBMC proliferation, and in addition both fluoxetine and citalopram tended to inhibit IL-1β production by the Langerhans-like cell line. This also concords with earlier data in which

SERT inhibitors were described to have an inhibitory effect on inflammatory cells (Pellegrino and Bayer, 1998; Pellegrino and Bayer, 2002).

In addition to the above-mentioned association between SERT and 5-HT2AR, a connection between SERT and 5-HT2CR has been described in relation to stress. Chronic treatment with fluoxetine reversed an altered 5-HT2CR function in stressed mice (Englander *et al*., 2005). In our murine ACD model 5-HT2CR expression was detected on epidermal I-A dendritic cells (LCs). The number of these cells was significantly increased in the eczematous skin in contrast to control skin, thus indicating the contribution of the serotonergic system to murine ACD mediated by an action of 5-HT2CR on Langerhans cells. In humans we also have indications with the same antibody that dendritic cells in the epidermis may express this receptor (unpublished study).

Furthermore, adding the 5-HT2CR agonist RO60-0175/007, at the strongest concentrations, to the cultured XS52 Langerhans-like cells resulted in a significant increase in their IL-1β secretion and a tendency to a decrease in IL-1β and cell proliferation with the lowest tested concentration. There was no effect of the agonist on the basic high level of mRNA for IL-1β, suggesting that the effect was mainly on preformed or secretory levels of IL-1β. These *in vitro* findings support that the 5-HT2C receptor has a proinflammatory effect.

In paper IV, there was no difference between the staining for the 5-HT3 receptor between the eczematous and control skin, suggesting that this receptor has no evident impact on the allergic contact eczema.

5.3 Innervation (PGP 9.5 and GAP-43)

The present study has demonstrated a general contribution of nerve fibres to the allergic reaction, already at 24 h in mice and at 72 h in humans. In both cases there was an increased number of regenerating GAP-43 positive fibres, being markers of axonal growth. Regarding the general neuronal marker PGP 9.5, there was a tendency to an increase in the murine model. In the study by Kinkelin *et al*. (2000) there was an increase in epidermal PGP 9.5 and GAP-43, 96 h after challenge of human allergic contact reactions. Our findings indicate a contribution of the nervous system to human ACD already at 72 h postchallenge, which is evident in the form of increased GAP-43 immunoreactive fibres. Cutaneous GAP-43 positive

fibres were also reported to be increased by stressful stimuli (Peters *et al*., 2005), and in the setting of inflammatory oral mucosa (Ramieri *et al*., 2004).

5.4 Sensory neuropeptides (CGRP, substance P and galanin)

In the present study we determined a tendency to an increase in the number of CGRP and substance P positive fibres during the murine contact allergic reaction, whereas no difference was recorded in the human specimens. In human skin we found a colocalization of CGRP and GAP-43 on immunoreactive nerve fibres and bundles. This finding indicates an earlier sprouting of CGRP-containing nerve fibres compared to substance P, which was not colocalized. In a report by Wu *et al*. (2002), colocalization of CGRP and GAP-43 positive fibres was demonstrated in adjuvant arthritic rats. Interestingly, there was an increase of substance P and NK1R positive cells in the human inflammatory skin, which indicates a major contribution of nonneuronally located substance P to the inflammatory phase. This is consistent with the finding of an increased substance P positive mast cells in the inflamed dermis of a mouse model of atopic dermatitis (Ohmura *et al*., 2004).

Using RIA to measure the neuropeptide concentration, there was a trend of a decrease for substance P and a significant decrease for CGRP. This might be explained by the rapid enzymatic degradation of these sensory neuropeptides as a result of the action of neutral endopeptidases, which was also suggested in the work by Ek and Theodorsson (1990) and Scholzen *et al*. (2001), respectively.

Regarding galanin, there was an increase in the number of positive fibres in the eczematous murine in contrast to control skin, suggesting a neuronal contribution of galanin to this inflammatory process. Using the same porcine antibody in humans, we observed very few positively stained fibres (unpublished study). At present we are testing a galanin antibody specific for human galanin.

RIA, as for CGRP, demonstrated a significant decrease in the concentration of galanin, which might be explained by consumption and degradation of galanin during inflammation.

The *in situ* hybridization analyses did not detect mRNA for galanin in the murine skin. This stands in contrast to the finding by Ji *et al*. (1995) who reported an upregulation of galanin mRNA signal in epidermal cells in response to the severe carrageenan-induced

inflammation. It is possible that these two different means of inducing inflammation could have generated different responses. It may also be the case that the technique was not sensitive enough to detect a nonneuronal galanin mRNA signal in a contact eczematous reaction.

5.5 Colocalization of serotonin and sensory neuropeptides

Colocalization of serotonin and sensory neuropeptides has been shown in the central nervous system. In the dorsal raphe nucleus the effect of substance P might be mediated through 5-HT1A receptors (Valentino *et al*., 2003). Our colocalization studies indicate that in the skin, sensory neuropeptides such as substance P and its receptor NK1, and serotonin receptors might be expressed on the same inflammatory cells (mast cells and lymphocytes) making such an interaction possible.

6. CONCLUSIONS

The aim of this study was to investigate the neuronal contribution to ACD in humans and mice, focusing on the contribution of 5-HT, innervation and sensory neuropeptides.

5-HT immunoreactive platelets were increased in both mice and human allergic contact eczematous skin. The number of 5-HT1AR immunoreactive mast cells was decreased in the eczematous human compared to the control skin, while the numbers of 5-HT2AR and SERT positive cells were increased. Both 5-HT2AR and SERT immunoreactive cells were colocalized with the lymphocyte markers CD4 and CD8. In addition, SERT positive cells also expressed CD1a (LCs marker) or $CD56^{+}CD3^{-}$ (NK cell marker). 5-HT2CR positive epidermal Langerhans cells were increased in the inflamed in contrast to the control murine skin.

High concentrations of the 5-HT2AR agonist DOI decreased nickel-stimulated lymphocyte proliferation and tended to decrease their IL-2 production. 5-HT2CR agonist RO60-0175/007, at high concentrations, increased the production of IL-1β by the XS52 Langerhans cell-like cells. The cellular proliferation was inhibited by a low concentration of the 5-HT2CR agonist.

Epidermal and dermal GAP-43 immunoreactive nerve fibres were increased in both the murine and human ACD eczematous skin. The density of the PGP 9.5 immunreactive nerve fibres in the inflamed mouse skin tended to increase in the epidermis as well as in the dermis.

CGRP and substance P positive nerve fibres tended to increase in the murine inflamed skin, whereas galanin positive fibres were increased. Conversely, the concentrations of CGRP and galanin were decreased in the eczematous skin, substance P only tending to decrease. Substance P immunoreactive cells as well as NK1R positive cells were increased in the eczematous human skin and these cells were determined to be mast cells or lymphocytes.

Our findings indicate the contribution of the serotonergic system, innervation and sensory neuropeptides to allergic contact dermatitis in humans and mice.

7. FUTURE PERSPECTIVES

In vivo and *in vitro* experiments strongly indicate that 5-HT1AR and 5-HT2AR and the neurokinin 1 receptor are all important receptors for the induction of murine allergic contact dermatitis. In addition, the 5-HT2C receptor might be of importance. SERT has also been shown to modulate the function of immune cells. It is therefore necessary to further elucidate the role of these receptors and SERT in the pathophysiological mechanisms in allergic contact dermatitis by using genetically modified mice. We are planning to utilise serotonin receptors, NK-1 receptor and SERT knockout mice to further investigate the contribution of the serotonergic system to murine contact allergy and to study the neuroimmune interactions in this inflammatory process. These knockout mice strains should preferentially be suitable to being sensitized.

A role for stress in ACD has been proposed earlier by several researchers in the field. It is within our scope to investigate neuroimmune interactions in ACD in mice subjected to chronic mild stress. This model consists of various types of mild stressors that simulate psychosocial and environmental stress.

In addition, pharmacological modulation of the allergic contact dermatitis response using these mentioned receptor agonists or antagonists will be performed, by using *in vivo* methods giving a steady tissue concentration of these modulators, as adapted from psychopharmacological studies.

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