

Reduced expression of nicotinic α subunits 3, 7, 9 and 10 in lesional and nonlesional atopic dermatitis skin but enhanced expression of α subunits 3 and 5 in mast cells

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Summary

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Conflicts of interest

None declared.

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Background The skin cholinergic signalling system is modulated in atopic dermatitis (AD).

Objectives To investigate of the role of nicotinic acetylcholine receptors (nAChRs) in the pathogenesis of AD.

Methods We investigated the expression and localization of nAChR α subunits in AD by quantitative reverse transcription-polymerase chain reaction and immunohistochemistry of biopsies from lesional and nonlesional areas of AD skin and of skin biopsies from healthy control persons.

Results Our data demonstrate the presence of mRNA and protein of the nAChR α subunits 3, 5, 7, 9 and 10 in keratinocytes and mast cells in healthy and AD skin. Expression of the α subunits 3, 7, 9 and 10 was generally reduced in the skin of patients with AD whereas mast cells in AD but not in healthy skin showed α 3 and α 5 subunit immunoreactivity. Differences in the subunit mRNA levels between lesional and nonlesional skin were obtained for the α subunits 3, 9 and 10 with higher levels of α 3 but lower levels of α 10 subunit mRNA in lesional areas. No differences in the expression of the α subunits was found between the groups of extrinsic, intrinsic or mixed AD types, between genders and between smokers and nonsmokers.

Conclusions This supports the idea that the cholinergic system is dysregulated independently from inflammation in AD and that inflammation further modulates individual nAChR subunits.

The skin contains a cholinergic signalling system that consists of the synthesizing enzymes and transporters for acetylcholine (ACh) and the ACh-sensitive muscarinic (MR) and nicotinic ACh receptors (nAChRs). High amounts of ACh are present in the skin,¹ and ACh, synthesized and released from keratinocytes, nerve fibres, eccrine glands, vascular endothelial cells and leucocytes, interacts with MR and nAChRs on keratinocytes, skin fibroblasts, sebocytes and eccrine glands.^{2–12} MR and nAChRs regulate motility, growth and differentiation of keratinocytes and fibroblasts.^{11,13–16}

Mammalian nonmuscular nAChRs are ligand-gated ion channels that are built up of either heteropentamers (α 2–6 with β 2–4 subunits or α 7 β 2 and α 9/ α 10 subunits) or α 7 and α 9 homopentamers.^{17–19} The subunit composition of nAChRs determines the function of the receptors including their Ca^{2+}

permeability.^{17,20} nAChR subtypes with high calcium permeability are α 7 subunit homopentamers^{17,20} or heteropentamers of α 9 and α 10 or α 7 and β 2 subunits.^{17–19} nAChRs modulate cell–cell contacts, the differentiation and proliferation of keratinocytes and fibroblasts and the cornification of the epidermis.^{6,9,13,16}

Atopic dermatitis (AD) is a chronic inflammatory skin disease characterized by eczematous skin lesions and pruritus and is often found in patients with a familial history of atopic diseases such as asthma and allergic rhinitis.^{21,22} The cholinergic system of the skin is modulated in AD. Patients with AD have higher amounts of ACh in their skin.²³ Furthermore, the sensitivity to ACh is changed in AD. ACh application into the skin induces pain in healthy subjects but itch in patients with AD.^{24–26} Therefore, not only differences in ACh synthesis but

also changes in its properties and therefore changes at the receptor level may be of importance in the pathogenesis of AD.

With this background, we investigated the expression of neuronal nAChR α subunits in AD by real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and immunohistochemistry of biopsies from lesional and non-lesional areas of AD skin and of skin biopsies from healthy control persons.

Materials and methods

Patients and tissues

Skin biopsies (3 mm²) were taken from 16 patients (nine men and seven women, mean $\pm$ SD age 30.47 $\pm$ 8.4 years) with AD. The diagnosis of AD was based on the criteria of Hanifin and Rajka²⁷ and patients had SCORAD²⁸ > 20 (Table 1). Clinical characteristics of the patients with AD and controls are shown in Table 1. Patients and healthy controls were included after providing written informed consent within a study protocol approved by the University of Giessen ethics committee. From each patient a blood sample was taken and the levels of total IgE, and specific IgEs of inhalant allergens (SX₁) and of food allergens (FX₅) were determined (Cap-FEIA;

Phadiatop Pharmacia, Freiburg, Germany). Age, sex, AD type, smoker/nonsmoker, SCORAD, presence of atopic syndrome, IgE, SX₁ and FX₅ values were recorded (Table 1). The AD type was classified according to Wüthrich and Schmid-Grendelmeyer^{29,30} as 'extrinsic' (IgE-mediated), 'intrinsic' (non-IgE-mediated) or 'mixed' (IgE-mediated with concomitant respiratory allergies). One biopsy was taken from the lesional site (forearm, cubital region, popliteal region, neck) and one biopsy from a nonlesional area, if possible from the nonlesional contralateral forearm, knee or elbow. In addition, biopsies (forearm, cubital region, popliteal region, neck) were taken from healthy control persons (five men and six women, mean $\pm$ SD age 26.8 $\pm$ 7.3 years). The biopsies were cut into two pieces of 1.5 mm² each. One, destined for RT-PCR analysis, was shock-frozen in liquid nitrogen and stored at -80 °C, and the other was shock-frozen in melting isopentane and stored at -20 °C or -80 °C until processing for immunohistochemistry. Sections of lesional and nonlesional skin were fixed in 4% paraformaldehyde and routinely processed for haematoxylin and eosin (H&E) staining. The stained skin specimens were evaluated by a dermatologist (M.N.). The presence of the inflammatory characteristics spongiosis, parakeratosis, hyperkeratosis, acanthosis and perivascular leucocytes was estimated in lesional and nonlesional skin specimens as: 0, not present; (1), intermediate; 1, present.

Table 1 Clinical characteristics of the patients (P) with atopic dermatitis (AD) and controls (C)

Subject	Sex	Age (years)	Smoker	SCORAD ²⁸	AD type	IgE (kU L ⁻¹)	FX ₅ (kUA L ⁻¹)	SX ₁ (kUA L ⁻¹)
P2	F	43	Ex	74	Extrinsic	> 5000	> 100	0.42
P5	M	42	No	78	Intrinsic	40	6.28	< 0.35
P6	F	32	Ex	39.5	Mixed	914	48.1	15.7
P7	M	28	Yes	90.3	Intrinsic	> 5000	> 100	1.98
P8	F	47	Yes	47.5	Extrinsic	396	3.29	0.44
P9	M	34	Yes	46.5	Mixed	1945	74.8	1.27
P10	F	25	No	82.9	Extrinsic	447	47.3	< 0.35
P11	M	33	No	48.4	Mixed	1411	> 100	0.51
P12	F	22	Ex	56.4	Mixed	> 5000	> 100	2.7
P20	M	24	Yes	37.7	Extrinsic	30.8	0.71	< 0.35
P21	F	20	Ex	26.6	Intrinsic	19.8	< 0.35	< 0.35
P23	F	23	Yes	69.9	Mixed	173	1.56	< 0.35
P30	M	23	Yes	52.1	Extrinsic	74.1	< 0.35	< 0.35
P31	M	20	No	71.1	Mixed	684	< 0.35	11
P32	M	30	Yes	50.1	Mixed	956	1.58	39.3
P35	M	33	No	42.3	Mixed	> 5000	82.1	> 100
C1	F	25	Yes			13.3	< 0.35	< 0.35
C2	F	47	Yes			37.8	< 0.35	< 0.35
C3	F	28	Yes			42.3	< 0.35	< 0.35
C4	M	32	No			13.4	< 0.35	< 0.35
C7	M	24	No			5.57	< 0.35	< 0.35
C12	F	24	Yes			16.2	< 0.35	< 0.35
C14	F	24	Yes			27.6	< 0.35	< 0.35
C20	M	24	Yes			31.2	< 0.35	< 0.35
C21	F	23	No			16.4	< 0.35	< 0.35
C30	M	25	No			10.2	< 0.35	< 0.35
C32	M	18	Yes			49.9	< 0.35	< 0.35

SX₁, specific IgE of inhalant allergens; FX₅, specific IgE of food allergens.

Table 2 Primer pairs for nicotinic acetylcholine receptor (nAChR) subunits and housekeeping genes

nAChR subunit (accession no.)	Primer pairs	Product length (bp)
$\alpha 3$ (M37981)	F: CAGAGTCCAAAGGCTGCAAG R: AGAGAGGGACAGCACAGCAT	148
$\alpha 4$ (L35901)	F: CTCACCGTCCTTCTGTGTC R: CTGGCTTTCTCAGCTTCCAG	110
Intron spanning $\alpha 5$ (NM 000745)	F: CTTACACGCTTCCCAAACCT R: CTTCAACAACCTCAGGAACA	186
Intron spanning $\alpha 7$ (U40583)	F: GTACGCTGGTTTCCCTTTGA R: CCACTAGGTCCCATTCTC	138
$\alpha 9$ (NM 017581)	F: GAAAGCAGCCAGGAACAAAG R: GCACTTGGCGATGTACTCAA	156
$\alpha 10$ (AF199235)	F: ACACAAGTGCCCTGAAGACCT R: TCCCATCGTAGGTAGGCATC	159
Intron spanning 18S rRNA	Ambion, Huntingdon, U.K.	324

F, forward; R, reverse.

Reverse transcription-polymerase chain reaction

For RT-PCR studies, human skin specimens were transferred into lysis buffer (Qiagen, Hilden, Germany) and homogenized using a mixer mill at a frequency of 300 Hz (Qiagen). Total RNA was isolated using spin columns according to the protocol recommended by the manufacturer (RNeasy kit; Qiagen). Contaminating DNA was removed using DNase (1 U μg^{-1} total RNA; Gibco-BRL, Life Technologies, Karlsruhe, Germany) in the presence of 20 mmol L⁻¹ Tris-HCl (pH 8.4), 2 mmol L⁻¹ MgCl₂, 50 mmol L⁻¹ KCl for 15 min at 25 °C. Equal amounts of RNA were reverse transcribed with Superscript RNase H⁻ reverse transcriptase (Gibco-BRL) for 50 min at 42 °C. For the PCR reaction, buffer II, 2 mmol L⁻¹ MgCl₂, 0.25 mmol L⁻¹ deoxyribonucleoside triphosphates (10 mmol L⁻¹ each), 0.5 U/25 μL AmpliTaq Gold polymerase (all reagents from Perkin Elmer, Rodgau, Germany) and 20 μmol L⁻¹ of each primer (Table 2; MWG Biotech, Ebersberg, Germany) were mixed. PCR primers specific for human nAChR $\alpha 2$ – $\alpha 7$, $\alpha 9$ and $\alpha 10$ subunits were designed using PRIMER EXPRESSTM software (Applied Biosystems, Foster City, CA, U.S.A.). The primers for 18S rRNA were purchased from Ambion (Huntingdon, U.K.) (Table 2). Cycling conditions for PCR were 10 min at 95 °C, 40 cycles of 30 s at 94 °C, 20 s at 62 °C and 30 s at 73 °C, followed by 7 min at 73 °C. The RT-PCR products were identified by sequencing (MWG Biotech).

Real-time quantitative reverse transcription-polymerase chain reaction

qRT-PCR (iCycler; Bio-Rad, Munich, Germany) was used to quantify levels of nAChR $\alpha 3$, $\alpha 5$, $\alpha 7$, $\alpha 9$ and $\alpha 10$ subunit mRNAs in specimens from lesional and nonlesional sites of AD skin and from healthy controls. Preparation of cDNA was done as described for RT-PCR. All PCR reactions were

prepared in triplicate using a ready-to-use kit according to the manufacturer's protocol (SYBR green, QuantiTectTM; Qiagen). Primers specific for 18S rRNA were used for standardization. The data were normalized by subtracting the threshold cycle (CT) levels between the α subunits and 18S rRNA. Real-time qRT-PCR was performed in triplicate for each sample. The relative α subunit expression in the control skin compared with lesional and nonlesional skin was determined by calculating $2^{(50 - \Delta\text{CT}_{\text{lesional area}}) - (50 - \Delta\text{CT}_{\text{nonlesional area}})}$.³¹

Statistics

The qRT-PCR data are presented as box plots that show percentiles 0, 25, 50 (median), 75 and 100. The ΔCT values were subtracted from 50, resulting in higher values in samples with higher expression. Matched pairs (lesional, nonlesional) were evaluated by Wilcoxon's rank sum test or by Student's paired t-test. In the case of more than two nonmatched groups, the Mann-Whitney U-test for comparison between two groups was conducted only when statistically significant differences were reached by the global Kruskal-Wallis test that was performed first. Differences were considered as statistically significant when $P < 0.05$.

Immunohistochemistry

Shock-frozen specimens were serially cut into 10- μm thin sections. They were fixed with acetone at -20 °C, air dried for 15 min followed by blockade of nonspecific binding sites with phosphate-buffered saline (PBS) containing 5% bovine serum albumin and 5% normal goat serum. Indirect immunofluorescence was performed by overnight incubation with primary antisera directed against nAChR α subunits or by incubation with combinations of antisera directed against α subunits diluted in PBS at room temperature (Table 3) followed by

Table 3 Primary and secondary antisera

Antigen/antiserum	Host	Dilution	Source
Primary antisera			
nAChR $\alpha 3$	Guinea-pig	1 : 1000	⁷
nAChR $\alpha 5$	Guinea-pig	1 : 2000	⁷
nAChR $\alpha 7$	Guinea-pig	1 : 2000	⁷
nAChR $\alpha 9$	Guinea-pig	1 : 2000	⁷
nAChR $\alpha 10$	Rabbit	1 : 1000	³²
Secondary antisera			
Nf68	Mouse monoclonal, clone NR4	1 : 300	Sigma, St Louis, MO, U.S.A.
Cy3-conjugated antiguinea-pig IgG	Donkey	1 : 800	Jackson, Soham, U.K.
FITC-conjugated antimouse IgG	Donkey	1 : 400	Dianova, Hamburg, Germany

nAChR, nicotinic acetylcholine receptor.

washing in PBS and subsequent incubation for 1 h at room temperature with appropriate secondary reagents (Table 3). Then the slides were washed in PBS, coverslipped in carbonate-buffered glycerol at pH 8.6 and evaluated either by epifluorescence microscopy using the appropriate filter combinations (Axioplan2; Zeiss, Oberkochen, Germany) or by sequential confocal laser scanning microscopy (TCS SP2; Leica, Bensheim, Germany) using argon or HeNe laser. The specificity of the antisera directed against the $\alpha 3$, $\alpha 5$, $\alpha 7$, $\alpha 9$ and $\alpha 10$ subunits has previously been demonstrated in human skin⁷ or rat dorsal root ganglia.³²

Toluidine blue staining

After the determination of $\alpha 3$, $\alpha 5$ and $\alpha 10$ subunit immunoreactivities, pictures were taken from regions with immunoreactive putative mast cells. Subsequently, the slides were washed in PBS, stained for 45 s in 0.1% toluidine blue, washed again, coverslipped in carbonate-buffered glycerol at pH 8.6 and re-evaluated under the microscope for positive staining of mast cells.

Results

Reverse transcription-polymerase chain reaction

Comparison between healthy and atopic dermatitis skin

In accordance with recently published data⁷ the analysis of human healthy skin showed the presence of mRNA for the

α subunits 3, 5, 7, 9 and 10 in healthy skin (Fig. 1). The nAChR α subunits 3, 5, 7, 9 and 10 were detected in healthy control skin and in nonlesional and lesional AD skin. The relative mRNA expression of the α subunits 3 and 9 was the highest, and that of the $\alpha 5$ subunit the lowest, in skin of healthy controls (Table 4 and Tables S1–S5; see Supplementary material). The lesional and nonlesional areas of AD skin showed a significant reduction in the expression of the α subunits 3, 7, 9 and 10 but not of the $\alpha 5$ subunit (Mann–Whitney test, Fig. 1, Table 4 and Tables S1–S5; see Supplementary material). The strongest reduction and largest difference between healthy and AD skin was obtained for the $\alpha 9$ subunit. The relative mRNA expression of this subunit was 23.1/12.7-fold higher in the healthy skin than in lesional/nonlesional areas of AD skin (control $n = 10$, lesional/nonlesional AD $n = 14$; Fig. 1, Table S4; see Supplementary material).

Comparison between lesional and nonlesional atopic dermatitis skin areas

Significant differences between the lesional and nonlesional areas of AD skin were measured for the $\alpha 3$, $\alpha 10$ and $\alpha 9$ subunits (Wilcoxon matched pairs test and t-test; Fig. 2, Tables S1–S5; see Supplementary material). The relative expression of the $\alpha 3$ subunit was lower in atopic skin compared with healthy controls but was significantly higher (up to a maximal 4.9-fold, patient 5) in the lesional site compared with nonlesional areas in nine of 16 patients (Fig. 2, Table 5) (relative

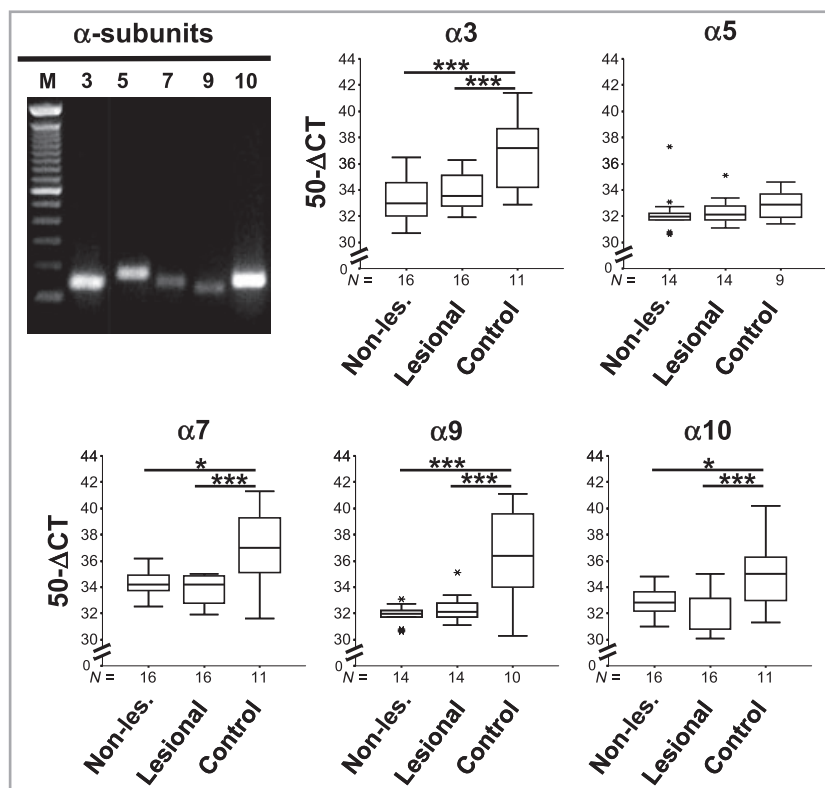


Fig 1. Reverse transcription-polymerase chain reaction (RT-PCR) analysis of nicotinic acetylcholine receptor (nAChR) α subunit expression in nonlesional, lesional and healthy atopic dermatitis (AD) skin. RT-PCR analysis using specific primers for the nAChR α subunits 3, 5, 7, 9 and 10 in human healthy skin. The ethidium bromide gel shows PCR products corresponding to the nAChR α subunits 3, 5, 7, 9 and 10. M = 100-bp marker. The box plots of quantitative RT-PCR analysis of the nAChR α subunit expression in human skin show percentiles 0, 25, 50 (median), 75 and 100. The relative expression of individual α subunit mRNAs in relation to the housekeeping gene 18S rRNA is shown for nonlesional and lesional AD skin and for the skin of healthy control persons. The ΔCT values were subtracted from 50, resulting in higher values in samples with higher expression. N = number of patients. *** $p < 0.001$, * $p < 0.05$.

Table 4 Expression levels of nicotinic acetylcholine receptor subunit mRNA in nonlesional and lesional atopic dermatitis skin and in healthy skin

	Nonlesional 50 – Δ CT (n)	Lesional 50 – Δ CT (n)	Control 50 – Δ CT (n)
α 3 subunit	33.42 $\pm$ 1.27 (16)	34.04 $\pm$ 2.05 (16)	36.46 $\pm$ 1.34 (11)
α 5 subunit	26.05 $\pm$ 10.75 (14)	32.04 $\pm$ 2.05 (14)	30.95 $\pm$ 0.78 (9)
α 7 subunit	34.34 $\pm$ 1.91 (16)	33.89 $\pm$ 0.28 (16)	36.15 $\pm$ 1.27 (11)
α 9 subunit	32.93 $\pm$ 1.2 (14)	32.86 $\pm$ 1.77 (14)	36.46 $\pm$ 2.26 (10)
α 10 subunit	32.98 $\pm$ 0.99 (16)	32.03 $\pm$ 1.56 (16)	35 $\pm$ 6.29 (11)

Results are shown as mean $\pm$ SD. The Δ CT values were subtracted from 50, resulting in higher values in samples with higher expression.

expression lesional vs. nonlesional, 1 vs. 1.95 ± 1.35 -fold, $P < 0.005$, Wilcoxon matched pairs test). In contrast, the α 10 mRNA was reduced in the lesional site in 13 of 16 patients (Wilcoxon matched pairs test; Fig. 2, Table 5, Table S5; see Supplementary material) with a maximal reduction to 0.15-fold expression (patient 30) (relative expression lesional vs. nonlesional, 1 vs. 0.7 ± 0.3 -fold). Significant differences in the α 9 mRNA expression between lesional/nonlesional areas were present only when skin from corresponding body regions was compared (Fig. 2). Skin taken from the cubital or popliteal region showed a slight but significant upregulation of α 9 mRNA in lesional areas (nonlesional $50 - \Delta$ CT 32.54 ± 2.0 ; lesional $50 - \Delta$ CT 34.26 ± 1.37 , $n = 7$, $P = 0.018$, *t*-test, Fig. 2 and Table S4; see Supplementary material).

Reduced values for the α 7 mRNA were observed in the lesional skin from 11 of 16 patients (Table S3; see Supplementary material) but statistical analysis revealed no significant difference between lesional and nonlesional areas (Wilcoxon matched pairs test, Table 5).

Two of the three housekeeping genes investigated in this study (β_2 -microglobulin and β -actin) were differentially expressed in nonlesional and lesional skin areas. Therefore 18S rRNA was used as housekeeping gene.

Comparison between atopic dermatitis types

Patients with AD were classified into groups based on the levels of IgE, FX₅, SX₁ and the SCORAD. No statistically significant differences in the mRNA expression of the α subunits investigated in this study were observed between patient groups with extrinsic, intrinsic or mixed AD, between genders or between smokers and nonsmokers. There were between three and seven patients in individual patient groups.

Haematoxylin and eosin staining

Evaluation of the H&E-stained sections showed that characteristics of inflammation were present in the lesional skin from 15 of 16 patients and in nonlesional skin from four of 16. The lesional skin of patients with AD showed uneven psoriasiform hyperplasia, focal slight hypergranulosis, and compact orthokeratosis which was absent in the nonlesional and healthy skin (data not shown).

Immunohistochemistry

Comparison between healthy and atopic dermatitis skin

nAChR α 3 subunit. The localization and intensity of this subunit immunoreactivity were similar in skin from healthy controls and in nonlesional AD skin. Immunoreactivity for the α 3 subunit was strongly present in the basal cell layer of the epidermis and decreased towards the granular layer (Fig. 3).

When compared with the nonlesional areas, immunoreactivity for the α 3 subunit in the lesional skin was unchanged in one patient, decreased in two patients and extended into suprabasal layers of the epidermis of lesional areas from nine of 12 patients (Fig. 3). In nine of 15 patients the dermis of lesional skin areas contained strongly α 3 subunit-immunoreactive, toluidine blue-positive mast cells that we could not detect in healthy or nonlesional AD skin (Fig. 4, Table 5).

nAChR α 5 subunit. The localization and intensity of the immunoreactivity for the subunit were similar in epidermis from healthy controls and in nonlesional AD skin. At best weak immunoreactivity for the α 5 subunit was present in all layers of the epidermis, with highest intensity in the spinous cell layer (Fig. 3).

The α 5 subunit immunoreactivity was, compared with the nonlesional areas, slightly increased and extended to upper epidermal cell layers in the epidermis of lesional areas in three of 12 patients, and decreased in nine. Dermal mast cells were identified by toluidine blue staining. They exhibited strong immunoreactivity for the α 5 subunit in the lesional area from five of 11 patients (Fig. 4, Table 5) but could not be detected in the skin of healthy controls or nonlesional AD skin.

nAChR α 7 subunit. The α 7 antiserum labelled the membrane of keratinocytes of the granular and upper spinous layer (Fig. 3). The α 7 subunit immunoreactivity was, compared with the nonlesional area, increased in the epidermis of the lesional area from one of nine patients, unchanged in four and retracted to the granular layer in four (Table 5).

nAChR α 9 subunit. In healthy skin, the keratinocytes of the suprabasal layers showed moderate to intense cell membrane staining that decreased towards the granular layers whereas almost all layers of nonlesional skin from patients with AD

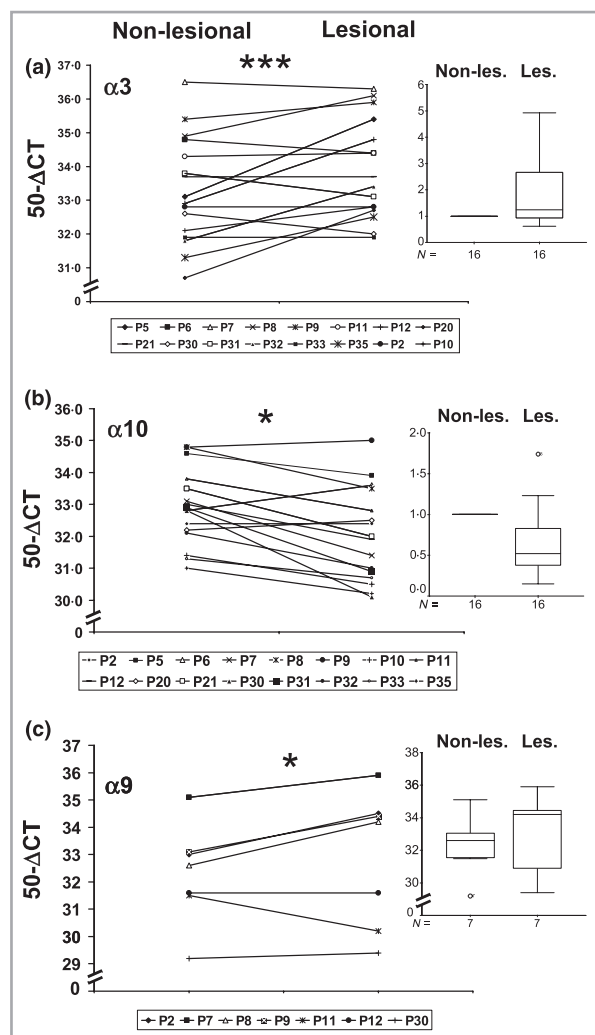


Fig 2. Quantitative reverse transcription-polymerase chain reaction analysis of the expression of nicotinic acetylcholine receptor (nAChR) $\alpha 3$, $\alpha 10$ and $\alpha 9$ subunits in nonlesional and lesional atopic dermatitis skin. The diagrams show the direct comparison and box plots (insets) of the $\alpha 3$ (a), $\alpha 10$ (b) and $\alpha 9$ (c) subunit expression in lesional and nonlesional skin areas of individual patients. The biopsy localizations were the cubital or popliteal region. The ΔCT values were subtracted from 50, resulting in higher values in samples with higher expression. N = number of patients. *** $P < 0.001$, * $P < 0.05$. The box plots of the relative expression of the nAChR α subunit expression in lesional in relation to nonlesional sites show percentiles 0, 25, 50 (median), 75 and 100. The relative α subunit expression in the control skin compared with lesional and nonlesional skin was determined by calculating $2^{(50 - \Delta CT_{\text{lesional area}}) - (50 - \Delta CT_{\text{nonlesional area}})}$.

exhibited weaker $\alpha 9$ subunit staining, in individual patients accompanied by a reduction of immunoreactivity to the basal and spinous layer (Fig. 3).

The localization and intensity of the epidermal $\alpha 9$ subunit immunoreactivity was similar between healthy and nonlesional skin areas. The $\alpha 9$ subunit immunoreactivity in lesional areas was, compared with the epidermis of the nonlesional area, decreased in the epidermal granular layer from eight of 13 patients, and unchanged in five. Comparison of the skin speci-

mens taken from the cubital or popliteal region showed a reduction in epidermal $\alpha 9$ immunoreactivity in the lesional skin areas but an increase in the number of $\alpha 9$ -immunoreactive cells in the dermis in 10 patients (Fig. 3, Table 5).

nAChR $\alpha 10$ subunit. Immunoreactivity for the $\alpha 10$ subunit was present in all layers of the healthy epidermis but was reduced to the basal layer in nonlesional areas of patients with AD. Dermal mast cells identified by toluidine blue-staining cells exhibited strong immunoreactivity for the $\alpha 10$ subunit in the lesional and nonlesional skin areas. Individual $\alpha 10$ subunit-immunoreactive mast cells were also found in skin from healthy controls (Fig. 4).

The $\alpha 10$ immunoreactivity was, compared with the nonlesional area, extended to the complete epidermis in lesional areas in seven of 13 patients, unchanged in five and decreased in one (Fig. 3, Table 5).

Discussion

The skin contains the non-neuronal cholinergic system that contributes to a variety of different functions in cutaneous cells such as keratinocytes and dermal fibroblasts.^{11,13,14,33,34} In analogy to other epithelial cells such as the respiratory epithelium proteins of the ACh-synthesizing machinery, keratinocytes contain^{11,14,35,36} and release ACh that subsequently interacts with MR and nAChRs on different target cells. Previous studies showed that the non-neuronal cholinergic system is affected in AD in that skin biopsies from patients with AD contain increased levels of ACh in the epidermis.²³

nAChR α subunit mRNA in human skin

In the present study we tested the hypothesis that changes in the cholinergic system especially in the nAChR expression may participate in the pathogenesis of AD. To address this question we analysed the expression and localization of nAChR α subunits in lesional and nonlesional skin biopsies of patients with AD and in skin biopsies of healthy control persons. This allowed us to distinguish between general effects in patients with AD and effects that are restricted to the lesional area.

Using qRT-PCR, we demonstrated for the first time AD-dependent changes in the expression of individual nAChR α subunits in human skin. Surprisingly, mRNA downregulation of almost all observed α subunits (except the $\alpha 5$ subunit) was not restricted to the affected area but was present also in intact skin of patients with AD. The extension of regulatory processes to nonlesional areas has been shown for the synthesis of the endogenous ligand of nAChR, ACh, demonstrating an upregulation of ACh production in eczematous and noneczematous areas.²³ Hence the reduction in nAChR subunit mRNA is not related to the acute inflammation but is a general phenomenon of AD skin and may be coupled to a higher susceptibility of AD skin to factors that trigger inflammation, eczema and changes in the skin barrier function. A reduction of mRNA for nAChR α subunits may have a major impact on

Table 5 Summary and comparison of results with healthy skin. Changes in the mRNA expression and localization of nicotinic acetylcholine receptor (nAChR) subunit proteins in lesional and nonlesional areas of patients with atopic dermatitis (AD) compared with the skin of healthy controls

nAChR subunit	AD (nonlesional)		AD (lesional)	
	mRNA	Immunoreactivity	mRNA	Immunoreactivity
$\alpha 3$	$\downarrow\downarrow$	Epidermis, basal > granular	$\downarrow$	Epidermis, extension to suprabasal layers, mast cells
$\alpha 5$	–	Epidermis, spinous	–	Epidermis, decrease in all layers, mast cells
$\alpha 7$	$\downarrow$	Epidermis, granular = spinous	$\downarrow$	Epidermis granular = spinous
$\alpha 9$	($\downarrow$) $\downarrow$	Epidermis, supragranular > granular	$\downarrow$	Epidermis, decrease in granular layer, increase in dermis
$\alpha 10$	$\downarrow$	Epidermis, basal, mast cells	$\downarrow\downarrow$	Epidermis, extension, mast cells

$\downarrow$ = reduction in mRNA expression.

skin function as epidermal nAChRs modulate and mediate processes such as adhesion, differentiation and growth of keratinocytes.^{6,9,16,33,37–40} Inhibition of nAChR $\alpha 3$ and $\alpha 9$ subunits is followed by loss of cell–cell contacts and by the detachment of cells.⁶ In particular, blockade of the $\alpha 9$ nAChR subunit with strychnine has been shown to have a potent influence on both terminal differentiation and epidermal cell adhesion in organotypically cultured keratinocytes.⁴¹ In mice, blockade of the homopentameric $\alpha 7$ nAChR reduces apoptosis in the granular layer.^{9,33,37–40} Our study demonstrates that independent of the localization, AD skin contains lower amounts of the nAChR α subunits 3, 7, 9 and 10 in lesional and nonlesional areas. The most prominent difference was found for the $\alpha 9$ subunit. This subunit is a unique member of the nAChR subunit family in that $\alpha 9$ nAChRs show mixed nicotinic-muscarinic receptor pharmacology.⁴² Blockade of the $\alpha 9$ subunit leads to acantholysis, indicating a pivotal role in the maintenance of the keratinocyte cell–cell contact.^{6,16} Differences in the expression levels of the α subunits between lesional and nonlesional skin were also present for the $\alpha 3$ and $\alpha 10$ subunits. Expression of the $\alpha 3$ subunit mRNA was increased in the lesional areas of AD skin whereas the $\alpha 10$ subunit mRNA expression was downregulated in these areas. The increase in $\alpha 3$ subunit mRNA was accompanied by an extension of immunoreactivity for the $\alpha 3$ subunit to all epidermal layers of lesional skin. These reciprocal changes of the mRNA levels of the $\alpha 3$ and $\alpha 10$ subunits might be related to the inflammation of the skin in lesional areas. To our knowledge this is the first description of a differential regulation of $\alpha 3$ and $\alpha 10$ nAChR subunits under inflammatory conditions in mammals.^{43,44} The functional role of these changes remains to be determined and, to our knowledge, the only coupling of the $\alpha 3$ nAChR subunit to inflammation has been shown in mice lacking the $\alpha 3$ nAChR subunit, that exhibit a marked inflammation in the bladder.⁴⁴

nAChR α subunit presence in human skin

Based on the qRT-PCR experiments, we tried to determine by immunohistochemistry the cell types that are responsible for the changes in the expression levels. The presence of nAChR

or nAChR subunits in keratinocytes has been described by us and others at a transcriptional, translational and functional level using immunohistochemistry, PCR, epidermal organotypic cocultures, Ca^{2+} imaging or migration assays.^{11,12,44} However, changes in the localization pattern of the α subunits in human skin had not been investigated so far, and they provide direct evidence for the involvement of individual nAChRs in the process of AD.

nAChR α subunits in human skin mast cells

We demonstrated in this study for the first time the presence of the nAChR $\alpha 3$, $\alpha 5$ and $\alpha 10$ subunits on human skin mast cells. Mast cells containing $\alpha 3$ and $\alpha 5$ immunoreactivity were restricted to the lesional skin area of patients with AD and were not detected in healthy or nonlesional skin. In contrast, immunoreactivity for the $\alpha 10$ subunit was found in mast cells of healthy and AD skin. The presence of $\alpha 3$ - and $\alpha 5$ -immunoreactive mast cells in lesional skin points to an upregulation of nAChR in these cells in response to changes in the structure and function of the epidermis. Mast cells hold a central position in atopic diseases^{45–47} and stimulation of mast cell $\alpha 3\alpha 5\beta^*$ -nAChR heteropentamers by non-neuronally released ACh could modulate mast cell degranulation. The effect of ACh on mast cells is variable. ACh has been shown to degranulate rat bladder mast cells⁴⁸ whereas human heart mast cells failed to respond to ACh.⁴⁹ Given that keratinocytes are able to synthesize and release ACh and that atopic skin contains a higher level of ACh, our experiments support a role for ACh in the regulation of mast cell degranulation in the skin, especially in AD skin. Further studies will have to determine the effects of ACh on human skin mast cells.

Differences between lesional and non-lesional skin

The $\alpha 3$ nAChR subunit

Although we obtained differences in the mRNA expression levels between healthy skin and nonlesional areas of patients with AD, we could not detect dramatic changes in the immunohistochemical staining intensities of α subunits between

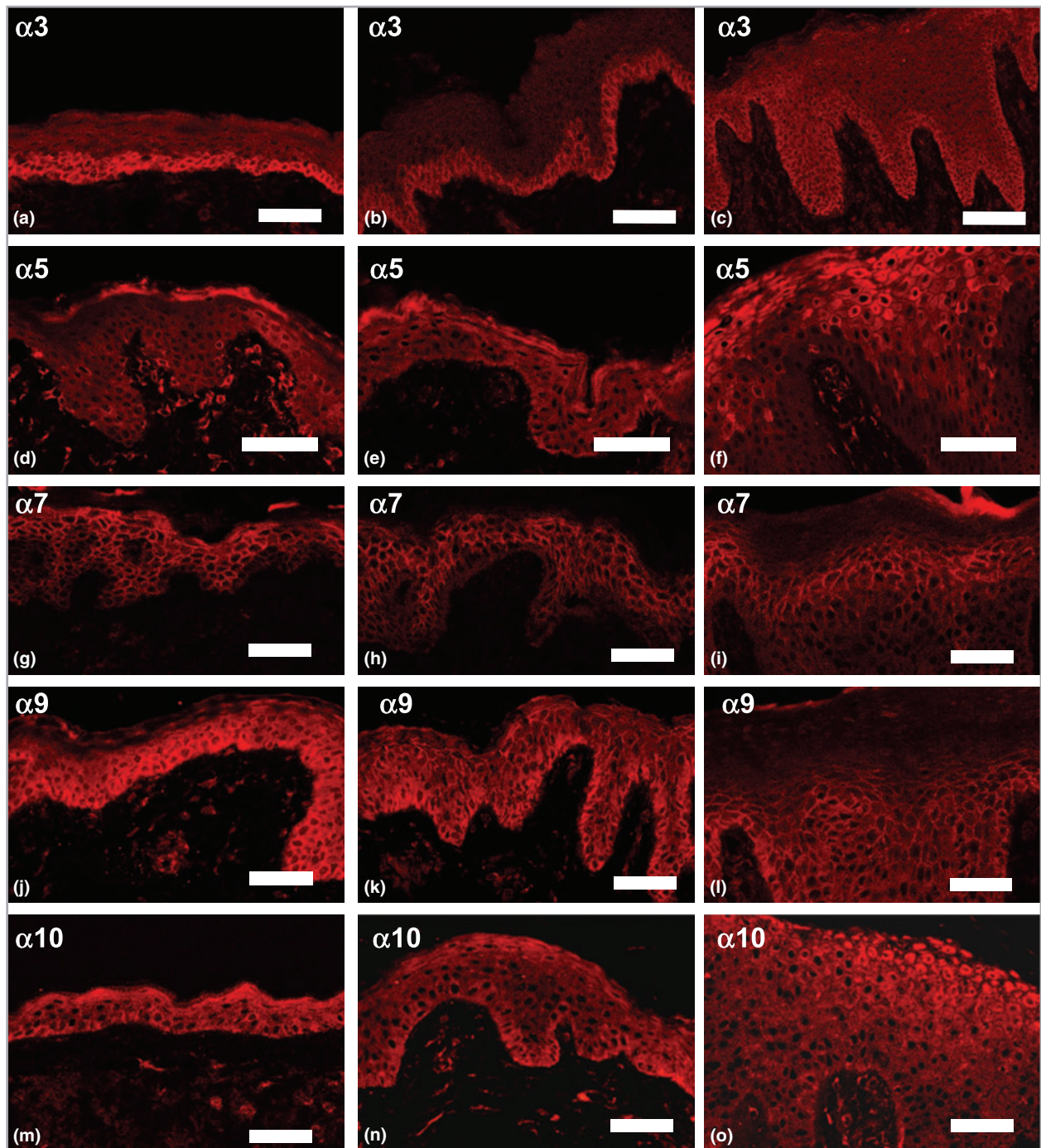


Fig 3. Localization of the nicotinic acetylcholine receptor $\alpha 3$, $\alpha 5$, $\alpha 7$, $\alpha 9$ and $\alpha 10$ subunits in human skin: (a, d, g, j, m) healthy skin; (b, e, h, k, n) nonlesional atopic dermatitis (AD) skin; (c, f, i, l, o) lesional AD skin. Indirect immunohistochemistry demonstrates $\alpha 3$ immunoreactivity in keratinocytes of the epidermis of healthy control skin (a), and nonlesional (b) and lesional (c) AD skin. Immunoreactivity for the $\alpha 3$ subunit is present in the basal cell layer of the epidermis (a, b) and decreases towards the granular layer and extends in lesional areas (c) to suprabasal layers. Immunoreactivity for the $\alpha 5$ subunit is also present in keratinocytes of the epidermis of healthy control skin (d), and nonlesional (e) and lesional (f) AD skin. Weak staining for the $\alpha 5$ subunit is present in all layers of the epidermis, with highest intensity in the spinous cell layer. The $\alpha 7$ antiserum labels the membrane of keratinocytes of the granular and upper spinous layer (g, h) with a reduction of $\alpha 7$ subunit immunoreactivity to the granular layer in lesional areas (i). In healthy skin and nonlesional areas, the keratinocytes of the suprabasal layers showed moderate to intense $\alpha 9$ subunit immunoreactivity in the cell membrane that decreased towards the granular layers (j, k). Lesional skin from patients with AD exhibited weaker $\alpha 9$ subunit staining (l). Immunoreactivity for the $\alpha 10$ subunit is present in all layers of the healthy epidermis (m) and is reduced to the basal layer in nonlesional areas of patients with AD (n). In the lesional areas $\alpha 10$ subunit reactivity extends to all layers (o). Bars = 50 μ m.

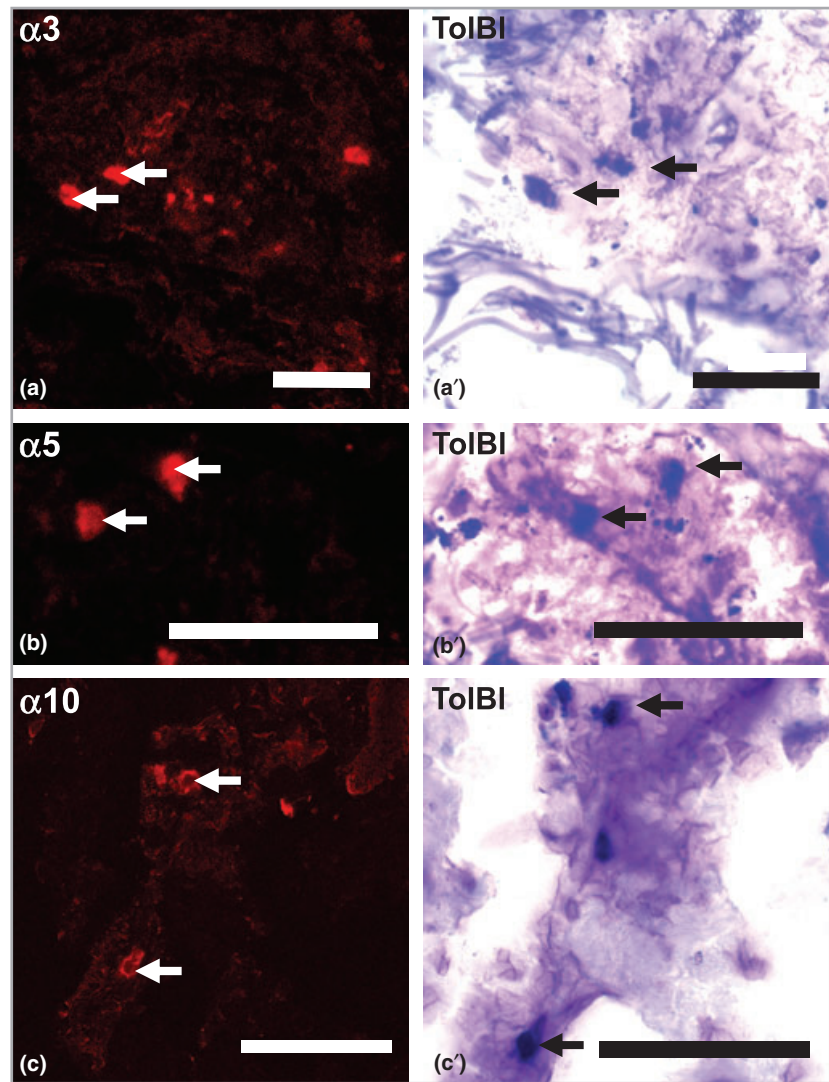


Fig 4. Indirect immunohistochemistry and toluidine blue (TolBl) staining demonstrated the presence of the $\alpha 3$, $\alpha 5$ and $\alpha 10$ subunits in dermal mast cells. The arrows in (a–c) and (a'–c') show α -immunoreactive mast cells in lesional atopic dermatitis skin (a–c) that were TolBl positive after subsequent staining (a'–c'). Note the different magnification of the pictures. Bars = 50 μm .

these areas. In addition to that in mast cells, changes of nAChR subunit proteins in nonlesional AD skin were found for the epidermal $\alpha 3$ subunit.

The extension of keratinocyte $\alpha 3$ immunoreactivity to suprabasal levels in lesional areas of AD skin resembles data reported by Hagforsen *et al.*⁴⁴ where an increase in staining intensity for the $\alpha 3$ subunit was found in the epidermis of palmoplantar pustulosis skin in smokers compared with nonsmoking controls.⁴⁴ The increase in immunoreactive cell numbers found in our study was – compared with healthy skin – not accompanied by increased levels of mRNA, suggesting a possible downregulation of the $\alpha 3$ subunit expression in individual cells. Another possibility is the upregulation of the nAChR proteins as a result of an increased stability of the receptors in the cell membrane based on a decrease in receptor turnover which has been described for $\alpha 3$ subunit-containing nAChRs in transfected cells and in the human brain.^{50–52} This mechanism was seen in response to chronic nAChR stimulation by chronic nicotine exposure. A similar process might

operate in AD skin as increased ACh levels have been shown in skin biopsies from patients with AD²³ thus leading to a prolonged chronic nAChR stimulation. This could explain the discrepancy between our qRT-PCR measurements and the immunohistochemistry. The extension of immunoreactivity for the $\alpha 3$ subunit to suprabasal layers supports the results of studies on keratinocytes in the agarose keratinocyte outgrowth system that indicated that $\alpha 3$ subunit-containing nAChRs are present in motile and nondifferentiated keratinocytes.¹⁰ The increased number of $\alpha 3$ -immunoreactive and probably nondifferentiated keratinocytes could indicate a proliferative process that finally leads to the increased thickness of human AD skin.

The $\alpha 9$ nAChR subunit

Surprisingly, by comparison of lesional and nonlesional areas taken from regions that are mainly affected by AD, the cubital and popliteal region, we found a slight but significant increase in $\alpha 9$ mRNA in lesional areas. This was accompanied by a

decrease in the epidermal $\alpha 9$ immunoreactivity but an increase in the number of $\alpha 9$ -immunoreactive cells in the dermis. Further studies will further characterize the population of dermal $\alpha 9$ subunit immunoreactive cells.

Taken together, our data demonstrate the presence of the nAChR α subunits 3, 5, 7, 9 and 10 in non-neuronal cells of healthy and AD skin. The expression of the mRNA for the α subunits 3, 7, 9 and 10 is, independent of extrinsic, intrinsic or mixed AD types, gender and smoking habits, generally reduced in AD skin. Differences in the subunit mRNA levels between lesional and nonlesional skin were obtained for the α subunits 3, 9 and 10 with an increase of the $\alpha 3$ and $\alpha 9$ but a decrease of the $\alpha 10$ subunit mRNA level. An increase of the $\alpha 9$ subunit mRNA was restricted to areas that are mainly affected by AD. The higher $\alpha 3$ mRNA level of lesional areas was accompanied by an increase in the number of $\alpha 3$ -immunoreactive suprabasal keratinocytes, whereas despite higher mRNA levels, the immunoreactivity for the $\alpha 9$ and $\alpha 10$ subunits was decreased in the lesional epidermis.

The general reduction of nAChR α subunit mRNA in AD points to a general dysregulation of the cholinergic system of the skin independent from the inflammation. The functional consequence of the downregulation of nAChR α subunits may be a higher susceptibility of the skin to mediators and factors that lead to eczema or influence the barrier function of the skin. It further supports the idea that modulation of the nAChR expression is coupled to changes in the epidermal morphology rather than to the inflammatory reaction. Nevertheless, acute inflammation has additional influence on the mRNA and protein levels of individual nAChR subunits.

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References

- Klapproth H, Reinheimer T, Metzen J *et al.* Non-neuronal acetylcholine, a signalling molecule synthesized by surface cells of rat and man. *Naunyn Schmiedeberg Arch Pharmacol* 1997; **355**:515–23.
- Hagforsen E, Einarsson A, Aronsson F *et al.* The distribution of choline acetyltransferase- and acetylcholinesterase-like immunoreactivity in the palmar skin of patients with palmoplantar pustulosis. *Br J Dermatol* 2000; **142**:234–42.
- Kirkpatrick CJ, Bittinger F, Nozadze K *et al.* Expression and function of the non-neuronal cholinergic system in endothelial cells. *Life Sci* 2003; **72**:2111–16.
- Nagata N, Oshida T, Yoshida NL *et al.* Analysis of highly expressed genes in monocytes from atopic dermatitis patients. *Int Arch Allergy Immunol* 2003; **132**:156–67.
- Buchli R, Ndoeye A, Rodriguez JG *et al.* Human skin fibroblasts express m2, m4, and m5 subtypes of muscarinic acetylcholine receptors. *J Cell Biochem* 1999; **74**:264–77.
- Nguyen VT, Chernyavsky AI, Arredondo J *et al.* Synergistic control of keratinocyte adhesion through muscarinic and nicotinic acetylcholine receptor subtypes. *Exp Cell Res* 2004; **294**:534–49.
- Kurzen H, Berger H, Jager C *et al.* Phenotypical and molecular profiling of the extraneuronal cholinergic system of the skin. *J Invest Dermatol* 2004; **123**:937–49.
- Kurzen H, Schallreuter KU. Novel aspects in cutaneous biology of acetylcholine synthesis and acetylcholine receptors. *Exp Dermatol* 2004; **13** (Suppl. 4):27–30.
- Arredondo J, Nguyen VT, Chernyavsky AI *et al.* Functional role of alpha7 nicotinic receptor in physiological control of cutaneous homeostasis. *Life Sci* 2003; **72**:2063–7.
- Zia S, Ndoeye A, Lee TX *et al.* Receptor-mediated inhibition of keratinocyte migration by nicotine involves modulations of calcium influx and intracellular concentration. *J Pharmacol Exp Ther* 2000; **293**:973–81.
- Grando SA. Biological functions of keratinocyte cholinergic receptors. *J Invest Dermatol Symp Proc* 1997; **2**:41–8.
- Grando SA, Pittelkow MR, Schallreuter KU. Adrenergic and cholinergic control in the biology of epidermis: physiological and clinical significance. *J Invest Dermatol* 2006; **126**:1948–65.
- Arredondo J, Hall LL, Ndoeye A *et al.* Central role of fibroblast alpha3 nicotinic acetylcholine receptor in mediating cutaneous effects of nicotine. *Lab Invest* 2003; **83**:207–25.
- Grando SA, Kist DA, Qi M *et al.* Human keratinocytes synthesize, secrete, and degrade acetylcholine. *J Invest Dermatol* 1993; **101**:32–6.
- Nguyen VT, Hall LL, Gallacher G *et al.* Choline acetyltransferase, acetylcholinesterase, and nicotinic acetylcholine receptors of human gingival and esophageal epithelia. *J Dent Res* 2000; **79**:939–49.
- Kurzen H, Berger H, Jager C *et al.* Alpha 9 acetylcholine receptors are essential for epidermal differentiation. *Exp Dermatol* 2005; **14**:155.
- Lindstrom J, Anand R, Gerzanich V *et al.* Structure and function of neuronal nicotinic acetylcholine receptors. *Prog Brain Res* 1996; **109**:125–37.
- Elgoyhen AB, Vetter DE, Katz E *et al.* alpha10: a determinant of nicotinic cholinergic receptor function in mammalian vestibular and cochlear mechanosensory hair cells. *Proc Natl Acad Sci USA* 2001; **98**:3501–6.
- Khiroug SS, Harkness PC, Lamb PW *et al.* Rat nicotinic ACh receptor alpha7 and beta2 subunits co-assemble to form functional heteromeric nicotinic receptor channels. *J Physiol* 2002; **540**:425–34.
- Lindstrom JM. Nicotinic acetylcholine receptors of muscles and nerves: comparison of their structures, functional roles, and vulnerability to pathology. *Ann NY Acad Sci* 2003; **998**:41–52.
- Spergel JM. Atopic march: link to upper airways. *Curr Opin Allergy Clin Immunol* 2005; **5**:17–21.
- Cookson W. The immunogenetics of asthma and eczema: a new focus on the epithelium. *Nat Rev Immunol* 2004; **4**:978–88.
- Wessler I, Reinheimer T, Kilbinger H *et al.* Increased acetylcholine levels in skin biopsies of patients with atopic dermatitis. *Life Sci* 2003; **72**:2169–72.
- Vogelsang M, Heyer G, Hornstein OP. Acetylcholine induces different cutaneous sensations in atopic and non-atopic subjects. *Acta Derm Venereol (Stockh)* 1995; **75**:434–6.
- Heyer G, Vogelsgang M, Hornstein OP. Acetylcholine is an inducer of itching in patients with atopic eczema. *J Dermatol* 1997; **24**:621–5.
- Heyer GR, Hornstein OP. Recent studies of cutaneous nociception in atopic and non-atopic subjects. *J Dermatol* 1999; **26**:77–86.
- Hanifin J, Rajka G. Diagnosis features of atopic dermatitis. *Acta Derm Venereol (Stockh)* 1980; **92** (Suppl.):44–7.
- Consensus Report of the European Task Force on Atopic Dermatitis. Severity scoring of atopic dermatitis: the SCORAD index. *Dermatology* 1993; **186**:23–31.

- 29 Wüthrich B, Schmid-Grendelmeier P. Intrinsic vs. extrinsic atopic dermatitis. In: *Atopic Dermatitis* (Bieber T, Leung DYM, eds). New York: Marcel Dekker, Inc., 2002; 1–20.
- 30 Wüthrich B, Schmid-Grendelmeier P. The atopic eczema/dermatitis syndrome. Epidemiology, natural course, and immunology of the IgE-associated ('extrinsic') and the nonallergic ('intrinsic') AEDS. *J Invest Allergol Clin Immunol* 2003; **13**:1–5.
- 31 Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2^{-(delta delta C(T))} method. *Methods* 2001; **25**:402–8.
- 32 Lips KS, Pfeil U, Kummer W. Coexpression of alpha 9 and alpha 10 nicotinic acetylcholine receptors in rat dorsal root ganglion neurons. *Neuroscience* 2002; **115**:1–5.
- 33 Arredondo J, Nguyen VT, Chernyavsky AI *et al.* Central role of alpha7 nicotinic receptor in differentiation of the stratified squamous epithelium. *J Cell Biol* 2002; **159**:325–36.
- 34 Grando SA. Receptor-mediated action of nicotine in human skin. *Int J Dermatol* 2001; **40**:691–3.
- 35 Pfeil U, Haberberger RV, Lips KS *et al.* Expression of the high-affinity choline transporter CHT1 in epithelia. *Life Sci* 2003; **72**:2087–90.
- 36 Haberberger RV, Pfeil U, Lips KS *et al.* Expression of the high-affinity choline transporter, CHT1, in the neuronal and non-neuronal cholinergic system of human and rat skin. *J Invest Dermatol* 2002; **119**:943–8.
- 37 Proksch E, Holleran WM, Menon GK *et al.* Barrier function regulates epidermal lipid and DNA synthesis. *Br J Dermatol* 1993; **128**:473–82.
- 38 Madison KC. Barrier function of the skin: 'la raison d'être' of the epidermis. *J Invest Dermatol* 2003; **121**:231–41.
- 39 Gotti C, Clementi F. Neuronal nicotinic receptors: from structure to pathology. *Prog Neurobiol* 2004; **74**:363–96.
- 40 Burkhart CG, Burkhart HR. Contact irritant dermatitis and anti-pruritic agents: the need to address the itch. *J Drugs Dermatol* 2003; **2**:143–6.
- 41 Kurzen H, Henrich C, Booken D *et al.* Functional characterization of the epidermal cholinergic system in vitro. *J Invest Dermatol* 2006; **126**:2458–72.
- 42 Verbitsky M, Rothlin CV, Katz E *et al.* Mixed nicotinic-muscarinic properties of the alpha9 nicotinic cholinergic receptor. *Neuropharmacology* 2000; **39**:2515–24.
- 43 Xu W, Gelber S, Orr-Urtreger A *et al.* Megacystis, mydriasis, and ion channel defect in mice lacking the alpha3 neuronal nicotinic acetylcholine receptor. *Proc Natl Acad Sci USA* 1999; **96**:5746–51.
- 44 Hagforsen E, Edvinsson M, Nordlind K *et al.* Expression of nicotinic receptors in the skin of patients with palmoplantar pustulosis. *Br J Dermatol* 2002; **146**:383–91.
- 45 Tsai M, Grimaldeston MA, Yu M *et al.* Using mast cell knock-in mice to analyze the roles of mast cells in allergic responses in vivo. *Chem Immunol Allergy* 2005; **87**:179–97.
- 46 Novak N, Kwiek B, Bieber T. The mode of topical immunomodulators in the immunological network of atopic dermatitis. *Clin Exp Dermatol* 2005; **30**:160–4.
- 47 D'Amato G, Liccardi G, Noschese P *et al.* Anti-IgE monoclonal antibody (omalizumab) in the treatment of atopic asthma and allergic respiratory diseases. *Curr Drug Targets Inflamm Allergy* 2004; **3**:227–9.
- 48 Spanos C, el-Mansoury M, Letourneau R *et al.* Carbachol-induced bladder mast cell activation: augmentation by estradiol and implications for interstitial cystitis. *Urology* 1996; **48**:809–16.
- 49 Patella V, de Crescenzo G, Ciccirelli A *et al.* Human heart mast cells: a definitive case of mast cell heterogeneity. *Int Arch Allergy Immunol* 1995; **106**:386–93.
- 50 Peng X, Gerzanich V, Anand R *et al.* Nicotine-induced increase in neuronal nicotinic receptors results from a decrease in the rate of receptor turnover. *Mol Pharmacol* 1994; **46**:523–30.
- 51 Wang F, Nelson ME, Kuryatov A *et al.* Chronic nicotine treatment up-regulates human alpha3 beta2 but not alpha3 beta4 acetylcholine receptors stably transfected in human embryonic kidney cells. *J Biol Chem* 1998; **273**:28721–32.
- 52 Ficklin MB, Zhao S, Feng G. Ubiquitin-1 regulates nicotine-induced up-regulation of neuronal nicotinic acetylcholine receptors. *J Biol Chem* 2005; **280**:34088–95.

Supplementary material

The following supplementary material is available online for this article:

Table S1 Nicotinic acetylcholine receptor $\alpha 3$, mRNA expression levels, relative expression

Table S2 Nicotinic acetylcholine receptor $\alpha 5$, mRNA expression levels, relative expression

Table S3 Nicotinic acetylcholine receptor $\alpha 7$, mRNA expression levels, relative expression

Table S4 Nicotinic acetylcholine receptor $\alpha 9$, mRNA expression levels, relative expression

Table S5 Nicotinic acetylcholine receptor $\alpha 10$, mRNA expression levels, relative expression

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Supplementary material

The expression of nicotinic acetylcholine receptor subunit mRNA in the lesional and nonlesional skin of patients with atopic dermatitis (P) and in the skin of healthy controls (C). The relative nAChR subunit expression in the lesional skin compared with nonlesional skin was determined by calculating $2^{(50 - \Delta CT_{\text{lesional area}}) - (50 - \Delta CT_{\text{nonlesional area}})}$. The ΔCT values were subtracted from 50, resulting in higher values in samples with higher expression.

Table S1 Nicotinic acetylcholine receptor $\alpha 3$, mRNA expression levels, relative expression

Patient	Lesional	Nonlesional	Relative expression	Control	
P2	32.8	32.8	1.00	C1	41.4
P5	35.4	33.1	4.92	C2	37.2
P6	34.4	34.8	0.76	C3	33.5
P7	36.3	36.5	0.87	C4	34.6
P8	36.1	34.9	2.30	C7	39.1
P9	35.9	35.4	1.41	C12	35.9
P10	32.8	32.1	1.62	C14	32.9
P11	34.4	34.3	1.07	C20	33.8
P12	34.8	32.9	3.73	C21	38.2
P20	32.7	30.7	4.00	C30	41.0
P21	33.7	33.7	1.00	C32	37.2
P30	32.0	32.6	0.66		
P31	33.1	33.8	0.62		
P32	33.4	31.8	3.03		
P33	31.9	31.9	1.00		
P35	32.5	31.3	2.30		

Table S2 Nicotinic acetylcholine receptor $\alpha 5$, mRNA expression levels, relative expression

Patient	Lesional	Nonlesional	Relative expression	Control	
P2	32.1	31.7	1.3	C2	33.7
P5	31.1	31.7	0.7	C4	31.4
P6	32.1	32.2	0.9	C7	32.9
P8	35.1	37.3	0.2	C12	34.6
P9	33.2	31.9	2.5	C14	31.6
P10	32.8	31.8	2.0	C20	31.9

P11	33.4	33.1	1.2	C21	32.1
P12	31.7	30.8	1.9	C30	33.6
P20	31.2	30.6	1.5	C32	33.7
P21	32.2	32.2	1.0		
P30	31.3	32.1	0.6		
P31	31.8	32.7	0.5		
P32	32.1	32.0	1.1		
P35	32.1	30.7	2.6		

Table S3 Nicotinic acetylcholine receptor $\alpha 7$, mRNA expression levels, relative expression

Patient	Lesional	Nonlesional	Relative expression	Control	
P2	32.3	33.9	0.3	C1	40.4
P5	34.7	32.5	4.6	C2	37.0
P6	32.5	33.7	0.4	C3	35.2
P7	34.9	35.7	0.6	C4	33.3
P8	35.0	36.2	0.4	C7	39.7
P9	35.0	33.6	2.6	C12	31.6
P10	34.7	34.2	1.4	C14	35.0
P11	34.8	34.0	1.7	C20	35.3
P12	33.0	33.8	0.6	C21	38.9
P20	33.1	34.6	0.4	C30	41.3
P21	34.9	34.9	1.0	C32	37.0
P30	32.4	34.2	0.3		
P31	34.1	34.4	0.8		
P32	34.3	34.9	0.7		
P33	31.9	32.8	0.5		
P35	34.1	35.2	0.5		

Table S4 Nicotinic acetylcholine receptor $\alpha 9$, mRNA expression levels, relative expression

Patient	Lesional	Nonlesional	Relative expression	Control	
P2	32.0	28.5	11.3	C1	40.0
P5	34.5	33.0	2.8	C2	36.4
P8	35.9	35.1	1.7	C3	34.0
P9	34.2	32.6	3.0	C7	39.6

P10	35.7	32.1	12.1	C12	35.9
P11	34.4	33.1	2.5	C14	30.3
P12	33.1	33.4	0.8	C20	33.5
P20	30.2	31.5	0.4	C21	38.6
P21	31.6	31.6	1.0	C30	41.1
P30	31.7	32.6	0.5	C32	36.4
P31	32.5	32.5	1.0		
P32	29.4	29.2	1.1		
P33	29.6	31.3	0.3		

Table S5 Nicotinic acetylcholine receptor $\alpha 10$, mRNA expression levels, relative expression

Patient	Lesional	Nonlesional	Relative expression	Control	
P2	32.4	32.4	1.00	C1	40.2
P5	33.9	34.6	0.62	C2	35.5
P6	33.6	32.8	1.74	C3	34.9
P7	31.4	33.1	0.31	C4	35.7
P8	33.5	34.8	0.41	C7	32.4
P9	35.0	34.8	1.15	C2	33.4
P10	30.5	31.4	0.54	C14	37.1
P11	32.8	33.8	0.50	C20	32.6
P12	31.9	33.0	0.47	C21	35
P20	32.5	32.2	1.23	C30	36.9
P21	32.0	33.5	0.35	C32	31.3
P30	30.1	32.8	0.15		
P31	30.9	32.9	0.25		
P32	31.0	32.1	0.47		
P33	30.7	31.3	0.66		
P35	30.2	31.0	0.57		