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Association of neuropeptides with Th1/Th2 balance and allergic sensitization in children

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Clinical and Experimental Allergy

Summary

Background Among neurogenic factors, the neuropeptides have an important regulatory influence on immune system activity and may lead to allergic sensitization.

Objective The aim of our study was to investigate the relationship of the neuropeptides vasoactive intestinal peptide (VIP), somatostatin (SOM) and substance P (SP) on modulation of Th1/Th2 balance and allergic sensitization in children.

Methods Within the LISApplus (Life style–Immune system–Allergy) study, blood samples of 321 six-year-old children were analysed for concentration of neuropeptides, Th1 and Th2 cytokines, transcription factors for T cell regulation and suppressors of cytokine signalling. In addition, samples were screened for specific IgE against inhalant and food allergens.

Results Children with high SOM values showed a Th2 polarization and a reduced expression of FOXP3, the marker for regulatory T cells. High (VIP) levels correlated inversely with the expression of T cell transcription factors (Tbet and SOCS3). In contrast, elevated levels of SP were associated with reduced GATA3 and SOCS3 expression and with increased IFN- γ concentrations. Allergic sensitization was more prevalent in children with higher SOM and VIP concentrations but not associated with SP levels.

Conclusion Our data reveal an association between neuropeptides and modulatory effects on immune cells *in vivo*, especially on Th1/Th2 balance with a correlation to allergic sensitization in children. We suggest that elevated SOM and VIP concentrations and the inducing factors should be considered as allergy risk factors.

Keywords allergic sensitization, children, neuropeptides, somatostatin, substance P, Th1/Th2 balance, vasoactive intestinal peptide

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Introduction

Increasing evidence suggests that mediators of the nervous system play a role in the regulation of immune system function and may influence the development of immune system-related diseases. The neuro-immune axis is a bidirectional pathway of intersystem communication: immune responses alter neural function and, in turn, neural activity modifies immunologic function. It is clear that exchange of information is facilitated by the release of neuromediators and cytokines by either of these systems and by the shared expression of reciprocal receptors for these mediators. Besides the well-known neurogenic factors, glucocorticoids and catecholamines, which can modulate immune responses, neuropeptides are also discussed for their immunological effects. As an example, T lymphocytes and dendritic cells express receptors for the neuropeptides substance P (SP), somatostatin (SOM) and vasoactive intestinal peptide (VIP).

Several *in vitro* studies and, moreover, *in vivo* mouse models were able to demonstrate the immunomodulatory effects of these neuropeptides. In general, an immunosuppressive, anti-inflammatory mode of action is reported for VIP [1]. Thus, VIP is a suppressive neuropeptide for T cell proliferation and for the production of IL-2 and IFN- γ , acting as an anti-inflammatory mediator [2, 3]. Similarly, SOM is also inhibitory for T cell proliferation and has an anti-inflammatory capacity inhibiting IFN- γ production from human and murine T cells [4]. In contrast, SP has an immunostimulatory function with direct effects on T cell activation [5, 6].

In particular, the risk for the development of an allergic hyperreactivity depends directly on the differentiation of functional active T cell subpopulations. Thus, it can be speculated that the presence of neuropeptides in the microenvironment of differentiating T cells might have an influence on allergy risk. The aim of the present study was to investigate whether the concentration of neuropeptides in the serum of healthy children is associated with Th1/Th2 balance and allergic sensitizations. We analysed the concentration of the neuropeptides SOM, VIP and SP in whole-blood samples of 6-year-old children and correlated the neuropeptide levels in blood with specific immuneparameters of relevance for Th1/Th2 regulation: IFN- γ as an indicator for Th1 reactivity and IL-4, IL-5 and IL-9 as indicators for Th2 reactivity. In addition, the expression of the T cell transcription factors Tbet (expressed in Th1 cells) GATA3 (expressed in Th2 cells), and FOXP3 (expressed in regulatory T cells), and the suppressors of cytokine signalling (SOCS) SOCS1 and SOCS3 were analysed from mRNA samples by quantitative polymerase chain reaction (PCR).

Methods

Study design and subjects

Whole-blood samples were drawn from 6-year-old children from the LISApplus (Life style Immune System Allergy) study. This study was designed to investigate the influence of life style and environmental factors on the maturation of the immune system and the atopic risk in early childhood. Three thousand ninety-seven newborns, born between December 1997 and January 1999 in the four German cities Munich, Leipzig, Wesel and Bad Honnef, were recruited for the LISA study. Only healthy-term neonates of German descent were included. Newborn children whose mothers suffered from autoimmune disease or infectious disorders during pregnancy were excluded. The design of LISA has been described in detail elsewhere [7].

At the age of 6, a clinical investigation was performed in a children's hospital. Blood samples were taken for the determination of specific IgE concentrations, and in the subcohort from the city of Leipzig for further determinations of immunological parameters and neuropeptides. For the present analysis, we used only data from the city of Leipzig cohort, as the measurement of neuropeptides and T cell regulatory factors was performed only in this subgroup. Five hundred sixty-five 6-year-old children from the city of Leipzig participated in the study. For 324 of these children, parents gave consent for with blood collection.

Participation in the study was voluntary and informed consent was obtained from the parents of all children. The study was approved by the Ethics Committees of the University of Leipzig.

Blood Sampling

Heparinized blood (7 mL) was obtained by venipuncture and prepared within 4 h for further analysis. For neuropeptide ELISA, 500 μ L of whole blood was diluted with 500 μ L of RPMI-1640 medium without supplements and centrifugated at 800 *g* for 5 min at 4 °C. Collected cell-free supernatants were stored at –80 °C until analysis. For cytokine measurement, blood samples (1 mL heparinized blood) were diluted 1 : 1 with RPMI-1640 medium without supplements and incubated for 4 h at 37 °C to equilibrate. After centrifugation, the supernatant was collected and stored at –80 °C until analysis. Blood samples for RNA extraction (1 mL heparinized blood) were prepared without stimulation according to a standardized protocol until further handling [8].

RNA extraction and cDNA synthesis

RNA extraction was performed with an automated standardized protocol for human whole blood using the MagNA

Pure LC system and the MagNA Pure LC mRNA Isolation Kit I (Roche Applied Science, Mannheim, Germany).

Blood samples (1 mL) were diluted 1 : 1 with cell culture medium (RPMI-1640 supplemented with 10% heat-inactivated foetal bovine serum, 0.01% Penicillin, 0.0085% Streptomycin, 2 mM L-glutamine, 0.35% β -mercaptoethanol) and left to equilibrate for 3 h at 37 °C and 5% CO₂. Lysis of erythrocytes was performed twice with hypotonic buffer (0.83% NH₄Cl, 0.1% KHCO₃, 0.1 mM ethylenediaminetetraacetic acid, pH 7.2). Pelleted cells were lysed with the guanidinium thiocyanate lysis buffer from the isolation kit containing additional 1% dithiothreitol and stored at – 80 °C.

cDNA was synthesized using the First-Strand cDNA Synthesis Kit (Roche Applied Science).

Real-time polymerase chain reaction

Reverse transcriptase products were amplified by real-time PCR with a LightCycler Fast Start DNA SYBR Green kit according to the manufacturer's instructions (Roche Applied Science). Primers and standards for GATA3, Tbet, FOXP3, SOCS1, SOCS3 and β -actin and the amplification protocol were obtained from Search-LC (Heidelberg, Germany). Primers for IL-4 were synthesized by MWG Biotech (Ebersberg, Germany). The primer sequences for IL-4 were sense 5'-AGA AGA CTC TGT CAC CGA TTGA-3' and antisense 5'-CTC TCA TGA TCG TCT TTA GCC TTT-3'. IL-4 standard was synthesized by GenExpress (Berlin, Germany). The amplification protocol used for IL-4 primers was: 5 °C, 1 s; 68 °C, 10 s; 72 °C, 16 s, with a lowered annealing temperature of 0.5 °C per cycle until reaching the final annealing temperature of 58 °C at cycle 17. Quantification of transcript concentration for the measured genes was calculated by external standardization. Data are presented as the ratio between the copy number of target gene and copy number of the housekeeping gene β -actin.

Neuropeptide ELISA

The ELISA kits for VIP, SOM, and SP were purchased from Phoenix Europe (Karlsruhe, Germany) and are based on the principle of competitive enzyme immunoassay. All measurements were performed in duplicate. The concentration of neuropeptides has been determined with the *EasyWin* kinetics 32 (TECAN, Crailsheim, Germany) software by extrapolation to the standard curve. To correct interassay variation, a control sample has been included on each plate. Neuropeptide concentrations were normalized by multiplication with the calculated factor for the control sample.

The detection limit for each neuropeptide was 7 pg/mL.

Cytometric bead array

Concentrations of the cytokines IFN- γ , IL-4, IL-5 and IL-9 were measured by flow cytometry using the BD CBA

Human Soluble Flex Set system (Becton Dickinson, Heidelberg, Germany) according to the manufacturer's instructions.

In brief, cytokine-specific antibody-coated beads were incubated for 1 h with 25 μ L of blood samples or standard solution. Thereafter, samples were incubated with the corresponding PE-labelled detection antibodies for 2 h. After one washing step, samples were measured by flow cytometry. Analysis of data and quantification of cytokines were performed using FCAP ArrayTM software (Becton Dickinson) on the basis of corresponding standard curves. Finally, the plasma dilution factor was determined.

The detection limits were 4 pg/mL for IFN- γ and IL-9 and 3 pg/mL for IL-5 and IL-4, respectively.

Specific IgE

Specific IgE was determined by the Pharmacia CAP System (Pharmacia Diagnostics, Freiburg, Germany). To establish atopic status, sera were screened for specific IgE against inhalant (SX1) and food allergens (Fx5) by two multiallergen panels. SX1 (CAP FEIA) includes house dust mite (*Dermatophagoides pteronyssinus*), cat, dog, timothy, rye, birch, mugwort and *Cladosporium herbarum*; Fx5 CAP FEIA contains the food allergens hen's egg, cow's milk, fish, wheat, peanut and soy. Samples with specific IgE concentrations >0.35 kU/L were regarded as positive. Sera with positive results in the multiallergen panels were tested for the single allergens.

Statistical analysis

Owing to the fact that the functional relationship between neuropeptide levels, T cell regulatory factors and allergic sensitization is not yet clear, neuropeptide concentrations were categorized by using quartiles (Q1, Q2, Q3, Q4) as cut-off values and included in the models with the lowest quartile as the reference. Data for T cell regulatory factors are presented as median with 25th–75th percentiles. Cytokine and neuropeptide concentrations below the limit of detection were assigned a value of one half of the detection limit and were taken into account for calculation. Trend tests were applied to detect dose-response relations. The Jonckheere–Terpstra test was used for trend calculation for the association between neuropeptides and T cell regulatory factors. Multiple logistic regression models were used to determine adjusted OR with 95% CI for the association between neuropeptide concentrations and allergic sensitization. OR were adjusted for family atopy history (FAH), gender, social economic status, pet keeping, smoking during pregnancy, breastfeeding and season of birth. The Cochran–Armitage test was used for trend calculation for the association between neuropeptides and allergic sensitization. Correlation between T cell regulatory factors (cytokines and transcription factors)

and specific IgE was analysed using the non-parametric Mann–Whitney Rank sum test. A Bonferroni correction, for multiple testing has been performed for all results. After this correction the significant *P*-value for the association of neuropeptides and T cell regulatory factors changed from 0.05 to *P* < 0.005 and from 0.05 to *P* < 0.004 for neuropeptides and allergic sensitization. Statistical analysis was performed with STATISTICA for Windows Version 7.1 (Statsoft Inc.) and StatXact version 6.2 (Cytel Inc., Cambridge, MA, USA).

Results

Characteristics of study subjects

From the initial 565 six-year-old participants from Leipzig, 324 blood samples were obtained. In cases where no sufficient amount of blood was available, serum for IgE measurements was collected with the highest priority, followed by heparinized blood for neuropeptide analysis, cytometric bead array and mRNA preparation. Therefore, reduced case numbers resulted for some parameters. Complete data for IgE, neuropeptides and cytokines were available from 321 children [154 girls (47.9%) and 167 boys (52.0%)]. The subsample of children with mRNA measurements was smaller (*n* = 277) because the amount of blood available was not sufficient or parents did not give consent for genetic (DNA/RNA) analysis.

The plasma concentration of neuropeptides ranged from 3.5 to 233.6 pg/mL for SOM, from 3.5 to 561.2 pg/mL for VIP and from 3.5 to 1274.2 pg/mL for SP. Measured cytokine concentrations varied between 2 and 152.4 pg/mL IFN- γ , 1.5 and 38.2 pg/mL IL-4, 1.5 and 4.3 pg/mL IL-5 and 2 and 102.2 pg/mL IL-9.

Neuropeptides and T cell regulation

In Tables 1–3, the relations between SOM, VIP and SP and T cell regulatory factors are shown. Trend tests were

applied to detect dose–response relations. A positive association was found between high SOM values and GATA3, IL-4 (protein) and SOCS1 expression (*P* for trend: 0.0008, 0.001 and 0.003, respectively, Table 1). In contrast, IFN- γ correlated negatively with SOM (*P* for trend: 0.001, Table 1). After Bonferroni correction, only a tendency for a negative correlation between FOXP3 and SOM has been observed (*P* for trend: 0.007, Table 1). No correlation has been found between SOM and transcription factor Tbet and the cytokine IL-9 (Table 1). VIP was negatively correlated with SOCS3 mRNA expression (*P* for trend: 0.003, Table 2) and showed a tendency for a negative correlation to the Th1 transcription factor Tbet. Although not significant, IL-4 and GATA mRNA expression increased with higher VIP concentrations, whereas FOXP3 tended towards a reversed relationship (Table 2). SP revealed a positive relationship with IFN- γ concentration (*P* for trend: 0.0005) and a negative relationship with the Th2 transcription factor GATA3 and SOCS3 expression (*P* for trend: 0.0009 and 0.001, respectively, Table 3).

Neuropeptides and allergic sensitization

Among the 321 investigated children, 92 (28.7%) reacted against inhalant (SX1) and 41 (12.8%) against food (Fx5) allergens. The results of the multiple regression analysis for the association between neuropeptides and allergic sensitization are presented in Tables 4 (SOM) and 5 (VIP). Owing to low numbers of positive cases, associations between neuropeptide levels and sensitization to the single food allergens fish (1.5%), wheat (3.1%) and soy (2.8%) and the inhalative allergen *Cladosporium herbarum* (1.9%), could not be calculated. Thus, these allergens are not included in the presentation of the results.

SP concentrations were not associated with any allergic sensitization (data not shown). In contrast, there was a positive relationship between SOM and VIP levels and

Table 1. Associations between SOM concentrations and T cell regulatory factors

	1st quartile (3.50–21.50 pg/mL)	2nd quartile (21.50–30.30 pg/mL)	3rd quartile (30.30–42.80 pg/mL)	4th quartile (42.80–233.60 pg/mL)	<i>P</i> *
IL-4 mRNA	5.99 (2.52–18.47)	9.97 (5.18–34.97)	8.36 (4.79–18.76)	8.54 (4.55–20.85)	0.06
IL-4 protein	1.50 (1.50–7.40)	6.30 (1.50–9.20)	6.64 (1.50–11.8)	6.30 (1.50–9.40)	0.001
IL-5 protein	1.50 (1.50–2.08)	1.50 (1.50–2.36)	2.08 (1.50–2.50)	2.00 (1.50–2.34)	0.02
IL-9 protein	6.74 (2.00–14.28)	11.22 (2.00–17.2)	9.60 (2.00–20.14)	8.74 (2.00–15.76)	0.100
IFN- γ protein	8.58 (2.00–15.18)	9.38 (2.00–13.66)	6.72 (2.00–14.41)	2.00 (2.00–12.86)	0.001
GATA3 mRNA	4.12 (2.36–9.98)	5.10 (3.33–9.90)	6.07 (3.83–10.58)	7.12 (4.07–16.44)	0.0008
Tbet mRNA	0.21 (0.06–0.46)	0.17 (0.04–0.38)	0.20 (0.10–0.35)	0.16 (0.08–0.29)	0.220
FOXP3 mRNA	0.51 (0.24–1.11)	0.58 (0.32–1.13)	0.52 (0.29–0.85)	0.37 (0.13–0.67)	0.007
SOCS1 mRNA	4.00 (2.13–13.37)	5.31 (2.72–17.75)	5.62 (3.49–8.97)	6.00 (3.90–17.28)	0.003
SOCS3 mRNA	2.77 (1.81–4.78)	3.37 (2.15–5.67)	3.94 (3.04–6.13)	2.97 (0.81–4.06)	0.224

Medians for SOM quartiles (25th – 75th percentile) and *P* for trend (*P**) are shown. Significant *P*-values after Bonferroni's correction are highlighted. SOM, somatostatin.

Table 2. Associations between VIP concentrations and T cell regulatory factors

	1st quartile (3.50–141.20 pg/mL)	2nd quartile (141.20–196.7 pg/mL)	3rd quartile (197.7–249.2 pg/mL)	4th quartile (249.2–561.2 pg/mL)	<i>P</i> *
IL-4 mRNA	8.33 (3.19–21.54)	7.30 (3.91–15.25)	9.40 (4.11–23.4)	9.57 (5.63–27.13)	0.06
IL-4 protein	5.94 (1.50–10.08)	4.80 (1.50–9.56)	5.85 (1.50–8.45)	5.76 (1.50–9.40)	0.374
IL-5 protein	1.50 (1.50–2.36)	1.50 (1.50–2.34)	1.12 (1.50–2.36)	1.50 (1.50–2.34)	0.463
IL-9 protein	9.60 (2.00–17.22)	9.90 (2.00–15.76)	9.17 (2.00–17.22)	7.80 (2.00–15.76)	0.493
IFN- γ protein	9.55 (2.00–15.68)	5.68 (2.00–10.77)	7.31 (2.00–13.07)	8.54 (2.00–13.88)	0.353
GATA3 mRNA	4.69 (3.36–9.58)	6.55 (3.09–13.32)	6.82 (3.70–12.42)	5.69 (3.30–12.05)	0.09
Tbet mRNA	0.22 (0.10–0.44)	0.21 (0.12–0.34)	0.12 (0–0.29)	0.16 (0.07–0.30)	0.03
FOXP3 mRNA	0.53 (0.25–1.07)	0.52 (0.30–1.04)	0.47 (0.13–0.83)	0.43 (0.16–0.83)	0.06
SOCS1 mRNA	5.00 (3.38–14.85)	4.89 (3.28–9.50)	5.59 (3.31–13.37)	5.96 (2.97–18.92)	0.155
SOCS3 mRNA	4.09 (2.68–6.25)	3.26 (2.21–4.56)	2.68 (0.58–4.54)	3.17 (2.00–4.33)	0.003

Medians for VIP quartiles (25th – 75th percentile) and *P* for trend (*P**) are shown. Significant *P* values after Bonferroni's correction are highlighted. VIP, vasoactive intestinal peptide.

Table 3. Associations between SP concentrations and T cell regulatory factors

	1st quartile (3.50–128.4 pg/mL)	2nd quartile (128.4–193.0 pg/mL)	3rd quartile (193.0–254.7 pg/mL)	4th quartile (254.7–1274.0 pg/mL)	<i>P</i> *
IL-4 mRNA	8.56 (4.03–25.71)	7.90 (3.96–19.9)	7.79 (3.08–16.7)	8.62 (4.47–23.40)	0.353
IL-4 protein	5.94 (1.50–11.46)	5.00 (1.5–7.68)	6.30 (1.50–9.22)	5.80 (1.50–9.48)	0.492
IL-5 protein	2.16 (1.50–2.50)	1.50 (1.50–2.16)	1.50 (1.50–2.34)	1.50 (1.50–2.14)	0.097
IL-9 protein	9.60 (2.00–20.14)	6.82 (2.00–14.28)	9.60 (2.00–15.76)	9.60 (2.00–16.49)	0.331
IFN- γ protein	5.68 (2.00–11.6)	7.32 (2.00–11.66)	7.36 (2.00–14.79)	10.36 (1.00–16.2)	0.0005
GATA3 mRNA	6.71 (4.27–14.42)	7.57 (3.36–13.06)	4.12 (2.93–10.86)	4.64 (3.13–8.78)	0.0009
Tbet mRNA	0.18 (0.11–0.40)	0.16 (0.05–0.40)	0.19 (0.02–0.35)	0.21 (0.08–0.33)	0.220
FOXP3 mRNA	0.51 (0.33–1.07)	0.56 (0.24–0.87)	0.39 (0.21–0.85)	0.48 (0.29–0.98)	0.176
SOCS1 mRNA	5.55 (3.34–10.83)	5.60 (3.85–12.34)	5.07 (2.87–13.81)	5.17 (3.06–14.15)	0.238
SOCS3 mRNA	4.00 (2.69–6.14)	3.24 (0.99–5.38)	3.08 (1.52–5.62)	2.77 (1.88–4.08)	0.001

Medians for SP quartiles (25th – 75th Percentile) and *P* for trend (*P**) are shown. Significant *P*-values after Bonferroni's correction are highlighted. SP, substance P.

sensitizations against the tested allergens. Multiple logistic regression models confirmed that atopic sensitization to the food allergen mix Fx5 (OR_{Q3} 4.71, 95% CI 1.39–15.91 and OR_{Q4} 4.93, 95% CI 1.57–15.47; *P* for trend = 0.001) and the single food allergen cow's milk (OR_{Q3} 11.0, 95% CI 1.25–97.44 and OR_{Q4} 8.81, 95% CI 1.01–76.72; *P* for trend = 0.002) was influenced by SOM concentration, Table 4. Also, for peanut, a higher allergy risk (OR_{Q4} 16.0, 95% CI 1.67–154.5) was observed, but after Bonferroni correction, the calculated trend failed to be significant (*P* for trend = 0.008). Although a significant association for SOM and the inhalative allergen mix was not found, single inhalative allergens correlated with SOM concentrations. The risk for allergic sensitization against timothy-grass-pollen and rye increased in the third and fourth quartile of SOM concentration (timothy: OR_{Q3} 4.58, 95% CI 1.47–14.28 and OR_{Q4} 4.13, 95% CI 1.33–12.85; rye: OR_{Q3} 3.34, 95% CI 1.01–11.09 and OR_{Q4} 4.17, 95% CI 1.35–12.87). Similar to SOM, estimated risks of sensitization to the food allergen mix Fx5 (OR_{Q4} 3.5, 95% CI

1.25–9.82; *P* for trend = 0.004) were influenced by VIP concentration, Table 5. For cow's milk, an increased allergy risk was observed in children with the highest VIP values compared with children with the lowest values (OR_{Q4} 4.0, 95% CI 1.02–15.67). In addition, we found a positive relationship between high VIP concentrations and rye-pollen sensitization (OR_{Q4} 3.22, 95% CI 1.12–9.29). Adjusted ORs differ in all cases only slightly from the calculated crude ORs, indicating that the observed effects were not confounded by FAH, gender, social economic status, cat allergens, smoking during pregnancy, breastfeeding and season of birth.

T cell regulatory factors and allergic sensitization

As expected, T cell regulatory factors supporting a Th2-driven immune response were positively correlated with specific IgE levels. GATA3 mRNA expression correlated with sensitization to the inhalative allergen Derp1 (*P* = 0.03). The expression of IL-4 also correlated positively

Table 4. Frequency [% (n)] and risk (adjusted* OR with 95% CI) of allergic sensitization (specific IgE) according to SOM concentrations

	SOM				P for trend
	1st quartile (3.5–21.5 pg/mL)	2nd quartile (21.5–30.3 pg/mL)	3rd quartile (30.3–42.8 pg/mL)	4th quartile (42.8–233.6 pg/mL)	
Food allergens	6.25% (5)	7.40% (6)	16.45% (13)	20.98% (17)	0.001
	1.00	1.45 (0.39–5.41)	4.71 (1.39–15.91)	4.93 (1.57–15.47)	
Hen's egg	2.50% (2)	1.23% (1)	6.23% (5)	4.39% (4)	0.191
	1.00	0.57 (0.04–8.08)	4.28 (0.67–27.40)	2.74 (0.43–17.34)	
Cow's milk	1.25% (1)	2.46% (2)	10.12% (8)	11.11% (9)	0.002
	1.00	2.39 (0.18–30.78)	11.0 (1.25–97.44)	8.81 (1.01–76.72)	
Peanut	1.25% (1)	1.23% (1)	3.79% (3)	8.64% (7)	0.008
	1.00	1.54 (0.07–34.21)	6.28 (0.54–72.01)	16 (1.67–154.5)	
Inhalative allergens	22.5% (18)	25.9% (2)	35.4% (28)	30.9% (25)	0.127
	1.00	1.30 (0.56–3.00)	2.48 (1.10–5.59)	1.99 (0.90–4.39)	
Derp1	13.75% (11)	16.04% (13)	18.98% (15)	17.28% (14)	0.467
	1.00	1.27 (0.47–3.44)	2.02 (0.77–5.29)	1.48 (0.58–3.75)	
Cat	1.25% (1)	7.40% (6)	7.59% (6)	8.64% (7)	0.064
	1.00	7.29 (0.79–66.62)	6.75 (0.67–67.56)	8.49 (0.93–77.24)	
Dog	1.25% (1)	4.93% (4)	3.79% (3)	6.17% (5)	0.166
	1.00	3.04 (0.29–31.50)	4.91 (0.41–58.35)	5.53 (0.59–51.29)	
Timothy	6.25% (5)	11.11% (9)	21.51% (17)	18.51% (15)	0.007
	1.00	1.73 (0.50–6.11)	4.58 (1.47–14.28)	4.13 (1.33–12.85)	
Rye	6.25% (5)	9.87% (8)	13.92% (11)	18.51% (15)	0.012
	1.00	1.68 (0.47–6.03)	3.34 (1.01–11.09)	4.17 (1.35–12.87)	
Birch	5.00% (4)	11.11% (9)	15.18% (12)	12.34% (10)	0.095
	1.00	2.59 (0.71–9.46)	3.85 (1.07–13.82)	3.25 (0.90–11.69)	
Mugwort	6.25% (5)	3.70% (3)	5.06% (4)	11.11% (9)	0.192
	1.00	0.58 (0.12–2.73)	1.04 (0.24–4.40)	2.52 (0.75–8.49)	

Significant *P*-values after Bonferroni's correction are highlighted.

*Adjusted for family atopy history, gender, social economic status, pet keeping, smoking during pregnancy, breastfeeding and season of birth.

SOM, somatostatin; OR, odds ratio; CI, confidence interval.

with sensitization to Derp1 as well as with sensitization to cow's milk allergen ($P=0.02$ and 0.04 , respectively). Furthermore, children with sensitizations to food or inhalative allergens had higher SOCS1 mRNA levels ($P=0.03$ for food allergen mix and $P=0.03$ for inhalative allergen mix). In contrast, lower IFN- γ levels were observed in children sensitized to food allergen mix ($P=0.01$) and lower Tbet mRNA levels in children sensitized to inhalative allergen mix ($P=0.03$).

Discussion

The present study provides the first epidemiologic survey demonstrating an association of neuropeptides SOM, VIP and SP with immune system regulation and allergic sensitization in children. We were able to show that individuals with high levels of SOM revealed a higher expression of the Th2 transcription factor GATA3. The influence of GATA3 for Th2 differentiation and allergic sensitization is well documented in a number of *in vitro* and *in vivo* studies [9]. It was shown that up-regulation of GATA3 expression in T cells is a hallmark of the early phase of Th2 recall responses to specific antigens in

atopic individuals [10]. This process is accompanied by release of type 2 cytokines like IL-4 and IL-5 in allergen-specific immune responses in atopics. Thus, factors like SOM, which may up-regulate GATA3 expression, might alter the Th1/Th2 balance and lead to allergic sensitization in healthy individuals as well. Indeed, in our study, subjects with high levels of GATA3 were characterized by high IL-4 production (Spearman's correlation: $P < 0.001$, data not shown) and sensitization against inhalant allergens. Moreover, a direct correlation between increased SOM and IL-4 and a tendency towards increased IL-5 levels was observed. These data confirm results from earlier *in vitro* studies referring to the induction of a Th2-like pattern (IL-4, IL-10) of cytokine secretion by SOM [11]. Furthermore, we detected a reduced IFN- γ production in children with high SOM levels and with sensitization to food allergens. Reduced IFN- γ is discussed as a further risk factor for the development of IgE-mediated disorders in childhood [12, 13]. Th1-suppressive capacity of SOM has also been demonstrated in mouse studies where the authors could show that SOM acts directly on T cells to inhibit T cell receptor (TCR)-stimulated IFN- γ release [14].

Table 5. Frequency [% (*n*)] and risk (adjusted* OR with 95% CI) of allergic sensitization (specific IgE) according to VIP concentrations

	VIP				<i>P</i> for trend
	1st quartile (3.5–141.2 pg/mL)	2nd quartile (141.2–196.7 pg/mL)	3rd quartile (197.7–249.2 pg/mL)	4th quartile (249.2–561.2 pg/mL)	
Food allergens	7.40% (6)	8.86% (7)	12.5% (10)	22.22% (18)	0.0038
	1.00	1.34 (0.40–4.48)	1.48 (0.48–4.55)	3.50 (1.25–9.82)	
Hen's egg	3.70% (3)	2.53% (2)	1.25% (1)	7.40% (6)	0.297
	1.00	0.62 (0.09–4.30)	0.15 (0.01–2.03)	2.17 (0.46–10.25)	
Cow's milk	3.70% (3)	3.79% (3)	3.75% (3)	13.58% (11)	0.014
	1.00	1.19 (0.21–6.60)	0.88 (0.16–4.90)	4.00 (1.02–15.67)	
Peanut	1.23% (1)	3.79% (3)	6.25% (5)	3.70% (3)	0.297
	1.00	3.11 (0.29–32.74)	4.67 (0.48–45.11)	2.94 (0.28–31.01)	
Inhalative allergens	30.86% (25)	20.25% (16)	30.00% (24)	33.33% (27)	0.451
	1.00	0.61 (0.27–1.35)	0.91 (0.42–1.97)	1.07 (0.52–2.21)	
Derp1	18.51% (15)	13.92% (11)	16.25% (13)	17.28% (14)	0.937
	1.00	0.81 (0.32–2.05)	0.81 (0.33–1.98)	0.84 (0.36–1.96)	
Cat	8.64% (7)	2.53% (2)	5.00% (4)	8.64% (7)	0.842
	1.00	0.37 (0.06–2.01)	0.75 (0.19–2.94)	1.09 (0.33–3.53)	
Dog	4.93% (4)	2.53% (2)	3.75% (3)	4.93% (4)	0.903
	1.00	0.65 (0.10–4.15)	1.16 (0.21–6.16)	0.92 (0.20–4.03)	
Timothy	12.34% (10)	8.86% (7)	13.75% (11)	22.22% (18)	0.048
	1.00	0.82 (0.28–2.42)	1.41 (0.52–3.83)	2.32 (0.92–5.85)	
Rye	7.40% (6)	7.59% (6)	13.75% (11)	19.75% (16)	0.008
	1.00	1.12 (0.33–3.81)	2.23 (0.73–6.85)	3.22 (1.12–9.29)	
Birch	7.40% (6)	8.86% (7)	13.75% (11)	13.58% (11)	0.132
	1.00	1.14 (0.35–3.73)	1.62 (0.53–4.95)	1.95 (0.63–5.98)	
Mugwort	6.17% (5)	7.59% (6)	3.75% (3)	8.64% (7)	0.768
	1.00	1.38 (0.38–4.96)	0.57 (0.12–2.72)	1.44 (0.41–5.05)	

Significant *P*-values after Bonferroni's correction are highlighted.

*Adjusted for family atopy history, gender, social economic status, pet keeping, smoking during pregnancy, breastfeeding and season of birth.

VIP, vasoactive intestinal peptide; OR, odds ratio; CI, confidence interval.

Several recent studies indicate that besides Th2 cells, one other group of effector cells, the regulatory T cells, plays a pivotal role in the development of allergic diseases [15, 16]. In human studies, a suppressive function of regulatory T cells to allergen-driven T cell activation and development of allergic disease is also evident [17, 18]. Here, we assessed the transcription factor FOXP3 as a marker for regulatory T cells. Our data indicate that for children with high SOM levels, there is a tendency for reduced expression of FOXP3. Although this association is not significant after Bonferroni correction of data ($P = 0.007$), one can speculate that even a weak relationship may lead to a diminished control of Th2 cell activity and, among the other factors, would contribute to the observed allergic sensitization in association with elevated SOM levels.

Among regulatory molecules, which can influence an immune response, the SOCS family are key physiological regulators of the immune response [19–21]. By targeting signalling intermediates for degradation, these molecules attenuate cytokine signal transduction in a classic feedback loop [22]. Thus, signal down-regulation through SOCS members may have an important role in the balance

of cytokines that determine the onset of Th1- and Th2-mediated immune responses, and their altered expression may cause immune system-related diseases. Our data show that increased amounts of SOCS1 mRNA occur in individuals with elevated SOM levels, indicating that expression of this factor can be influenced by SOM. Furthermore, we found that high expression of SOCS1 correlates with high values of IL-4 and GATA3 expression mRNA (Spearman's correlation: $P < 0.001$, data not shown). In mouse models, it has been confirmed that SOCS1 is a crucial inhibitor of IFN- γ *in vivo* [23]. However, we could not find any association of SOCS1 with IFN- γ expression. On the other hand, there is evidence from TCR-transgenic T cell experiments that SOCS1 is mainly expressed in Th1 cells [24] and therefore negatively regulates Th1 differentiation, inducing a Th2 response [25].

In the current study, we also observed that the frequency of food allergen sensitization increases in children with higher SOM concentrations (P for trend = 0.001) and in children with the highest SOM concentrations an increased allergy risk for single inhalative allergens (timothy, rye) was observed (Table 4). Thus, taken together,

our data demonstrate that SOM is associated with the development of a Th2-driven T cell response. As IgE-mediated allergic reactions are based on a Th2-driven T cell response, SOM may also therefore be involved in allergy development. In fact, in our study we were able to demonstrate an association between SOM and allergy risk in healthy individuals.

Similar to SOM, the neuropeptide VIP also revealed a positive relationship for allergic sensitization with food allergens (P for trend = 0.004) and in addition children with high VIP concentrations (fourth quartile) had an increased allergy risk for the single inhalative allergen rye (Table 5). Interestingly, VIP was negatively associated with the suppressor of cytokine signalling member SOCS3. This molecule is selectively expressed in Th2 cells and has the ability to inhibit IL-12-mediated STAT4 activation, thereby blocking Th1 development [24] and is involved in the maintenance of Th2-mediated allergic response [26]. On the other hand, our results point to direction that VIP is associated with low expression of the Th1 transcription factor Tbet and has a tendency to increase IL-4 and GATA3 mRNA expression, which might lead to the allergic risk. Low expressions of Tbet at mRNA level correlated with low levels of IFN- γ in supernatants from unstimulated blood cells and with sensitization to inhalative allergens. One might speculate that inhibition of Tbet by VIP might overcome the 'beneficial' inhibition of SOCS3, leading to an allergic sensitization. Recent findings could show that VIP-generated dendritic cells induce functional regulatory T cells *in vitro* and *in vivo* [27]. These regulatory T cells (CD4 as well as CD8 positive) suppress primarily antigen-specific Th1-mediated responses [28]. Thus, in accordance with our data, the Th1-suppressive capacity of VIP, although it can be beneficial for the treatment of some autoimmune diseases, might be responsible for Th2 bias and in this way may also contribute to the allergic risk.

Somehow the results regarding the effect of VIP and SOM on IgE production *in vitro* are contradictory. Early studies from Kimata et al. [29, 30] showed that spontaneous IgE release from B cells is inhibited by VIP and SOM and not by SP, but only in the presence of T cells and monocytes. However, recent publications from the same group demonstrated that stress enhances allergen-specific IgE, with a concomitant increase of plasma levels of VIP and SP in patients with atopic dermatitis [31, 32]. It has also been reported by others that serum levels of VIP are elevated in patients with atopic dermatitis and may contribute to the pathology of this disease [33, 31]. These findings indicate that the results from *in vitro* studies may not reflect the *in vivo* situation for IgE production.

While SOM and VIP were associated with allergic sensitization, in our study SP had no effect on allergic responses. This might result from secondary effects. We showed that down-regulation of Th2 transcription factor

GATA3 is predominant in individuals with high SP levels. Furthermore, our data show that these individuals are characterized by high IFN- γ levels and by a reduction of SOCS3 expression. These findings support the idea that elevated IFN- γ secretion, as well as the reduced SOCS3 expression, has a protective effect against Th2 development, thereby diminishing the allergy risk. The fact that other studies were able to show that SP evoke pro-inflammatory responses, especially IFN- γ production, and may participate in development of inflammatory diseases, is in accordance with our data [34–36]. But, it is also noteworthy that SP can induce mast cell degranulation and thereby contributes to allergic inflammation [37, 38].

In summary, our data reveal an association between neuropeptides, modulation of Th1/Th2 balance and, for SOM and VIP, also a relation to allergic sensitization in children. As mentioned, some reports could show that neuropeptides are released after stress signals [31, 32]. Thus, future research on the influence of stress regarding the risk of allergic sensitization has to focus on factors influencing the level of neuropeptide expression.

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