The protective effect of farm animal exposure on childhood allergy is modified by *NPSR1* polymorphisms

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ABSTRACT

Background: Little is known about the asthma candidate gene neuropeptide S receptor 1 (*NPSR1*) in relation to environmental exposures, but recent evidences suggest its role as an effect modifier.

Objectives: To explore the interaction between *NPSR1* polymorphisms and environmental exposures related to farming lifestyle and to study the in vitro effects of lipopolysaccharide (LPS) stimulation on NPSR1 expression levels.

Methods: We studied 3113 children from PARSIFAL, a European cross-sectional study on environmental/lifestyle factors and childhood allergy, partly focused on children brought up on a farm. Information on exposures and outcomes was primarily obtained from parental questionnaires. Seven tagging polymorphisms were analysed in a conserved haplotype block of *NPSR1*. Multivariate logistic regression was used to evaluate a multiplicative model of interaction. NPSR1 protein and messenger RNA (mRNA) levels in monocytes were measured after LPS stimulation by fluorescence activated cell sorting (FACS) and quantitative real-time polymerase chain reaction (PCR).

Results: A strong interaction was seen between current regular contact to farm animals and several *NPSR1* polymorphisms, particularly rs323922 and rs324377 ($p<0.005$), with respect to allergic symptoms. Considering the timing of initiation of such current regular farm animal contact, significant interactions with these and two additional polymorphisms (SNP546333, rs740347) were revealed. In response to LPS, NPSR1-A protein levels in monocytes were upregulated ($p = 0.002$), as were *NPSR1-A* mRNA levels ($p = 0.02$).

Conclusions: The effect of farm animal contact on the development of allergic symptoms in children is modified by *NPSR1* genetic background.

Epidemiological studies have shown that children living on farms have a lower prevalence of IgE mediated allergic diseases.[1–5] The protective effects have specifically been correlated to contact with farm animals and farm milk consumption,[5–10] with exposure to endotoxins or moulds as possible molecular explanations.[7 11–13] The timing is important, with prenatal and early life exposures showing particularly strong protective effects.[6 14] Interestingly, several innate immunity related receptors, which directly interact with bacterial components, have been reported to have heterogeneous genetic effects suggestive of gene–environment interactions.[15–17]

The neuropeptide S (NPS) receptor 1 (*NPSR1*; earlier named *GPR154* or *GPRA*) was positionally cloned as an asthma candidate gene in families from Finland and Canada, and has been replicated for both childhood and adult populations of diverse geographic origin.[18–24] Neuropeptide S (*NPS*), the ligand, shows overlapping expression patterns with *NPSR1* in several tissue types, including the epithelium of human colon and bronchi.[25 26] NPSR1 is further expressed by macrophages and stimulation with NPS induces macrophage phagocytosis and chemotaxis.[27] Stimulation of peripheral blood mononuclear cells (PBMC) by lipopolysaccharide (LPS), a potential molecular proxy for farm animal exposure, has been shown to increase mRNA levels of *NPSR1*.[27] When the downstream target genes of NPSR1 signalling were studied, genes with ontology terms relating to immune response and chemotaxis were found to be overrepresented.[28] Interestingly, recent genetic studies of *NPSR1* have presented results suggesting gene–environment interactions.[23 24]

PARSIFAL (Prevention of Allergy Risk factors for Sensitisation In children related to Farming and Anthroposophic Lifestyle) is a European cross-sectional study designed to investigate the role of different lifestyles and environmental exposures in four groups: farm children, children from Steiner schools (who often have an anthroposophic lifestyle), and two corresponding reference groups.[4] The large sample size and wealth of information on environmental exposures makes this a unique dataset for investigating gene–environment interactions. We have previously demonstrated that *NPSR1* haplotypes are moderately associated with asthma and atopic sensitisation in this sub-sample of PARSIFAL.[19] Here we explore potential effect modification by *NPSR1* on environmental exposures related to the farming lifestyle. Since protective effects of the farming environment have been connected with endotoxins,[7 12 13] we also investigated the effects of LPS stimulation on NPSR1 protein levels and mRNA expression levels in monocytes.

METHODS

Study design

This work is based on a sub-sample of children from the cross-sectional PARSIFAL study intended for gene–environment analyses. Recruitment and patient characteristics are described in detail

elsewhere.[4] In brief, the study included 14 893 children between 5–13 years old from four groups: farm children, Steiner school children, and two appropriate reference groups (see below), in five European countries (Austria, Germany, Holland, Sweden and Switzerland). Ethical approval for the study, including genetic analyses, was obtained in each country. The parents completed a detailed questionnaire on their child's allergic diseases, infectious history and environmental exposures.[4 6 29–31] The present analysis included 3113 children with complete questionnaire data, adequate DNA material, and consent to genetic analyses. Information on non-participation and comparability between the whole PARSIFAL dataset and the genetic sub-sample can be found elsewhere.[4 19]

Criteria for study group inclusion

Farm children included those children who currently were living on a farm and whose parents were running the farm. Their reference group was recruited from children in the same area but who did not meet the inclusion criteria for farm children. The Steiner group was recruited among children attending Steiner schools, whose parents often have an anthroposophic lifestyle. Their reference group was recruited from other schools in similar suburban/rural areas. For further information see Alfvén *et al*.[4]

Health end point definitions and data on environmental exposures

Information on environmental exposures and health end points were reported by the parents of the children, except atopic sensitisation (see below). Children who had ever been diagnosed with asthma (by a physician), or with obstructive bronchitis more than once, were classified as having asthma, while children who reported sneezing, runny or stuffy nose in combination with itchy eyes in the last 12 months without having a cold at the same time were considered to have rhinoconjunctivitis.[4] Two common cut-off values for atopic sensitisation were used: allergen-specific IgE level $\geq$0.35 kU/l and $\geq$3.5 kU/l in either Phadiatop (a mix of common inhalant allergens) and/or fx5 (a mix of common food allergens; Phadia AB, Uppsala, Sweden) analyses. To increase the power of the interaction analyses, the term "allergic symptoms" was broadly defined as asthma and/or rhinoconjunctivitis based on the definitions above. The health endpoint definitions used in this study have been amply documented and used in several PARSIFAL reports.[4 9 10 14 19]

Genotyping

Detailed information on the genotyping methodology, polymorphism selection and quality assessment is given elsewhere.[19] In brief, seven tagging polymorphisms reported in the Laitinen *et al* study[18] were chosen in the conserved haplotype block of *NPSR1* and genotyped on a MALDI-TOF (matrix associated laser desorption ionisation time of flight) platform. The selected polymorphisms are rs323917, rs323922, rs324377, SNP546333, rs324384, rs324396, and rs740347.

Analysis of NPSR1 protein expression after LPS stimulation

Peripheral blood from 10 healthy volunteers was drawn into EDTA tubes and 200 µl was transferred to siliconised polypropylene FACS tubes, to minimise monocyte adherence. Blood samples were mixed with 800 µl RPMI-1640 medium containing l-glutamine (0.29 mg/ml), penicillin (100 U/ml) and streptomycin (100 µg/ml) (Gibco Lifetechnologies, Paisley, UK) and incubated for 16 h at 37°C, with or without 10 ng/ml LPS (Sigma-Aldrich, St Louis, Missouri, USA). For staining of intracellular receptor epitopes, red blood cells were lysed and leucocytes were fixed in 4% paraformaldehyde and permeabilised in 0.5% saponin. NPSR1-A and -B rabbit antibodies and pre-immune serum negative controls were raised and characterised as previously described[18 26 27] and used with a secondary PE labelled goat-anti-rabbit IgG antibody (Southern Biotech, Alabama, USA). Samples were analysed on a FACSCalibur flow cytometer with CellQuest software (Becton Dickinson, San Jose, California, USA). Cell populations were detected using laser side and forward scatter and the monocyte population was further confirmed using a CD14-fluorescein isothiocyanate (FITC) antibody (Beckton Dickinson). On average 2500 gated cells were analysed and results are shown as median fluorescence intensity (MFI) values with the pre-immune serum MFI subtracted. The study was approved by the ethical review board at Karolinska Institutet.

Analysis of *NPSR1* mRNA expression after LPS stimulation

Human monocytes were obtained from 10 healthy blood donors as previously described.[27] Briefly, peripheral blood cells were isolated by density gradient centrifugations using Ficoll-Paque Plus (Amersham Biosciences, Uppsala, Sweden). PBMCs were seeded onto cell culture bottles and incubated in complete RPMI 1640 medium supplemented with 0.29 mg/ml l-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin (Gibco Lifetechnologies) and 10% fetal bovine serum (Sera-Lab, Sussex, UK) for 10–20 min. Adherent monocytes were harvested by scraping, rested over night in complete RPMI medium at a density of 1.5×10^6 cells/ml, and cultured for 6 h and 24 h with or without 10 ng/ml LPS (Sigma) at 37°C. Total RNA was isolated with RNeasy Mini Kit (Qiagen, Hamburg, Germany) from $3–9\times10^6$ cells. cDNA was synthesised with TaqMan reverse transcription reagents (Applied Biosystems, Foster City, California, USA) and oligo dT primers. Quantitative real-time polymerase chain reaction (PCR) was performed with ABI PRISM 7500 Sequence Detection System applying SybrGreen chemistry (Applied Biosystems). The primer sequences for human *NPSR1-A* were 5′-CCCCCTCATCTACTGTGT CTTCA-3′ (forward) and 5′TCTCTCCCGGAACGTCA TTCT-3′ (reverse). The primer sequences for *NPSR1-B* and elongation factor 1 α (EF-1α) have been documented previously.[27] The relative gene expression differences were calculated with the comparative ΔΔCT method using EF-1α as a control.[32] The study was approved by the ethical review board at University of Helsinki.

Statistical analysis

Multivariate logistic regression was used for analyses and thus departure from a multiplicative model of interaction on the odds ratio (OR) scale was investigated. The statistical significance of effect modification was tested by a likelihood ratio (LR) test for the improvement of goodness of fit for the model when including the interaction term. All confidence intervals were calculated at the 95% level, $p = 0.05$.

The evaluated main determinants were focused on the children's lifetime or prenatal exposures, related to the farm environment. Throughout the manuscript, "current" refers to exposures that occurred during the last 12 months and "regular" refers to exposures that occurred at least once a week. The investigated exposures were: "ever regular farm

animal contact'', ''current regular farm animal contact'', ''current regular visits to stable or barn'', ''mother's farm animal contact during pregnancy'', ''mother worked regularly in stable or barn during pregnancy'', ''ever consumption of farm milk''. For ''current regular farm animal contact'', timing of the initiation of such regular exposure was further investigated (current regular contact with initiation in first year of life/after first year but more than a year ago/last 12 months), with no current contact as the reference category. The seven genotyped *NPSR1* polymorphisms were evaluated as potential effect modifiers. The polymorphisms were included as variables with two genotype groups with reference ''non-risk'' genotype(s), determined from a main effect logistic regression analysis of each polymorphism separately (supplemental table 1). Two polymorphisms (rs323917 and rs740347) had such low minor allele frequencies that the rare minor allele homozygotes for analytical reasons were pooled with the heterozygotes and a dominant model was used. For the other five polymorphisms, a recessive model was found to estimate the strongest main risk effect.

The following potential confounding factors were evaluated: country (Austria/Germany/Holland/Sweden/Switzerland), age (5–6/7–8/9/10–11/12–13 years), sex (male/female), heredity (mother's or father's reported asthma and/or rhinoconjunctivitis), parental education (low/medium/high), maternal smoking during pregnancy (yes/no), current smoking at home (yes/no), older siblings (no/one/two/three or more older siblings), and study group (farm/farm reference/Steiner school/Steiner reference). Relevant confounders were identified starting from the full model, with relevant confounding defined using the customary criterion of approximately 10% change in the investigated effect estimates. All analyses were carried out using the statistical software STATA 9.0 (College Station, Texas, USA).

Where evidence for interaction was detected on the genotype level, haplotype by environment analyses were performed in R using the haplo.glm algorithm in the haplo.stats package,[33] which includes an expectation maximisation (EM) step to estimate probabilities for haplotypes. Evidence for an overall haplotype–environment interaction was obtained through an LR test for the improvement of goodness of fit for the model when including versus excluding the interaction term.

The global significance for the interaction between *NPSR1* and timing of current regular contact with farm animals in determining risk of allergic symptoms was tested by permutation. The affection status was permuted 10 000 times, each followed by seven multivariate logistic regressions as described above, after which the number of nominally significant interactions was recorded (randomised χ^2 tests). The proportion of permutation tests where the number of nominally significant LR tests exceeded or was equal to the original number of significant tests provided the global p value.

Paired Wilcoxon signed rank tests were used to analyse the effect of LPS stimulation on NPSR1 protein and mRNA levels.

RESULTS

Descriptive analyses and main effects

The prevalence of farm related exposures and allergic diseases in the four study groups are presented in table 1. As expected, the exposures were most common among farm children, but a substantial proportion of children in the other groups was also exposed. Of the investigated potential confounder variables, only gender, heredity and study group were identified as confounders of the investigated associations and interactions and were thus included in all analyses described below. In an initial analysis, protective main effects of the farm related exposures on allergic symptoms and atopic sensitisation (most pronounced for ⩾3.5 kU/l) were observed in this sub-sample of PARSIFAL (table 2). Overall genetic associations of *NPSR1* haplotypes have previously been described in this population.[19] In the gene–environment interaction analyses, our focus was on the haplotype tagging polymorphisms for a number of reasons. First, because of the ease of interpretation when using binary genetic coding—that is, two levels of genetic exposure and two levels of environmental exposures, which also increases the

Table 1 Prevalences of allergic phenotypes and farm related exposures among the PARSIFAL children, by study group

	Farm children n = 1307	Steiner school children n = 686	Farm reference children n = 654	Steiner reference children n = 466
Allergic phenotypes (%)				
Doctor's diagnosis of asthma	6.2	7.0	11.6	12.9
Current symptoms of rhinoconjunctivitis	4.0	7.7	8.8	11.7
Allergic symptoms*	9.2	11.9	18.1	20.3
Atopic sensitisation (allergen specific IgE ⩾0.35 kU/l)	22.7	29.0	34.5	37.8
Atopic sensitisation (allergen specific IgE ⩾3.5 kU/l)	10.3	16.8	21.6	24.9
Farm related exposures (%)				
Ever regular farm animal contact	92.7	45.2	36.2	20.9
Current regular farm animal contact	82.7	30.2	26.3	12.0
Current regular visits to stable or barn	81.9	23.3	17.2	7.4
Mother's farm animal contact during pregnancy	79.9	18.7	15.5	6.6
Mother worked regularly in stable or barn during pregnancy	70.7	9.7	5.4	1.9
Ever consumption of farm milk	72.5	39.4	20.7	7.8
Current regular contact with farm animals, timing				
No current regular farm animal contact	17.3	69.8	73.7	88.0
Current farm animal contact initiated during first year	53.2	10.3	8.5	1.9
Current farm animal contact initiated after first year but >12 months ago	15.8	9.1	5.9	1.3
Current farm animal contact initiated within last 12 months	13.7	10.8	12.0	8.8

PARSIFAL, Prevention of Allergy Risk factors for Sensitisation In children related to Farming and Anthroposophic Lifestyle.
*Doctor's diagnosis of asthma and/or current symptoms of rhinoconjunctivitis.

Table 2 Main environmental effects of farm related exposures on allergic symptoms and atopic sensitisation in all PARSIFAL children

Exposures	Number of exposed/ unexposed children	Outcome			
		Doctor's diagnosis of asthma OR (95% CI)†	Current symptoms of rhinoconjunctivitis OR (95% CI)†	Allergic symptoms* OR (95% CI)†	Atopic sensitisation (⩾3.5 kU/l) OR (95% CI)†
Ever regular farm animal contact	1852/1252	0.91 (0.66 to 1.26)	0.71 (0.50 to 1.01)	0.82 (0.63 to 1.07)	0.71 (0.55 to 0.91)
Current‡ regular farm animal contact	1513/1591	0.80 (0.57 to 1.11)	0.70 (0.48 to 1.01)	0.78 (0.60 to 1.03)	0.74 (0.58 to 0.96)
Current regular visits to stable or barn	1369/1719	0.54 (0.37 to 0.79)	0.64 (0.42 to 0.97)	0.58 (0.43 to 0.79)	0.67 (0.51 to 0.89)
Mother's farm animal contact during pregnancy	1280/1730	0.72 (0.48 to 1.05)	0.58 (0.38 to 0.89)	0.65 (0.48 to 0.89)	0.75 (0.56 to 0.96)
Mother worked regularly in stable or barn during pregnancy	1028/2065	0.51 (0.34 to 0.78)	0.56 (0.34 to 0.90)	0.54 (0.39 to 0.77)	0.62 (0.45 to 0.85)
Ever consumption of farm milk	1319/1581	0.50 (0.35 to 0.70)	0.66 (0.45 to 0.97)	0.56 (0.42 to 0.74)	0.95 (0.74 to 1.22)

PARSIFAL, Prevention of Allergy Risk factors for Sensitisation In children related to Farming and Anthroposophic Lifestyle.
*Doctor's diagnosis of asthma and/or current symptoms of rhinoconjunctivitis.
†Odds ratios (OR) (with 95% confidence intervals (CI)) estimated from a logistic regression model, adjusting for gender, heredity and study group.
‡ Current refers to exposures that have occurred during the last 12 months (for more detailed information see Methods).

power of the analysis in that the cross-classification cell counts increase. Second, choosing a baseline in the regression becomes intuitive for single nucleotide polymorphisms (SNPs) where one allele or genotypic combination defines risk in relation to another, which is not true for haplotypes that are generally numerous and show complex association patterns. Third, since these polymorphisms were chosen as tags, they are actually fewer than the total number of haplotypes observed, reducing the risk of type I error. Minor allele frequencies and crude ORs for the genotypic model selected for each polymorphism for use in the interaction analyses are presented in supplemental table 1.

Interaction between farm animal exposure and *NPSR1* polymorphisms

First, we assessed whether the protective effects of farm related exposures was influenced by *NPSR1* polymorphisms. Strong effect modification for allergic symptoms was seen for regular farm animal contact (table 3). The strongest effect modification was seen for current regular farm animal contact, especially by rs323922 and rs324377 (p value for interaction = 0.001 and 0.002). The protective effects of the other variables against development of allergic symptoms were not dependent on genotype (supplemental table 2). In a separate analysis for asthma and rhinoconjunctivitis, rs324396 seemed to differ somewhat between the phenotypes. However, the overall trends were similar and the combined variable for allergic symptoms was consistently showing more significant effects (table 3). No gene–environment interactions were observed for any of the main environmental determinants when atopic sensitisation was used as outcome (neither ⩾0.35 kU/l nor ⩾3.5 kU/l, data not shown).

When investigating the effect of timing of initiation of current regular farm animal contact on allergic symptoms, significant effect modification were seen with rs323922,

Table 3 Effect modification of regular farm animal contact on allergic symptoms† by *NPSR1* genotypes, among all PARSIFAL children

SNPs with genotype strata	Ever regular farm animal contact			Current‡ regular farm animal contact		
	Asthma OR (95% CI)	Rhinoconjunctivitis OR (95% CI)	Allergic symptoms OR (95% CI)	Asthma OR (95% CI)	Rhinoconjunctivis OR (95% CI)	Allergic symptoms OR (95% CI)
rs323917						
CC (non-risk)	0.87 (0.62 to 1.22)	0.66 (0.46 to 0.97)	0.78 (0.59 to 1.03)	0.74 (0.52 to 1.04)	0.66 (0.44 to 0.98)	0.72 (0.54 to 0.96)
CG/GG	1.04 (0.47 to 2.33)	1.02 (0.45 to 2.28)	1.07 (0.56 to 2.03)	1.03 (0.46 to 2.28)	0.90 (0.39 to 2.05)	1.08 (0.57 to 2.07)
rs323922						
GG/CG (non-risk)	1.08 (0.76 to 1.54)	0.78 (0.53 to 1.15)	0.98 (0.73 to 1.31)*	0.99 (0.68 to 1.42)*	0.82 (0.55 to 1.23)*	0.96 (0.71 to 1.30)**
CC	0.67 (0.36 to 1.25)	0.48 (0.24 to 0.98)	0.51 (0.30 to 0.87)	0.37 (0.18 to 0.75)	0.31 (0.14 to 0.71)	0.33 (0.18 to 0.60)
rs324377						
AC/CC (non-risk)	1.08 (0.75 to 1.56)	0.81 (0.54 to 1.20)	1.00 (0.74 to 1.35)*	0.97 (0.67 to 1.41)*	0.80 (0.52 to 1.21)	0.95 (0.70 to 1.30)**
AA	0.70 (0.39 to 1.27)	0.48 (0.25 to 0.94)	0.53 (0.32 to 0.87)	0.43 (0.22 to 0.82)	0.36 (0.17 to 0.78)	0.38 (0.22 to 0.66)
SNP546333						
AA/AG (non-risk)	0.37 (0.17 to 0.83)*	0.28 (0.10 to 0.78)	0.38 (0.20 to 0.75)*	0.52 (0.23 to 1.17)	0.22 (0.06 to 0.76)	0.46 (0.23 to 0.92)
GG	1.03 (0.73 to 1.44)	0.77 (0.53 to 1.11)	0.89 (0.67 to 1.18)	0.84 (0.59 to 1.18)	0.77 (0.52 to 1.14)	0.83 (0.62 to 1.10)
rs324384						
CT/CC (non-risk)	1.01 (0.70 to 1.47)	0.74 (0.49 to 1.11)	0.93 (0.68 to 1.26)	0.91 (0.63 to 1.33)	0.76 (0.50 to 1.17)	0.90 (0.66 to 1.23)
TT	0.77 (0.47 to 1.25)	0.68 (0.39 to 1.16)	0.66 (0.44 to 1.00)	0.60 (0.36 to 1.00)	0.58 (0.32 to 1.04)	0.58 (0.38 to 0.90)
rs324396						
CT/TT (non-risk)	1.18 (0.77 to 1.79)	0.62 (0.39 to 0.98)	0.97 (0.68 to 1.37)	0.96 (0.62 to 1.48)	0.64 (0.39 to 1.06)	0.90 (0.63 to 1.29)
CC	0.73 (0.48 to 1.10)	0.76 (0.48 to 1.21)	0.68 (0.48 to 0.97)	0.66 (0.43 to 1.00)	0.68 (0.42 to 1.10)	0.64 (0.45 to 0.92)
rs740347						
GG (non-risk)	1.04 (0.73 to 1.49)	0.75 (0.50 to 1.11)	0.93 (0.69 to 1.25)	0.93 (0.64 to 1.34)	0.84 (0.56 to 1.27)*	0.92 (0.68 to 1.25)*
CC/CG	0.64 (0.37 to 1.11)	0.59 (0.32 to 1.09)	0.58 (0.36 to 0.92)	0.51 (0.29 to 0.92)	0.35 (0.17 to 0.73)	0.46 (0.28 to 0.77)

Odds ratios (OR) and 95% confidence intervals (CI) from a logistic regression model, adjusting for gender, heredity and study group.
†Doctor's diagnosis of asthma and/or current symptoms of rhinoconjunctivitis.
‡"Current" refers to exposures that have occurred during the last 12 months.
*p value for interaction <0.05 from a likelihood ratio (LR) test for the improvement of the regression model when including the interaction term.
**p value for interaction <0.005 from an LR test for the improvement of the regression model when including the interaction term.

rs324377, SNP546333, and rs740347 (tables 4 and for asthma and rhinoconjunctivitis separately). If the current regular farm animal contact was initiated during the first year of life, there was a tendency towards a protective effect regardless of genotype, while if the contact was initiated after early infancy, the environmental effects differed substantially depending on *NPSR1* background (fig 1). To investigate further the consistency of the interaction, a stratified analysis was performed for farm children and for all non-farm children in the study (Steiner school children combined with the two reference groups) separately, and a similar interaction trend was observed in both strata (table 4). The power was low for the separate analyses of asthma and rhinoconjunctivitis, resulting in broader confidence intervals and lack of cases for some combinations of exposure and genotype (table 5).

To get a composite view of the interaction on the haplotype level, the overall haplotype by environment interaction significance was calculated for ever regular farm animal contact ($p = 0.06$), current regular farm animal contact ($p = 0.14$), and timing of initiation of current regular farm animal contact ($p = 0.01$). Furthermore, the permuted p value for an overall interaction between *NPSR1* and timing of initiation of current regular farm animal contact was 0.008 (10 000 permutations), thus indicating a significant global gene–environment interaction.

NPSR1 expression after LPS stimulation

Since the protective effects of the farming environment have been attributed to high levels of endotoxins, for example, we hypothesised that the observed interaction between farm animal contact and *NPSR1* might be linked to the innate immune response. Therefore, the direct in vitro effects of LPS stimulation on NPSR1 protein and mRNA levels were investigated. $CD14^+$ PBMCs from 10 blood donors showed a definite pattern of upregulation of the NPSR1-A isoform after 16 h of LPS stimulation ($p = 0.002$, supplemental fig 1 A, B and D), as measured by FACS analysis, while we did not observe an upregulation for the B isoform (supplemental fig 1 A, C, and E). In the parallel mRNA investigations, where monocytes from 10 blood donors were stimulated with LPS, the *NPSR1-A* isoform was found to show a modest upregulation trend at 6 h ($p = 0.02$), while the B isoform response showed a similar but

Table 4 The effect of initiation of current regular farm animal contact on allergic symptoms* is modified by *NPSR1* genotypes, among all PARSIFAL children and in farmer and non-farmer subgroups

	Timing of initiation of current regular farm animal contact					
Exposure	Current farm animal contact initiated within last 12 months	Current farm animal contact initiated after first year but >12 months ago	Current farm animal contact initiated during first year	Overall interaction p value†		
SNPs with genotype strata	OR (95% CI), all children			All children	Non-farm children‡	Farm children
rs323917				0.4018	0.4929	0.4880
CC	0.74 (0.50 to 1.12)	0.77 (0.48 to 1.24)	0.66 (0.45 to 0.96)			
CG/GG	1.23 (0.49 to 3.12)	1.80 (0.69 to 4.71)	0.73 (0.31 to 1.75)			
rs323922				0.0028	0.0599	0.0844
CG/GG	1.08 (0.72 to 1.61)	1.10 (0.69 to 1.76)	0.84 (0.57 to 1.23)			
CC	0.19 (0.06 to 0.65)	0.37 (0.13 to 1.10)	0.39 (0.18 to 0.82)			
rs324377				0.0033	0.0325	0.1570
AC/CC	1.12 (0.74 to 1.69)	1.10 (0.67 to 1.79)	0.79 (0.53 to 1.18)			
AA	0.19 (0.06 to 0.62)	0.43 (0.16 to 1.14)	0.46 (0.23 to 0.90)			
SNP546333				0.0288	0.1076	0.0305
AA/AG	0.22 (0.05 to 0.97)	0.17 (0.02 to 1.30)	0.74 (0.33 to 1.66)			
GG	0.93 (0.63 to 1.37)	1.03 (0.66 to 1.60)	0.69 (0.48 to 1.00)			
rs324384				0.2599	0.7049	0.5939
CT/CC	0.99 (0.64 to 1.51)	1.08 (0.66 to 1.78)	0.76 (0.51 to 1.15)			
TT	0.53 (0.26 to 1.08)	0.59 (0.28 to 1.24)	0.58 (0.34 to 1.01)			
rs324396				0.1245	0.4186	0.3298
CT/TT	1.10 (0.66 to 1.82)	1.20 (0.70 to 2.09)	0.67 (0.41 to 1.09)			
CC	0.56 (0.33 to 0.96)	0.62 (0.33 to 1.16)	0.68 (0.44 to 1.06)			
rs740347				0.0006	0.0278	0.0351
GG	1.11 (0.75 to 1.67)	1.20 (0.76 to 1.91)	0.72 (0.48 to 1.07)			
CC/CG	0.22 (0.08 to 0.63)	0.26 (0.08 to 0.85)	0.70 (0.39 to 1.26)			

PARSIFAL, Prevention of Allergy Risk factors for Sensitisation In children related to Farming and Anthroposophic Lifestyle; SNP, single nucleotide polymorphism.
Odds ratios (OR) and 95% confidence intervals (CI) from a logistic regression model with all children, adjusting for gender, heredity and study group. The reference groups (no current regular farm contact, stratified by genotype, total n = 1622) are not shown.
*Doctor's diagnosis of asthma and/or current symptoms of rhinoconjunctivitis.
†Interaction as tested by the likelihood ratio (LR) test for the improvement of the model when including the interaction term.
‡Steiner school children combined with the two reference groups of the PARSIFAL study.

Table 5 The effect of initiation of current regular farm animal contact on asthma and rhinoconjunctivitis is modified by *NPSR1* genotypes, among all PARSIFAL children

		Timing of initiation of current regular farm animal contact			
	Exposure	Current farm animal contact initiated within last 12 months	Current farm animal contact initiated after first year but >12 months	Current farm animal contact initiated during first year	Overall interaction p value*
Outcome	SNPs with genotype strata	OR (95% CI)	OR (95% CI)	OR (95% CI)	
	rs323917				
Asthma	CC (non-risk)	0.73 (0.46 to 1.19)	0.69 (0.38 to 1.24)	0.75 (0.48 to 1.16)	0.272
	CG/GG	1.16 (0.36 to 3.73)	2.12 (0.70 to 6.46)	0.58 (0.18 to 1.85)	
Rhinoconjunctivitis	CC	0.77 (0.45 to 1.31)	0.75 (0.33 to 1.44)	0.51 (0.29 to 0.87)	0.915
	CG/GG	1.10 (0.34 to 3.52)	1.10 (0.29 to 4.13)	0.62 (0.19 to 2.00)	
	rs323922				
Asthma	CG/GG (non-risk)	1.02 (0.62 to 1.68)	1.00 (0.66 to 1.80)	0.95 (0.61 to 1.51)	0.060
	CC	0.34 (0.10 to 1.14)	0.44 (0.13 to 1.51)	0.36 (0.14 to 0.91)	
Rhinoconjunctivitis	CG/GG	1.06 (0.63 to 1.79)	0.97 (0.51 to 1.83)	0.60 (0.34 to 1.04)	0.038
	CC	0.12 (0.02 to 0.90)	0.38 (0.09 to 1.66)	0.39 (0.14 to 1.08)	
	rs324377				
Asthma	AC/CC (non-risk)	1.07 (0.65 to 1.76)	0.95 (0.52 to 1.76)	0.91 (0.57 to 1.46)	0.095
	AA	0.32 (0.10 to 1.08)	0.54 (0.18 to 1.59)	0.43 (0.19 to 0.99)	
Rhinoconjunctivitis	AC/CC	1.10 (0.64 to 1.88)	1.04 (0.54 to 2.01)	0.50 (0.27 to 0.92)	0.021
	AA	0.12 (0.02 to 0.87)	0.34 (0.08 to 1.50)	0.50 (0.20 to 1.22)	
	SNP546333				
Asthma	AA/AG (non-risk)	0.18 (0.02 to 1.40)	5e-8 (3e-8 to 8e-8)	0.99 (0.41 to 2.38)	0.005
	GG	0.93 (0.58 to 1.48)	1.01 (0.39 to 1.72)	0.73 (0.47 to 1.15)	
Rhinoconjunctivitis	AA/AG	0.21 (0.03 to 1.58)	0.35 (0.05 to 2.74)	0.16 (0.02 to 1.20)	0.160
	GG	0.92 (0.56 to 1.52)	0.88 (0.48 to 1.63)	0.60 (0.36 to 1.00)	
	rs324384				
Asthma	CT/CC (non-risk)	0.99 (0.59 to 1.66)	0.93 (0.49 to 1.75)	0.86 (0.53 to 1.39)	0.471
	TT	0.50 (0.21 to 1.22)	0.69 (0.30 to 1.61)	0.60 (0.31 to 1.14)	
Rhinoconjunctivitis	CT/CC	0.86 (0.48 to 1.54)	1.06 (0.55 to 2.05)	0.54 (0.29 to 0.98)	0.495
	TT	0.71 (0.31 to 1.64)	0.40 (0.12 to 1.35)	0.54 (0.25 to 1.15)	
	rs324396				
Asthma	CT/TT (non-risk)	1.19 (0.65 to 2.16)	0.98 (0.48 to 2.01)	0.84 (0.48 to 1.47)	0.327
	CC	0.53 (0.27 to 1.03)	0.71 (0.35 to 1.45)	0.70 (0.42 to 1.18)	
Rhinoconjunctivitis	CT/TT	0.72 (0.35 to 1.49)	1.18 (0.59 to 2.36)	0.34 (0.16 to 0.80)	0.128
	CC	0.79 (0.41 to 1.53)	0.42 (0.15 to 1.21)	0.66 (0.36 to 1.23)	
	rs740347				
Asthma	GG (non-risk)	1.07 (0.66 to 1.75)	1.27 (0.74 to 2.19)	0.75 (0.47 to 1.21)	NA†
	CC/CG	0.27 (0.08 to 0.90)	NA*	0.90 (0.47 to 1.72)	
Rhinoconjunctivitis	GG	1.11 (0.66 to 1.87)	0.93 (0.48 to 1.81)	0.61 (0.34 to 1.07)	0.063
	CC/CG	0.21 (0.05 to 0.88)	0.53 (0.16 to 1.79)	0.34 (0.13 to 0.93)	

PARSIFAL, Prevention of Allergy Risk factors for Sensitisation In children related to Farming and Anthroposophic Lifestyle.
Odds ratios (OR) and 95% confidence intervals (CI) from a logistic regression model, adjusting for gender, heredity and study group.
*Interaction as tested by the likelihood ratio (LR) test for the improvement of the model when including the interaction term.
†NA means that cases are missing for this combination, thus no OR or overall interaction p value can be calculated.

not significant trend (supplemental fig 1 F and G). The *NPRS1* mRNA levels after 24 h of LPS stimulation showed a large variation for both isoforms and no trends could be found (data not shown).

DISCUSSION

In this study we show how effects of an environment can differ depending on genetic background, with exposures to farm animals exerting protective or risk effects for allergic symptoms, depending on the inherited *NPSR1* variant. Robust results on interaction were also presented for a variable describing the timing of initiation of current regular farm animal contact. It is clear that independent replication is needed; however, the temporal phenomenon observed here suggests that an environmental exposure can show a more homogeneous effect if introduced early in life, while different mechanisms may act later on to modify such effects by, for example, a person's genetic setup. This was also supported by the finding that the protective effect of farm animal contact during pregnancy was not dependent on *NPSR1* genotypes.

NPSR1 is expressed by macrophages,[27] and thus has potential for direct or indirect interaction with environmental endotoxins.

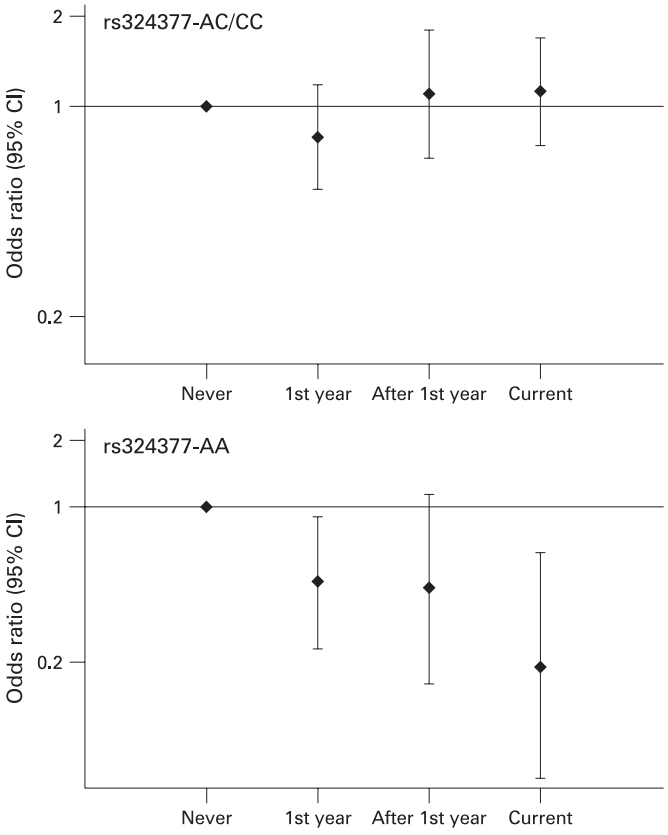

Figure 1 The effect of current regular farm animal contact on allergic symptoms is dependent on *NPSR1* polymorphism in the PARSIFAL (Prevention of Allergy Risk factors for Sensitisation In children related to Farming and Anthroposophic Lifestyle) children. Odds ratios (OR) and 95% confidence intervals (CI) for the effect of timing of current regular farm animal contact in strata of rs324377 are shown. The two reference groups (no current regular exposure to farm animals, stratified by genotype), are indicated at OR = 1. The following group includes those with current regular farm exposure that was initiated during the first year of life, the next group after the first year until recently, and the last group initiated their current regular farm animal contact recently (last 12 months). All groups are compared to the reference group (that has no current farm animal contact) in their genotype stratum.

Since the protective effects of farm animal exposure have been connected with higher levels of endotoxins, for example, we investigated the in vitro effects of LPS and found that the NPSR1-A (but not B) protein levels were specifically upregulated by LPS in $CD14^+$ PBMCs (monocytes). We further demonstrated that purified monocytes upregulate *NPSR1-A* (with a similar trend for *NPSR1-B*) mRNA levels after 6 h of LPS stimulation. Previously, the more heterogeneous cell population PBMCs have been shown to upregulate *NPSR1* mRNA levels after 16 h of LPS stimulation for both isoforms.[27]

A limitation of this experiment is its small sample size, although it is larger than the previous study.[27] It should also be noticed that several other environmental components typical for farm animal exposure might affect NPSR1 abundance in monocytes and macrophages, and would be worthy of study.[34–36] Further, the most relevant tissue or cell type for a functional study is not entirely clear. Here we focused on peripheral blood monocytes, since NPSR1 has been demonstrated to affect their function[27] and because innate immunity in general has been of interest in previous studies with similar scope.[14 15] Although more thorough studies are needed, all experimental results taken together suggest that NPSR1 levels are affected by LPS stimulation, and this can serve as a first molecular hypothesis for our epidemiological results.

The *NPSR1* polymorphisms that were investigated in this study are located in the second intron of the gene, comprising a block of tight linkage disequilibrium that was originally identified as associated to asthma related traits.[18] So far, no functional characteristics have been described for these polymorphisms, while many hypotheses can be proposed, including transcription factor binding, regulation of alternative splicing of the two isoforms A and B, and their potential linkage disequilibrium to a disease causing polymorphism. The heterogenic responses to LPS stimulation observed here may suggest that inherited *NPSR1* variants have an effect. Our limited sample size makes this difficult to assess, but further subject and data collections are ongoing to address this important issue.

The large sample size of the PARSIFAL study and its wealth of information on environmental exposures make it suitable for assessment of gene–environment interactions. The subset of the study available for genetic analyses has earlier been concluded essentially representative.[19] Further, we have previously noted that the frequencies of genetic variants across the countries were quite homogeneous, suggesting that population stratification is not expected.[19] In the present analyses we investigated typical farm related environmental exposure in all the four study groups. The finding that timing of initiation of farm animal contact is important when studying *NPSR1* modification was further analysed in farmer and non-farmer children, separately. It should be noted that when analysing only farmers, there are few individuals in the reference group (no current exposure), while there are fewer exposed for the non-farmers. Nevertheless, it is reassuring that both analyses present a similar interaction, despite the different general environments of the groups of children.

When effect modification between *NPSR1* and farm related variables was analysed, we used both atopic sensitisation and allergic symptoms as outcome. However, it was only for allergic symptoms that we found evidence for effect modification. This could be due to chance, but of note is that some studies have suggested that different environmental exposures related to the farming lifestyle are responsible for protection against different types of allergic phenotypes.[6 14] The definition of sensitisation by serology measurements may also be of importance. For completeness, we performed all analyses using two common cut-offs, $\geq$0.35 kU/l and $\geq$3.5 kU/l, but no genetic interaction effects were seen with either cut-off. We have also performed interaction analyses by stratifying for sensitisation (that is, allergic symptoms with or without detectable sensitisation) and there were trends for interaction in both groups, emphasising the independence of these two phenotypes (data not shown).

For allergic symptoms, we performed interaction analyses for asthma and rhinoconjunctivitis separately and also for a combined variable of the two. The incentive for the combination was to increase the number of cases, thus acquiring a higher statistical power to perform detailed gene–environment interaction analysis. Some of the observed small differences between the symptoms might be of biological interest, but we have to conclude that a larger, better powered study would be needed to make any such conclusions.

We have recently used this population to evaluate the overall association between *NPSR1* variants and childhood allergic disease.[19] Here, we chose to consider how environmental effects could be modified by an inherited *NPSR1* allele. This seemed intuitive, because the genetic setup is fixed at birth, while the

Electronic database information

"R" (http://www.r-project.org); haplo.stats package (http://mayoresearch.mayo.edu/mayo/research/schaid_lab/software.cfm)

environment is interchangeable. However, statistically the interaction effects could be viewed in both ways and the presented results implicitly mean that the genetic effects also differ depending on environment. Of interest is that the tag polymorphisms that showed association in our first study (rs324384, rs324396, distinguishing H1/H3 from rest) did not show significant interaction in this study. Further, when performing haplotype–environment analyses, the haplotypes showing effect modification (using H1 as reference) were H2 and H4, both of which did not show association in our previous analysis with the PARSIFAL children,[19] but were associated with risk in the original *NPSR1* identification study.[18] These new findings emphasise the importance of considering gene–environment effects in association studies of complex diseases such as asthma and atopic sensitisation. Of note, a recent association study of *NPSR1* presented flip-flop association patterns for different populations, possibly suggesting that different environments might cause the discrepant association patterns.[24] Consideration of environmental exposures and simultaneously the timing of exposures may be more important than earlier perceived.

In conclusion, this study adds *NPSR1* to the list of genes suggested to modify the protective effects of environmental exposures,[15–17] and further indicates that genetic effect modification of environmental exposures may be influenced by the timing of initiation of such exposures.

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The protective effect of farm animal exposure on childhood allergy is modified by *NPSR1* polymorphisms

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