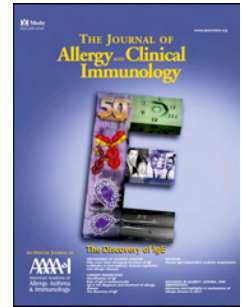


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Substance P represents a novel first-line defense mechanism in the nose

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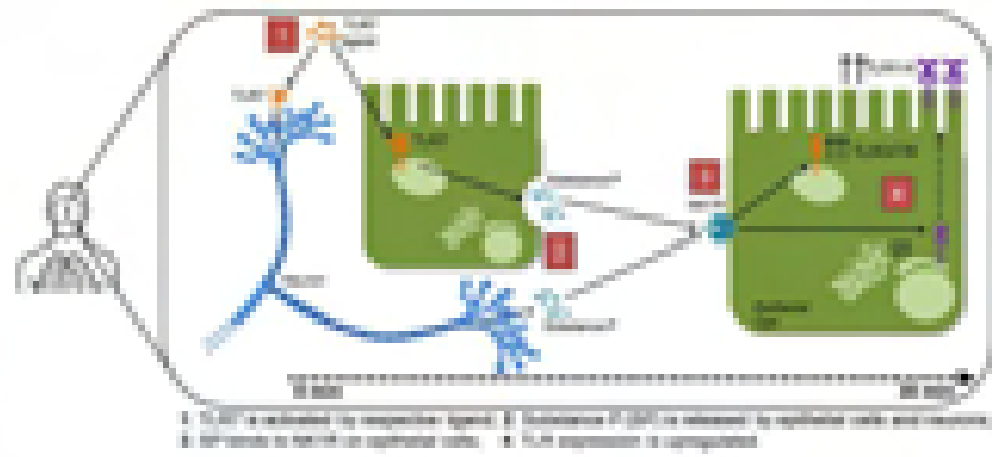
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Substance P represents a novel first-line defense mechanism in the nose

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Abstract

Background: Neuropeptides, such as substance P (SP), have long been seen as mediators of widespread, continuous airway inflammation, a process known as neurogenic inflammation. However, this has been difficult to demonstrate clinically, suggesting an alternative role for these signaling molecules.

Objectives: To examine the role of SP in nasal infection, by assessing the release of SP in response to viral stimulation and characterizing the effects of SP on innate immunity, the latter reflected in changes in local Toll-like receptor (TLR) expression.

Methods: The distribution of SP and TLRs in the nasal mucosa and in local airway neurons was assessed with immunohistochemistry. The TLR7 agonists R-837 and R-848 were used to mimic a viral insult in upper airways, represented by primary human and mouse nasal epithelial cells (HNEC, MNEC) and isolated murine trigeminal ganglia (TGN). SP release from HNEC, MNEC and TGN was quantified with EIA. The effects of SP on TLR expression on HNEC were determined using flow cytometry and confocal microscopy.

Results: SP was released from the sensory neurons, MNEC and HNEC within 15 minutes of local TLR7 stimulation. Subsequently, stimulation with SP induced upregulation of TLR expression in HNEC within 30 minutes, via induction of TLR movement within HNEC. Upregulation of TLR expression was not evident when cells were treated with the neurokinin 1 receptor (NK1R) antagonist aprepitant prior to SP stimulation.

Conclusions: This highlights a novel role for sensory neuropeptides as acute and local mediators of pathogen-driven inflammation, rapidly priming innate immune defenses in the airway.

43 **Key Words**

44 Toll-like Receptor

45 Substance P

46 TLR7

47 Viral Infection

48 Epithelial Cells

49 Nasal Mucosa

50 Innate immunity

51

52

Key Messages

- SP is rapidly released upon viral detection by Toll-like receptors in the nasal mucosa
- SP subsequently causes a prompt upregulation of mucosal TLRs, indicating a novel role for neuropeptides in innate immunity

Capsule Summary

Toll-like receptors (TLRs) and substance P (SP) have both been shown to contribute to airway disease, particularly during viral infection. The present paper demonstrates a novel role for SP in kick-starting the TLR-driven immune response.

Introduction

Substance P (SP), a member of the tachykinin family of neuropeptides, was first identified in the 1930s; however, SP wasn't the subject of intense research until the 1980s when neuropeptides such as SP, calcitonin gene-related peptide (CGRP) and vasoactive intestinal polypeptide (VIP) were identified as integral mediators of neurogenic inflammation¹. This process, by which certain neuropeptides induce vasodilation and increase vascular permeability, glandular activation and leukocyte recruitment, particularly in the airway, was shown to have an important contributory role in airway inflammation and associated pathologies in animal models². SP was identified as a key mediator of this process in guinea pigs, as animals treated with capsaicin to deplete SP, and animals treated with an SP antagonist, did not develop vagally induced neurogenic inflammation³. Neuropeptides, and their respective receptors, could be identified in human airways^{4,5}, and their release was shown to be induced by allergen⁶ or by hypertonic saline provocation⁷. However, the presence and relative contribution of neurogenic inflammation to human airway disease has been difficult to demonstrate⁸, suggesting an alternative clinically relevant role for neuropeptides.

Viruses are primarily recognized via their pathogen-associated molecular patterns (PAMPs) by the evolutionarily conserved family of Toll-like receptors (TLRs), of which TLR 3, 4, 7, 8, and 9 are responsible for viral recognition⁹. PAMP recognition by TLRs is known to activate a signalling cascade which results in cytokine production leading to recruitment of cells of the immune system¹⁰. The production of SP in response to viral infection of the airways is well established^{11, 12}, with current research revealing a significant contribution of SP to the clinical

87 symptoms of respiratory syncytial virus infection¹³. In addition, recent findings demonstrate the
88 ability for neuropeptides to modulate the mechanisms of innate immunity¹⁴⁻¹⁶. However, the
89 early and local production of SP in response to viral invasion in the nose has not previously been
90 studied.

91
92 Therefore, the present study was designed to investigate if SP could be rapidly and locally
93 released in response to a virus entering the nose, and, if so, if SP release could subsequently
94 affect the local immune defense, represented by mucosal TLR expression.

Methods

Animals

Six week female C57BL/6N mice were obtained from Charles River (Sulzfeld, Germany). They were housed in plastic cages with adsorbent bedding in temperature and light-dark cycle (12h:12h) controlled rooms. Food and water were available *ad libitum*. Animals were handled in accordance with the Federation for European Laboratory Animal Science Associations guidelines. All animal procedures were approved by the regional animal experimental ethical review board (ethical permit numbers: N153-11 and N258-13).

Isolation and Culture of Trigeminal Sensory Neurons (TGNs)

Murine trigeminal sensory neurons (TGNs) were isolated as previously described¹⁷, with modifications. A full description of the isolation and culture procedure is provided in the supplementary material.

Isolation and Culture of Human Nasal Epithelial Cells (HNECs)

Human nasal epithelial cells (HNECs) from nine healthy, non-allergic, non-smoking subjects (3 male, 6 female) were isolated by nasal brushing as previously described¹⁹. Cells were maintained in collagen-coated tissue culture flasks in Keratinocyte Serum-Free Medium (KSFM) (Gibco, Paisley, UK), supplemented with 0.005 µg/mL epidermal growth factor (Gibco), 0.05 mg/mL bovine pituitary extract (Gibco), 20 U/mL penicillin, 20 µg/mL streptomycin and 0.25 µg/mL Fungizone (complete KSFM). All cells were cultured in a humidified chamber at 37°C with 5%

CO₂. Cells from passages 2–5 were used, and were all positive for EpCAM (>90%), an epithelial-specific adhesion molecule.

Isolation and Culture of Murine Nasal Epithelial Cells (MNECs)

Isolation of murine nasal epithelial cells was carried out as previously described¹⁸. A full description of the isolation and culture procedure is provided in the supplementary material.

Cell Stimulation

HNEC were seeded on 24-well culture plates (250,000 cells/well) in 1 mL complete KSFM and incubated overnight. Cells were subsequently cultured for 15 min, 30 min or 4 hr with R-837 (Invivogen, San Diego, CA, USA) or R-848 (Invivogen) (both at 5, 10, 30 or 100 µg/mL). Cell-free supernatants were analyzed for SP release. Alternatively, cells were cultured for 30 min with SP (100 nM) (Sigma-Aldrich) following a 3-min pre-incubation with the neurokinin 1 receptor (NK1R) antagonist Aprepitant (10 nM, Selleck Chemicals, Rungsted, Denmark). Cells were analyzed for TLR expression using flow cytometry.

MNEC were cultured with R-837 (5, 10, 30 or 100 µg/ml) for 15 min, 30 min or 4 hr. Cell-free supernatants were analyzed for SP release.

TGN were cultured with R-837 (5, 10, 30 or 100 µg/mL) for 15 min, 30 min or 4 hr. Cell-free supernatants were analyzed for SP release.

Collection and immunohistochemistry of nasal biopsies

Human nasal biopsies from five healthy subjects were obtained in conjunction with nasal surgery (resection of the lower turbinate) or as previously described²⁰. Biopsies were post-fixed in 4% PFA embedded in paraffin and subsequently cut into 5µm sections on a microtome. A full description of the immunohistochemistry procedure can be found in the supplementary material.

Collection and immunohistochemistry of human trigeminal ganglia

Trigeminal ganglia were a kind gift from Janos Tajti at the University of Szeged. They were obtained during autopsy from adult subjects in accordance with the University of Szeged medical school guidelines for ethics in human tissue experiments. Ganglia were obtained from six subjects (4 males, 2 females) with an average age of 73 years. None of the patients suffered from any central nervous system disease. Tissue was collected within 24 hr after death. A full description of the immunohistochemistry procedure can be found in the supplementary material.

Flow Cytometry

For detection of TLRs, the conjugated mouse monoclonal antibodies TLR2-AF647 (Clone: TLR2.3, Serotec, Oxford, UK) TLR3-PE (Clone: 40C1285.6), TLR4-FITC (Clone: HTA125), TLR5-PE (Clone: 85B152.5), TLR6-FITC (Clone: 86B1153.2), TLR8-FITC (Clone: 44C143) and TLR9-AF647 (Clone: 26C593.2), as well as the conjugated rabbit antibodies TLR1-AF647 and TLR7-PE were used. The neurokinin 1 receptor (NK1R) was detected with a polyclonal rabbit NK1R-FITC antibody. All antibodies were purchased from Imgenex (San Diego, CA, USA), now Novus Biologicals (Abingdon, UK), unless otherwise specified. The IntraPrep Permeabilization Reagent kit (Immunotech, Beckman Coulter, Marseille, France) was used to

detect intracellular TLRs, as per the manufacturer's instructions. Epithelial cells were analyzed on an LSR Fortessa Flow Cytometer (BD, San Jose, USA) and gated based on forward and side-scatter properties (Fig. E1 in this article's online Repository at www.jacionline.org). Events in the range of 20 000–50 000 were collected. Data was analyzed using FlowJo Analysis Software (TreeStar Inc, Ashland, USA).

Stimulation, immunocytochemistry of confocal microscopy of HNEC

HNEC were seeded onto 96-well IbiTreat μ -plates (Ibidi GmbH, Martinsried, Germany) at 150,000 cells/ml in 300 μ L complete KSFM, and incubated overnight. Cells were cultured for 30 min with SP (100 μ M) or media and subsequently fixed with 4% PFA. A full description of the immunohistochemistry procedure can be found in the supplementary material. Imaging was performed on a Zeiss LSM800 confocal microscope.

Immunocytochemistry of murine neurons

A full description of the immunohistochemistry procedure can be found in the supplementary material.

Measurement of Substance P

Substance P from cell culture supernatant was measured using a Substance P EIA Kit allowing detection of human and mouse substance P (Cayman Chemical, Ann Arbor, MI, USA), according to the manufacturer's instructions.

Statistical Analysis

Data was analyzed using GraphPad Prism Software (San Diego, CA, USA). Results are expressed as individual dots with mean or mean $\pm$ SEM. In experiments involving human tissue, including biopsies and cultured cells, n is equal to the number of subjects. In experiments involving cultured murine epithelial cells or neurons, n is equal to the number of individual replicate measurements. SP levels were analyzed using a Kruskal-Wallis test (TGN, MNEC) or a Friedman test (HNEC) for non-parametric data followed by a Dunn's multiple comparison post-test. TLR expression on HNEC was analyzed using a repeated measures ANOVA followed by a Bonferroni's multiple comparison post-test. A p-value of 0.05 or less was considered statistically significant.

Ethics Statement

This study was approved by the Ethics Committee of Karolinska Institutet, Stockholm, Sweden (2011/1486-31/4, 2013/2075-31/3 and 2016/821-31/2). All participants gave their written informed consent. All procedures were conducted according to the principles expressed in the Declaration of Helsinki.

Results

Toll-like Receptor Expression in the Nasal Mucosa

The presence of viral recognizing TLRs in the upper airway was initially verified through immunohistochemical staining of nasal biopsies from healthy subjects. Widespread expression of TLR3, TLR4 and TLR9 was evident (Fig. 1A, B, D), whereas TLR7 expression was found to be limited to the nerve fibers (Fig. 1C, 3B) and the nasal epithelium (Fig. 3A). Neuronal TLR expression was further assessed using cadaveric human trigeminal ganglia, demonstrating a high expression of TLR7 and TLR9 (Fig. 2C-D) and a low expression of TLR3 and TLR4 (Fig. 2A-B). TLR7 expression was also shown to co-localize with SP on innervating nerve fibers in human nasal biopsies (Fig. 3C).

Due to the use of murine ganglia in subsequent functional experiments, expression of TLR7 was further investigated in murine ganglion-cell cultures. TLR7 was found to be expressed in cell bodies and axons of isolated neurons (Fig. 4D), but could not be detected in surrounding supporting cells (Fig. 4E).

TLR7 Stimulation of HNEC and TGN Induces SP Release

As TLR7 was mainly expressed on nerve fibers, which predominantly produce SP, and epithelial cells, which act as the first line of defense in the nose, the study focused on TLR7 to investigate the contribution of SP to viral recognition by TLRs in the airways. Stimulation of isolated trigeminal ganglion neurons (TGN) by R-837 resulted in a significant and concentration-

dependent increase in SP after 15 minutes (Fig. 4A). Levels remained significantly high after 30 minutes and 4 hours (Fig 4A, F).

Mouse nasal epithelial cells (MNEC) were similarly stimulated with the TLR7 agonist R-837. Stimulation resulted in a significant increase in the levels of SP after only 15 minutes compared to the control (Fig 4B). A concentration-dependent increase in SP levels was observed and the levels of SP obtained after 15 min were maintained at 30 min and at 4 hours (Fig. 4B, F).

To confirm if this release occurs in humans, nasal epithelial cells from human subjects (HNEC) were stimulated with the TLR7 agonist R-837 and the TLR7/8 agonist R-848. Stimulation by R-837 resulted in a significant increase in the levels of SP after only 15 minutes compared to control (Fig 4C). A concentration-dependent increase in SP levels was observed and the levels of SP obtained after 15 min were maintained at 30 min and at 4 hours (Fig. 4C, F). Stimulation with R-848 similarly resulted in SP release, but the effect was less prominent. However, a significant effect was observed at an R-848 concentration of 100 μ g/mL (Fig. 4C).

Due to the different seeding techniques used in the TGN, MNEC and HNEC cultures, the release of SP was quantified as a concentration per 1000 cells. The results show that stimulation of TGN resulted in 500-fold higher production of SP compared to stimulation of MNEC and HNEC (Fig. 4G).

SP affects TLR expression on HNEC

The function of the immediate release of SP from the nasal epithelia and sensory neurons was subsequently assessed by stimulating HNEC with different concentrations of SP. To confirm the

ability for SP to activate epithelial cells, expression of NK1R was measured. NK1R was found to be expressed on HNEC (Fig. 5A). SP stimulation resulted in an upregulation of TLR1, TLR3, TLR4, TLR7 and TLR9 (Fig 5B-F). TLR2, TLR5, TLR6 and TLR8 appeared to be unresponsive to stimulation with SP (see Fig. E2 in this article's online Repository at www.jacionline.org). Exposure to SP increased expression of TLR1, 3 and 4 in a concentration-dependent manner (Fig. 5B-D). The increase in expression reached statistical significance within 30 minutes at SP concentrations of 50 nM and higher. Upon exposure to SP, the increase in TLR7 and TLR9 expression reached statistical significance at only 10 nM of SP, after which it plateaued: exposure to higher SP concentrations did not lead to further TLR expression (Fig. 5E-F).

To determine how TLRs were upregulated, expression of TLR4 in HNEC was imaged following SP stimulation. In unstimulated HNEC, TLR4 was located mainly intracellularly around the nucleus (Fig. 6A, C). Following SP stimulation, higher expression of TLR4 could be detected on the cell surface (Fig. 6B, D). Some cells demonstrated polarized (white arrow) or punctate (yellow arrow) expression.

To evaluate if the upregulation of TLRs was specific to SP, cells were cultured in the presence of SP and its antagonist aprepitant. Expression of TLR1, TLR4, TLR7 and TLR9 remained at control levels (Fig. 7A, C, D, E). Comparatively, TLR3 upregulation was not blocked by aprepitant (Fig. 7B).

Discussion

The neuropeptide substance P (SP) was once demonstrated to play a critical role in animal models of chronic airway inflammation^{2, 3}. However, this was difficult to validate clinically⁸, suggesting an alternative role for this neuropeptide. The present study demonstrates that SP is released from epithelial cells and sensory neurons in the nasal mucosa in response to TLR7 stimulation. The released SP in turn rapidly upregulates TLR expression on epithelial cells. This suggests that SP plays a role in priming the innate immune system during viral infections.

SP production in response to airway viral infection is a well-established phenomenon^{11, 12}, with evidence suggesting a role for SP in the symptoms of infection¹³. As viruses are primarily and initially recognized by TLRs, the present study was designed to investigate if TLRs could contribute to the early production of SP during viral infection. As TLR7 was almost exclusively found on nerve fibers, with an ability to produce SP, and epithelial cells, which act as the first line of defense, the study focused on the relationship between TLR7 and SP.

Stimulation with the TLR7 agonist R-837 and the TLR7/8 agonist R-848 resulted in a rapid release of SP from sensory neurons, mouse nasal epithelial cells (MNEC) and human nasal epithelial cells (HNEC). Given that TLR8 has not been found to be expressed on HNECs²¹, the present results indicate that the observed SP increase was primarily related to TLR7 stimulation. Significantly, SP levels increased after just 15 minutes and were maintained after 4 hours, suggesting that both nasal epithelia and sensory neurons release SP in a single burst, within minutes of TLR7 stimulation. Although previous studies have demonstrated that TLR agonists

can heighten neuropeptide release by other stimulants (e.g. capsaicin)²², this is the first study to show that TLR stimulation can directly and rapidly trigger SP secretion.

SP was shown to be released from both sensory neurons and nasal epithelium, in a concentration-dependent manner. However, the former release was found to be about 500 times higher than the latter, when the release was expressed in relation to the number of cells involved. Although this demonstrates a profound capability of neurons to produce SP, nasal epithelial cells are local, and they far outnumber neurons, indicating their powerful role of producing SP in the nasal mucosa. The mechanism by which this occurs is beyond the scope of this study, but may be related to an interaction between TLR7 and transient receptor potential ankyrin 1 (TRPA1), as activation of the latter is known to induce SP release²³. TLR7 has also been shown to interact with TRPA1 on the surface of sensory neurons²⁴, as well as on airway epithelial cells²⁵. The ability for both cell types to rapidly secrete SP in response to TLR stimulation and viral invasion represents a novel first-line defense mechanism for the cells of the upper airway.

Neuropeptides have previously been identified as having immunomodulatory roles, able to alter the expression of TLRs in certain cell types^{16, 26}. For example, in a disease-model setting, treatment with VIP alters TLR expression in gut mucosa²⁶. Comparatively, within 24 hr, SP is able to up-regulate the expression of TLR2 and TLR8 on human mast cells *in vitro*¹⁶. In our study, HNEC demonstrated high expression of the NK1R, leading to the hypothesis that SP may have an influence on the innate host defense responses of nasal epithelial cells by altering TLR expression.

Stimulation of HNEC with SP resulted in an increase in the expression of TLR1, TLR3, TLR4, TLR7 and TLR9. Upregulation of all but TLR3 were demonstrated to be the result of interaction of SP with NK1R. As TLR3 was itself upregulated by the NK1R inhibitor aprepitant, an inhibition of TLR3 expression could not be verified. It is possible that other NK receptors are somewhat involved in SP induced TLR3 upregulation.

Significantly, the increase in the mucosal expression of TLRs was very rapid, evident after just 30 minutes of SP stimulation. Due to the speed at which the increased expression of TLRs occurs, it is likely due to alterations in trafficking, rather than *de novo* synthesis²⁷. In line with this, stimulation of HNEC with SP resulted in a movement of TLR4 from intracellular compartments close to the nucleus, to the cell surface. It has previously been shown that respiratory epithelial cells express intracellular TLR4 located in pools in the Golgi complex, which is transferred to the cell surface within minutes after stimulation^{28, 29}. Similarly, TLR3, 7 and 9 are trafficked from endoplasmic reticulum (ER) or Golgi pools, to endosomes, upon stimulation³⁰. Therefore, it is likely that the upregulation of TLRs shown in this study is due to SP-induced redistribution.

Our results show that SP can be released from epithelial cells and sensory neurons in the nose within 15 minutes of TLR7 stimulation. Since neuropeptides are pre-packaged in secretory vesicles, they are available for rapid secretion upon cell stimulation. Neuropeptides are known to have a slow turnover rate²⁷, indicating that widespread release of SP is an energy consuming process. Further, SP has a short half-life in tissues, ranging from seconds to tens of minutes³¹. The mechanism behind the short half-life is explained by the kinetics of enzymatic and/or

chemical degradation in the extracellular environment, by binding to cells, and by the cellular internalization of SP³². Therefore, our postulated release of SP in a single burst suggests a more efficient use of this mediator.

This study also demonstrates that SP induces up-regulation of TLRs in the nasal epithelium, and that the up-regulation of TLRs occurs at the protein level within 30 minutes of cell stimulation. Hence, it appears that in the case of viral stimuli, SP is released promptly after activation of TLR7, which in turn causes TLRs to be up-regulated. This rapidly prepares the epithelial cells against incoming viral antigens. SP is not continuously produced upon TLR7 stimulation, and after the immediate up-regulation of TLRs, the TLRs themselves take over and inflammation proceeds along the traditional lines. Therefore, neuropeptides may be designed for kick-starting the immune system, locally regulating or priming acute inflammation.

The presented data shows for the first time that the burst of neuronal and epithelial SP acts as an initial defensive response to viral stimuli in the upper airway, up-regulating TLRs in the nasal epithelia, rapidly preparing the airway for pathogenic insult. In regards to the lower airway previous studies have shown that circulating SP is up-regulated in asthmatic patients who present with an exacerbation³³. We have also shown that engagement of TLRs induces exacerbated hyperreactivity in a well-established murine model of asthma, in an inflammation-independent manner³⁴. This makes it tempting to suggest that the interplay between SP and TLRs is an important and novel mechanism not only preparing the nasal mucosa against incoming antigens, but also inducing augmented hyperreactivity and dysregulated inflammation in viral-induced

asthma exacerbation. Hence, this might be a key pathway in the acute and local pathogen-driven innate immune response that is of major clinical importance.

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References

1. Harrison S, Geppetti P. Substance p. *Int J Biochem Cell Biol* 2001; 33:555-76.
2. Joos G, Kips J, Pauwels R, Van der Straeten M. The respiratory effects of neuropeptides. *Eur J Respir Dis Suppl* 1986; 144:107-36.
3. Lundberg JM, Saria A, Brodin E, Rosell S, Folkers K. A substance P antagonist inhibits vagally induced increase in vascular permeability and bronchial smooth muscle contraction in the guinea pig. *Proc Natl Acad Sci U S A* 1983; 80:1120-4.
4. Baraniuk JN, Lundgren JD, Okayama M, Goff J, Mullol J, Merida M, et al. Substance P and neurokinin A in human nasal mucosa. *Am J Respir Cell Mol Biol* 1991; 4:228-36.
5. Baraniuk JN, Lundgren JD, Goff J, Mullol J, Castellino S, Merida M, et al. Calcitonin gene-related peptide in human nasal mucosa. *Am J Physiol* 1990; 258:L81-8.
6. Mosimann BL, White MV, Hohman RJ, Goldrich MS, Kaulbach HC, Kaliner MA. Substance P, calcitonin gene-related peptide, and vasoactive intestinal peptide increase in nasal secretions after allergen challenge in atopic patients. *J Allergy Clin Immunol* 1993; 92:95-104.
7. Baraniuk JN, Ali M, Yuta A, Fang SY, Naranch K. Hypertonic saline nasal provocation stimulates nociceptive nerves, substance P release, and glandular mucous exocytosis in normal humans. *Am J Respir Crit Care Med* 1999; 160:655-62.
8. Barnes PJ. Neurogenic inflammation in the airways. *Respir Physiol* 2001; 125:145-54.
9. Xagorari A, Chlichlia K. Toll-like receptors and viruses: induction of innate antiviral immune responses. *Open Microbiol J* 2008; 2:49-59.
10. Whitsett JA, Alenghat T. Respiratory epithelial cells orchestrate pulmonary innate immunity. *Nat Immunol* 2015; 16:27-35.
11. O'Connor TM, O'Connell J, O'Brien DI, Goode T, Bredin CP, Shanahan F. The role of substance P in inflammatory disease. *J Cell Physiol* 2004; 201:167-80.
12. Bost KL. Tachykinin-modulated anti-viral responses. *Front Biosci* 2004; 9:1994-8.
13. Haynes LM, Tonkin J, Anderson LJ, Tripp RA. Neutralizing anti-F glycoprotein and anti-substance P antibody treatment effectively reduces infection and inflammation associated with respiratory syncytial virus infection. *J Virol* 2002; 76:6873-81.
14. Harzenetter MD, Novotny AR, Gais P, Molina CA, Altmayr F, Holzmann B. Negative regulation of TLR responses by the neuropeptide CGRP is mediated by the transcriptional repressor ICER. *J Immunol* 2007; 179:607-15.
15. Jiang X, McClellan SA, Barrett RP, Zhang Y, Hazlett LD. Vasoactive intestinal peptide downregulates proinflammatory TLRs while upregulating anti-inflammatory TLRs in the infected cornea. *J Immunol* 2012; 189:269-78.
16. Tancowny BP, Karpov V, Schleimer RP, Kulka M. Substance P primes lipoteichoic acid- and Pam3CysSerLys4-mediated activation of human mast cells by up-regulating Toll-like receptor 2. *Immunology* 2010; 131:220-30.
17. Malin SA, Davis BM, Molliver DC. Production of dissociated sensory neuron cultures and considerations for their use in studying neuronal function and plasticity. *Nat Protoc* 2007; 2:152-60.
18. Arebro J, Tengroth L, Razavi R, Kumlien Georen S, Winqvist O, Cardell LO. Antigen-presenting epithelial cells can play a pivotal role in airway allergy. *J Allergy Clin Immunol* 2016; 137:957-60 e7.
19. Tengroth L, Millrud CR, Kvarnhammar AM, Kumlien Georen S, Latif L, Cardell LO. Functional effects of Toll-like receptor (TLR)3, 7, 9, RIG-I and MDA-5 stimulation in nasal epithelial cells. *PLoS One* 2014; 9:e98239.
20. Fransson M, Benson M, Erjefalt JS, Jansson L, Uddman R, Bjornsson S, et al. Expression of Toll-like Receptor 9 in nose, peripheral blood and bone marrow during symptomatic allergic rhinitis. *Respiratory Research* 2007; 8.
21. van Tongeren J, Roschmann KI, Reinartz SM, Luiten S, Fokkens WJ, de Jong EC, et al. Expression profiling and functional analysis of Toll-like receptors in primary healthy human nasal epithelial cells shows no correlation and a refractory LPS response. *Clin Transl Allergy* 2015; 5:42.
22. Diogenes A, Ferraz CC, Akopian AN, Henry MA, Hargreaves KM. LPS sensitizes TRPV1 via activation of TLR4 in trigeminal sensory neurons. *J Dent Res* 2011; 90:759-64.

23. Nakamura Y, Une Y, Miyano K, Abe H, Hisaoka K, Morioka N, et al. Activation of transient receptor potential ankyrin 1 evokes nociception through substance P release from primary sensory neurons. *J Neurochem* 2012; 120:1036-47.
24. Park CK, Xu ZZ, Berta T, Han Q, Chen G, Liu XJ, et al. Extracellular microRNAs activate nociceptor neurons to elicit pain via TLR7 and TRPA1. *Neuron* 2014; 82:47-54.
25. Mukhopadhyay I, Gomes P, Aranake S, Shetty M, Karnik P, Damle M, et al. Expression of functional TRPA1 receptor on human lung fibroblast and epithelial cells. *J Recept Signal Transduct Res* 2011; 31:350-8.
26. Zhongkai L, Jianxin Y, Weichang C. Vasoactive intestinal peptide promotes gut barrier function against severe acute pancreatitis. *Mol Biol Rep* 2012; 39:3557-63.
27. Chanput W, Mes J, Vreeburg RA, Savelkoul HF, Wichers HJ. Transcription profiles of LPS-stimulated THP-1 monocytes and macrophages: a tool to study inflammation modulating effects of food-derived compounds. *Food Funct* 2010; 1:254-61.
28. Hornef MW, Frisan T, Vandewalle A, Normark S, Richter-Dahlfors A. Toll-like receptor 4 resides in the Golgi apparatus and colocalizes with internalized lipopolysaccharide in intestinal epithelial cells. *J Exp Med* 2002; 195:559-70.
29. Guillot L, Medjane S, Le-Barillec K, Balloy V, Danel C, Chignard M, et al. Response of human pulmonary epithelial cells to lipopolysaccharide involves Toll-like receptor 4 (TLR4)-dependent signaling pathways: evidence for an intracellular compartmentalization of TLR4. *J Biol Chem* 2004; 279:2712-8.
30. Lee BL, Barton GM. Trafficking of endosomal Toll-like receptors. *Trends Cell Biol* 2014; 24:360-9.
31. McGregor GP, Bloom SR. Radioimmunoassay of substance P and its stability in tissue. *Life Sciences* 1983; 32:655-62.
32. Skidgel RA, Engelbrecht S, Johnson AR, Erdos EG. Hydrolysis of substance p and neurotensin by converting enzyme and neutral endopeptidase. *Peptides* 1984; 5:769-76.
33. Cardell LO, Uddman R, Edvinsson L. Low Plasma-Concentrations of Vip and Elevated Levels of Other Neuropeptides during Exacerbations of Asthma. *European Respiratory Journal* 1994; 7:2169-73.
34. Starkhammar M, Larsson O, Georen SK, Leino M, Dahlen SE, Adner M, et al. Toll-Like Receptor Ligands LPS and Poly (I:C) Exacerbate Airway Hyperresponsiveness in a Model of Airway Allergy in Mice, Independently of Inflammation. *PLoS One* 2014; 9.

Figure Legends**Figure 1.**

Expression of TLRs in human nasal mucosa. (A-E) Representative images from one healthy subject demonstrating expression of TLR3 (A), TLR4 (B), TLR7 (C), TLR9 (D) and negative control (E), as well as β -III tubulin in human nasal mucosa. Biopsies from 5 healthy subjects were stained. TLRs (green); β -III tubulin (red); co-localized (yellow). Scale bar: 50 μ m.

Figure 2.

Expression of TLRs in human trigeminal ganglia. Representative images from one healthy subject demonstrating expression of TLR3 (A), TLR4 (B), TLR7 (C), TLR9 (D) in human trigeminal ganglia sections. Biopsies from 6 healthy subjects were stained. Scale bar: 50 μ m.

Figure 3.

Expression of TLR7 in human nasal mucosa. Representative images from one healthy subject demonstrating expression of TLR7 (green) and EpCAM (red) (A), β -III tubulin (red) (B) and Substance P (blue), co-localization (light blue) (C). Biopsies from 6 healthy subjects were stained. Scale bar: 50 μ m.

Figure 4.

SP production by HNEC (n=5) (A), MNEC (n=4-5) (B) and TGN (n=4) (C)
* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, comparing unstimulated vs. stimulated cells. Black line: mean.
(D-E) TLR7 (green) in TGN (neurofilament/red); DAPI (blue); co-localization = yellow (n=10).

Scale bar: 50 μ m. (F) SP production after R-837 (100 μ g/mL), values: mean $\pm$ SEM (n=4-5). (G) SP production 15 min after R-837 (5 μ g/mL) (n=5). ***p<0.001. Black line: mean.

Figure 5.

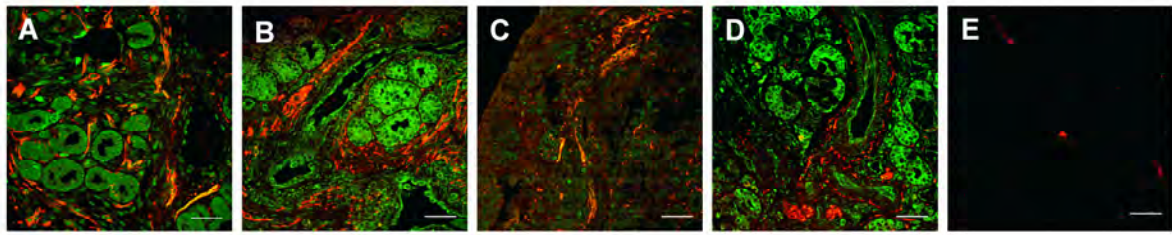
Epithelial expression of TLRs following stimulation with SP. (A) Unstained (black histogram), isotype (grey histogram) and NK1R (red histogram) (n=5). HNEC expression of TLR1 (B), TLR3 (C), TLR4 (D), TLR7 (E) and TLR9 (F) after stimulation with SP or vehicle for 30 minutes (n=5). *p<0.05, **p<0.01, comparing unstimulated vs. stimulated cells. Values: mean $\pm$ SEM

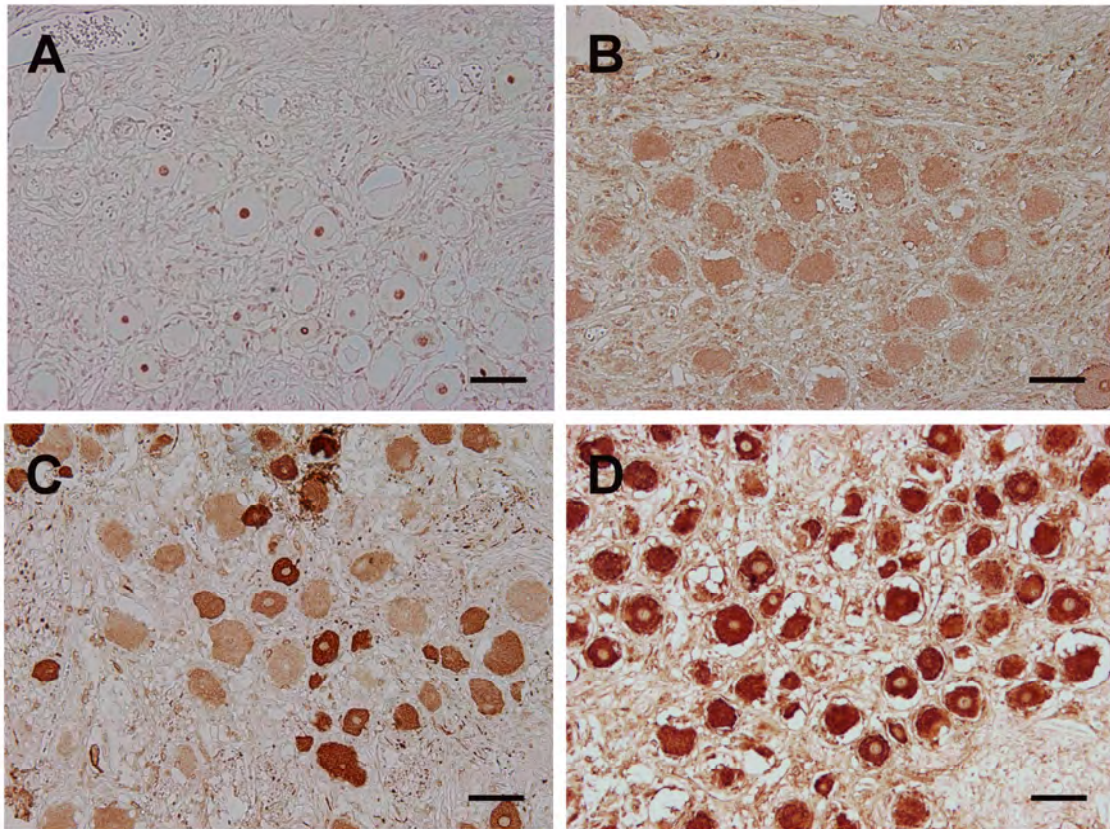
Figure 6.

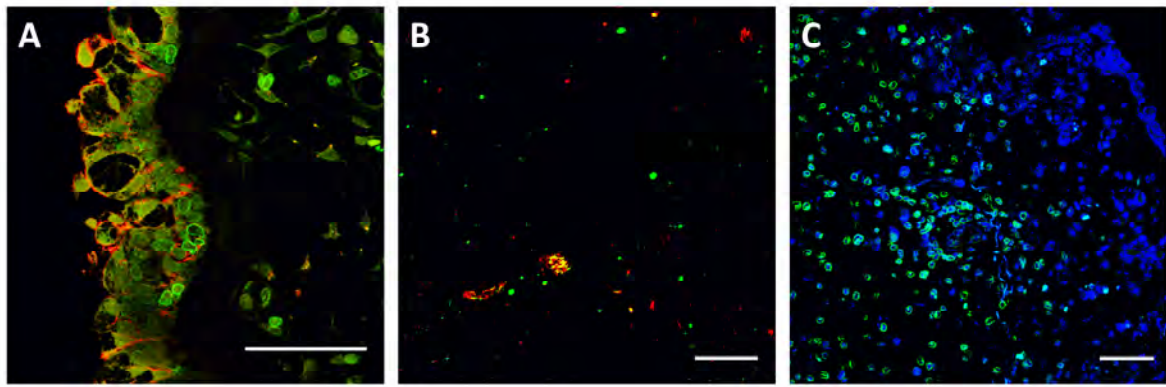
Epithelial expression of TLR4 following stimulation with SP. (A, C) Unstimulated HNEC, (B, D) SP stimulated HNEC (100nM SP for 30 min). Representative images from one healthy subject demonstrating expression of TLR4 (green) and nucleus (blue) (n=5). Scale bar: 50 μ m.

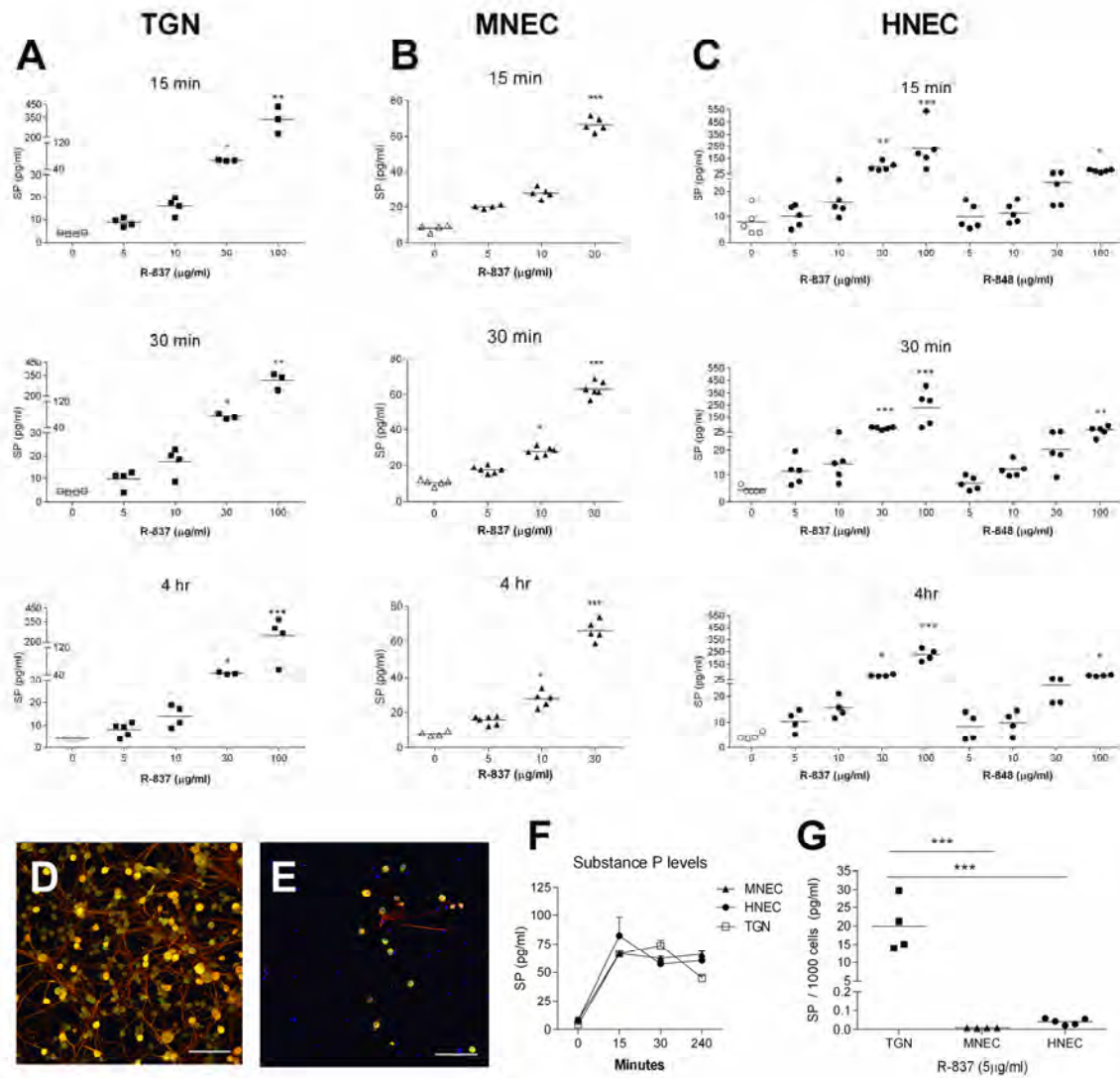
Figure 7.

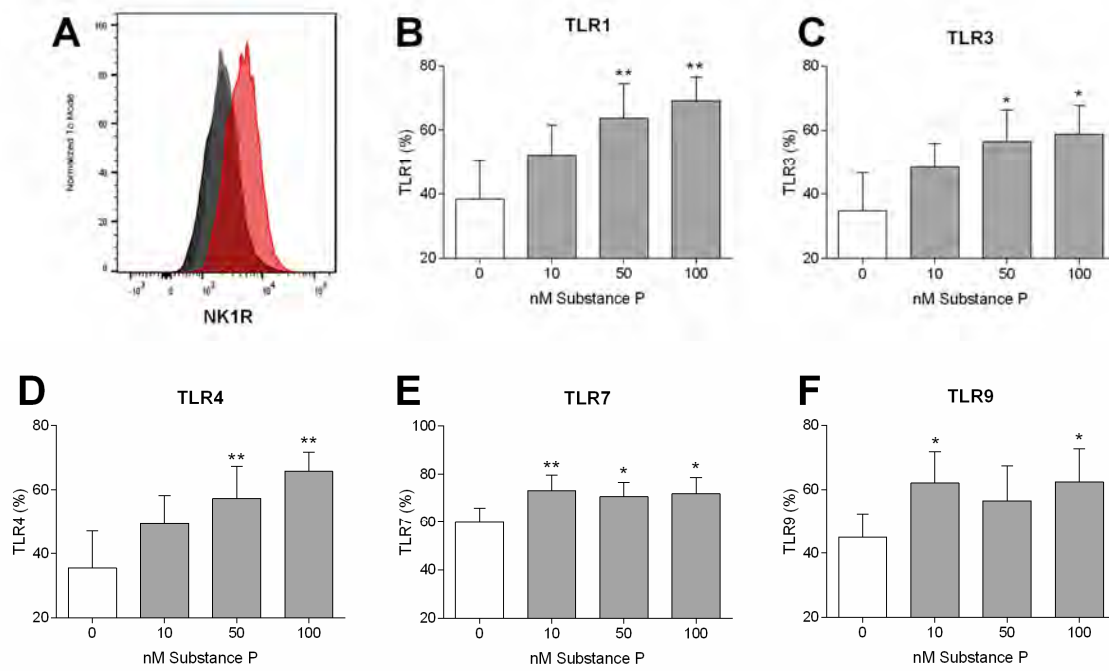
Inhibition of epithelial TLR expression following stimulation with SP. HNEC expression of TLR1 (A), TLR3 (B), TLR4 (C), TLR7 (D) and TLR9 (E) 30 min after SP stimulation (100 nM), with or without prior incubation (3 minutes) with Aprepitant (10 nM) (n=5). *p<0.05, **p<0.01, comparing all cells vs. SP stimulated cells. All values: mean $\pm$ SEM.

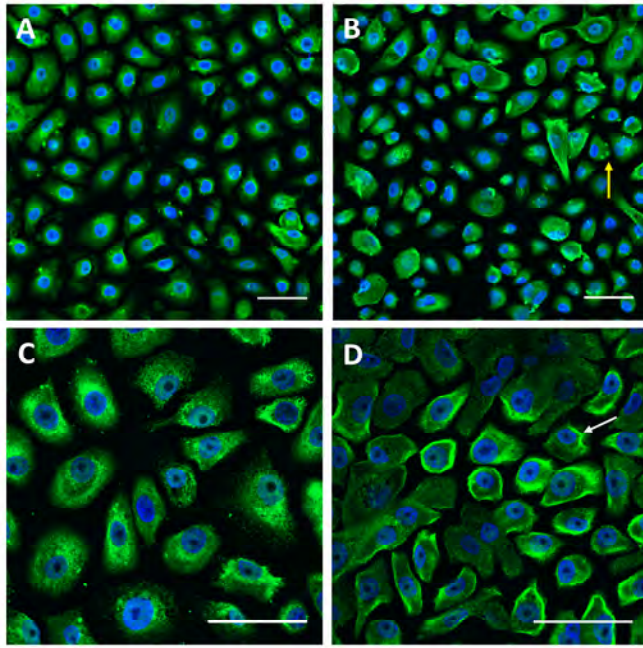


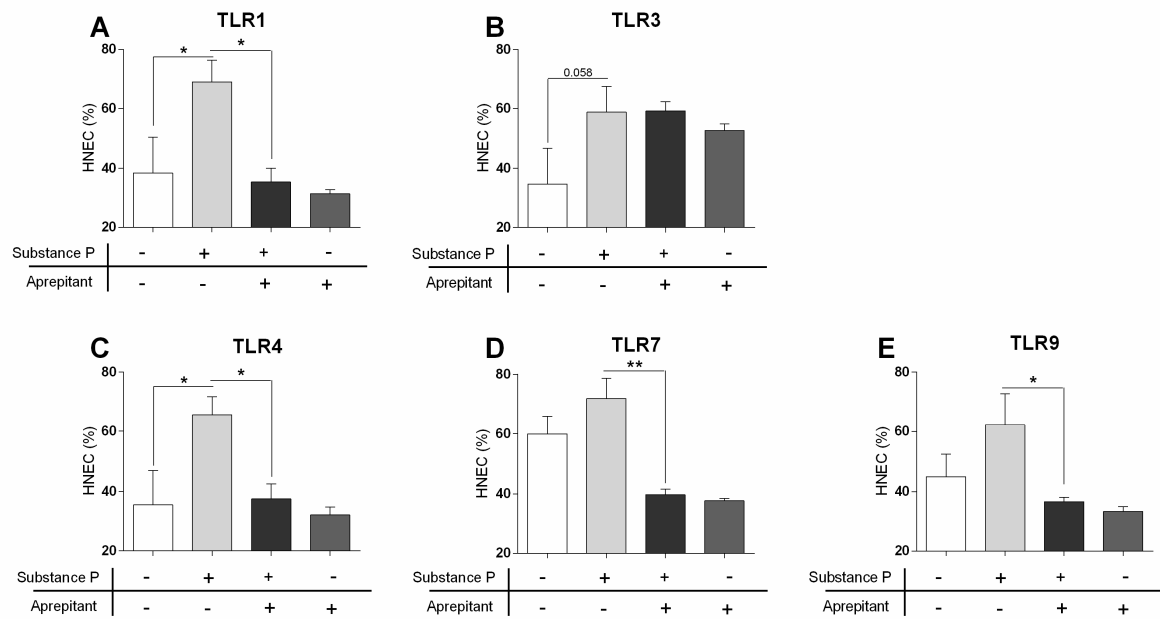








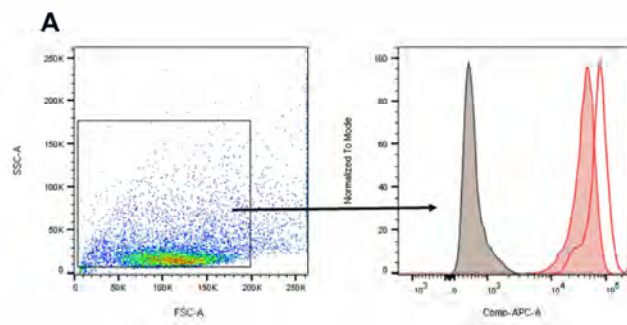




E Figure Legends

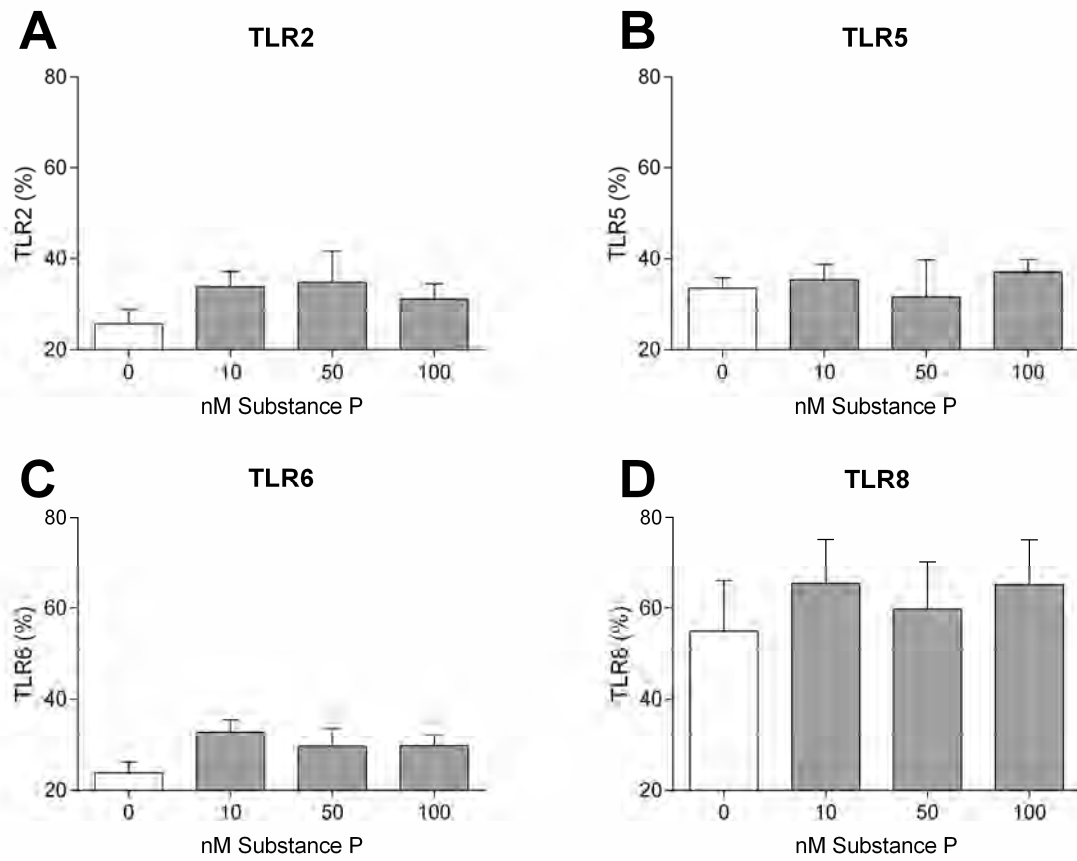
Figure E1. FACS gating strategy to detect TLR expression on HNEC. (A) Example of cultured HNEC gated on their forward-side properties and TLR9 expression. Unstained (gray histogram), Unstimulated (red histogram) and Substance P stimulated (open histogram). (B) Experimental designed color panel, including spectral overlap.

Figure E2. Epithelial expression of TLRs following stimulation with SP. (A-D) HNEC expression of TLR2 (A), TLR5 (B), TLR6 (C), TLR8 (D) after stimulation with SP (10 nM/50 nM/100 nM) or vehicle for 30 minutes (n=3-4). All values: mean $\pm$ SEM.



B

	FITC	APC/AF647	PE
FITC	100	0	0.042
APC	0.007	100	0
PE	0.589	0.017	100



Supplementary Materials

Supplementary Materials and Methods

Isolation and Culture of Trigeminal Sensory Neurons (TGNs)

Trigeminal ganglia were quickly removed and digested by incubation at 37°C with 40U/mL papain (Sigma-Aldrich, St. Louis, USA) for 30 minutes followed by 4 mg/mL Collagenase D (Roche, Mannheim, Germany) and 5 mg/mL dispase II (Sigma-Aldrich) for 30 minutes. Following mechanical dissociation, cells were separated from debris and enriched for neurons by two-step centrifugation (1800 x g, 10 min) through a 30% to 60% Percoll (GE Healthcare, Uppsala, Sweden) gradient. Isolation was carried out in L15 medium supplemented with 10mM HEPES, 10 mM glucose, 1000 U/mL penicillin, 1000 µg/mL streptomycin and 2.5 µg/mL Fungizone (Gibco, Paisley, UK). For immunocytochemistry, cells were seeded at a concentration of 500,000 cells/well onto poly-D-lysine/laminin-coated coverslips (BD Biocoat, VWR, Karlskoga, Sweden) in 24-well cell culture plates. For cell stimulation experiments, cells were seeded at a concentration of 500,000 cells/well in poly-D-lysine/laminin-coated 24-well plates (BD Biocoat). Cells were cultured in F12 medium (Gibco), supplemented with 10% Fetal Bovine Serum (FBS) (Gibco), 1000 U/mL penicillin, 1000 µg/mL streptomycin, 0.25 µg/mL Fungizone and 50 ng/mL recombinant β -Nerve Growth Factor (β -NGF) (Peprotech, London, United Kingdom) for 3–4 days. All cells were cultured in a humidified chamber at 37°C with 5% CO₂.

Isolation and Culture of Murine Nasal Epithelial Cells (MNECs)

Thirty-two mice were sacrificed by cervical dislocation and septal mucosa were dissected and placed in DMEM/F12 (Gibco). Nasal septa were pooled and transferred to dissociation medium [MEM (minimum essential medium) (Gibco), 100 IU/ml penicillin, 100 µg/ml streptomycin, 1.4 mg/ml Pronase (Sigma-Aldrich) and 0.1 µg/ml DNase (Sigma-Aldrich)] for 1 hour, after which DMEM/F12 containing 10% FBS was added to stop the reaction. Tissue was passed through a cell strainer twice and resuspended in DMEM/F12 containing 100 IU/ml penicillin, 100 µg/ml streptomycin, 0.25µg/ml Fungizone, 10% FBS, 2.5ng/ml EGF and 120 IU/ml ITS Universal Culture Supplement (Corning, Wiesbaden, Germany) (complete culture medium). Suspensions were incubated at 37°C for 2 hours on 100mm Primaria culture dishes (BD Biosciences). The cell suspensions were seeded onto collagen-coated 24-well well plates at 250,000 cells/ml and maintained in a humidified chamber at 37°C with 5% CO₂. Cells were grown in complete culture medium until confluency was reached, after which media was changed to maintenance medium (complete culture medium containing only 2% FBS).

Immunohistochemistry of nasal biopsies

Following deparaffinization, rehydration and heat-mediated antigen retrieval in citrate buffer, sections were permeabilized with 1% Triton X-100 (Sigma-Aldrich) and blocked for non-specific binding with 10% normal donkey serum (Abcam, Cambridge, UK) and 1% bovine serum albumin (BSA, Roche). Sections were incubated with primary anti-human antibodies against TLR3 (1:300, ab62566, Abcam) TLR4 (1:20, ab13556, Abcam), TLR7 (1:50, ab45371, Abcam), TLR9 (1:50, ab37154, Abcam), EpCAM (1:50, ab8601, Abcam), Substance P (1:30, ab7340, Abcam) or β III tubulin (1:300, ab78078, Abcam) at 4°C overnight and subsequently incubated with a secondary AlexaFluor 555-conjugated donkey anti-mouse antibody (1:200, Abcam), AlexaFluor 647-conjugated donkey anti-rabbit antibody (1:400, Abcam), AlexaFluor 488-

conjugated donkey anti-rabbit antibody (1:400, Abcam) or a secondary AlexaFluor 488-conjugated donkey anti-rat antibody (1:200, Abcam). Control sections were stained with secondary antibody only. Imaging was performed on a Zeiss LSM800 confocal microscope (Zeiss, Oberkochen, Germany).

Immunohistochemistry of human trigeminal ganglia

Specimens were embedded in paraffin, cut in 5 µm-thick sections and mounted onto glass slides. Ganglia sections were deparaffinized in xylene, rehydrated in decreasing concentrations of ethanol and incubated with a target retrieval solution. Sections were subsequently permeabilized with 1% Triton X-100, followed by hydrogen peroxidase to reduce endogenous peroxidase activity. Primary mouse antibodies against TLR1, TLR3 and TLR9, as well as primary rabbit antibodies against TLR4, TLR5 and TLR7 were applied to sections for 2 hr. As a negative control, antibody diluent or N-series Universal Negative Control Reagent (DakoCytomation, Copenhagen, Denmark) was used. Sections were subsequently incubated with horseradish peroxidase (HRP)-labelled goat anti-mouse or goat anti-rabbit polymer for 30 minutes followed by 3,3'-diaminobenzidine (DAB) substrate-chromogen for 10 minutes. Sections were dehydrated with increasing concentrations of ethanol, washed with xylene and subsequently mounted in Pertex (Histolab Products AB, Gothenburg, Sweden). Imaging was performed with an Olympus Provis AX70 microscope, with a Pixera Pro 150ES Camera (Pixera, San Jose, CA, USA).

Immunocytochemistry of HNEC

Cells were permeabilised with 0.25% Triton X-100 and blocked for non-specific binding with 10% normal donkey serum. Samples were incubated with a primary rabbit polyclonal antibody against TLR4 (1:25, Abcam) followed by a secondary AlexaFluor 488-conjugated donkey anti-

rabbit antibody (Abcam). Cells were stored in ProLong Diamond Antifade Mountant with DAPI (Life Technologies, Paisley, UK).

Immunocytochemistry of murine neurons

Cultured murine trigeminal neurons were fixed with 4% PFA, permeabilized with 0.25% Triton X-100 and blocked for non-specific binding with 5% normal goat serum and 5% normal donkey serum. Samples were incubated with a primary chicken polyclonal antibody against Neurofilament H (1:1500, Merck Millipore) and a primary rabbit polyclonal antibody against TLR7 (1:300, Abcam), followed by a secondary AlexaFluor555-conjugated goat anti-chicken antibody (1:200, Abcam) and a secondary AlexaFluor488-conjugated donkey anti-rabbit antibody (1:200, Abcam). Coverslips were mounted onto slides with ProLong Diamond Antifade Mountant with DAPI (Life Technologies). Imaging was performed using an Olympus Provis microscope, connected to an Olympus U-PS camera.