Nerve growth factor: a novel mediator in asthma

Annick de Vries

Cover design: Martijn Pieck en Annick de Vries
Dorsal root ganglia in culture

ISBN 90.393.2475.1

2

Nerve growth factor: a novel mediator in asthma

Nerve growth factor: een nieuwe mediator in astma (met een samenvatting in het Nederlands)

Proefschrift ter verkrijging van de graad van doctor
aan de Universiteit Utrecht
op gezag van de Rector Magnificus, Prof. dr. H.O. Voorma,
ingevolge het besluit van het College voor Promoties,
in het openbaar te verdedigen
op vrijdag 22 september 2000 des namiddags te 2.30 uur.

door

Annick de Vries
geboren op 2 december 1970 te Utrecht

promotor:	prof. dr. Frans P. Nijkamp, Universiteit Utrecht
co-promotoren:	dr. Ferdi Engels, Universiteit Utrecht dr. Paul A.J. Henricks, Universiteit Utrecht

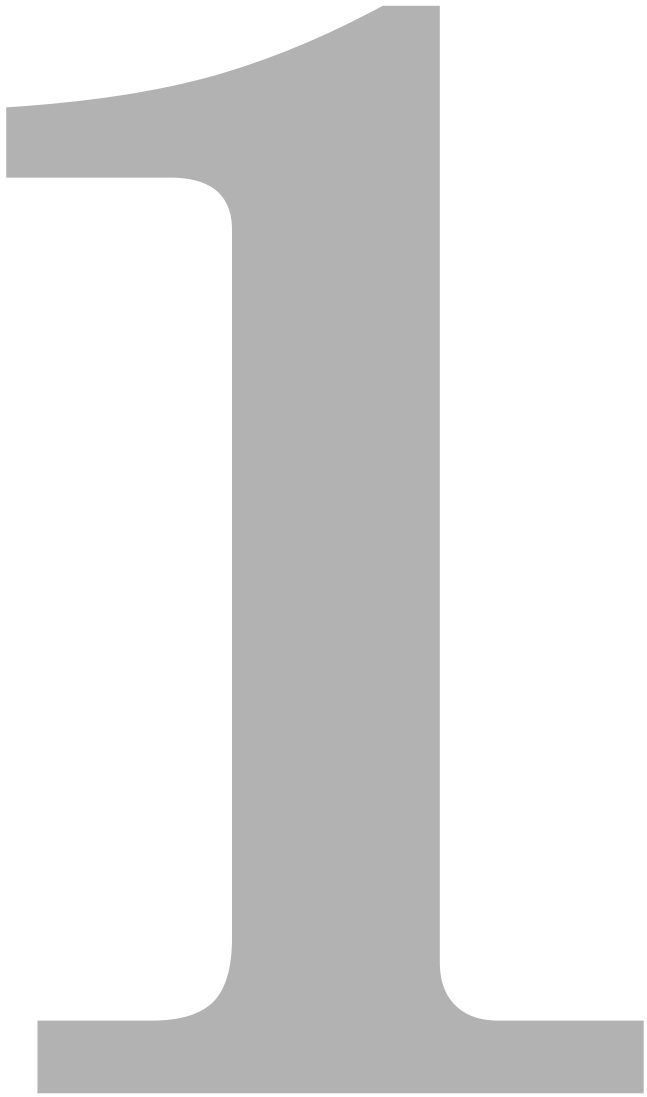
The studies presented in this thesis were performed at
the Department of Pharmacology and Pathophysiology,
Utrecht Institute for Pharmaceutical Sciences,
Faculty of Pharmacy,
Utrecht University, Utrecht,
The Netherlands

Publication of this PhD thesis was financially supported by:

Dr. Saal van Zwanenbergstichting
Netherlands Asthma Foundation

Contents

Introduction
Nerve growth factor:
Facts in relation to its possible role in asthma



Why study nerve growth factor in relationship to asthma?

In November 1996 a review article appeared carrying the title "Nerve growth factor: from neurotrophin to neurokine" (1). The review described a new role for nerve growth factor (NGF) in inflammatory responses. Hence, the classification of NGF as neurotrophin, a protein that induces outgrowth of neurons, was suggested to be changed into neurokine, indicating a similarity to cytokines and thus a role in immunological responses. This publication by Nobel Prize winner Rita Levi-Montalcini and colleagues was in view with our ideas on the possible role of NGF in allergic asthma. This introduction elaborates on the possible role of NGF in allergic asthma.

Allergic asthma

A reversible airway obstruction, airway inflammation and an increased sensitivity to bronchoconstrictive stimuli characterize allergic asthma, the latter phenomenon is also referred to as airway hyperresponsiveness (2-4). Two distinct phases can be discriminated: an early and a late asthmatic response. The early phase involves acute bronchoconstriction, extensive vascular leakage and mucus hypersecretion, immediately after allergen challenge; this usually resolves within 1-2 hrs. The early asthmatic response is mainly caused by products released from mast cells, as allergen specific IgE, induced during a preceding sensitization period, induces massive mast cell activation at the time of allergen challenge (5). One of the important mediators, released by mast cells and responsible for early phase effects is histamine (6). The early phase can be followed by a late phase several hours after the allergen challenge. Infiltration of inflammatory cells in the airways, epithelial shedding, mucus hypersecretion and bronchoconstriction characterize this phase (7, 8). The late response and airway hyperresponsiveness are associated with increases in airway eosinophils and release of inflammatory mediators and cytokines (4, 7, 9-11). The mechanism by which the allergic response relates to airway hyperresponsiveness and respiratory smooth muscle reactivity is still a matter of debate (recently discussed in 12).

Neurogenic inflammation

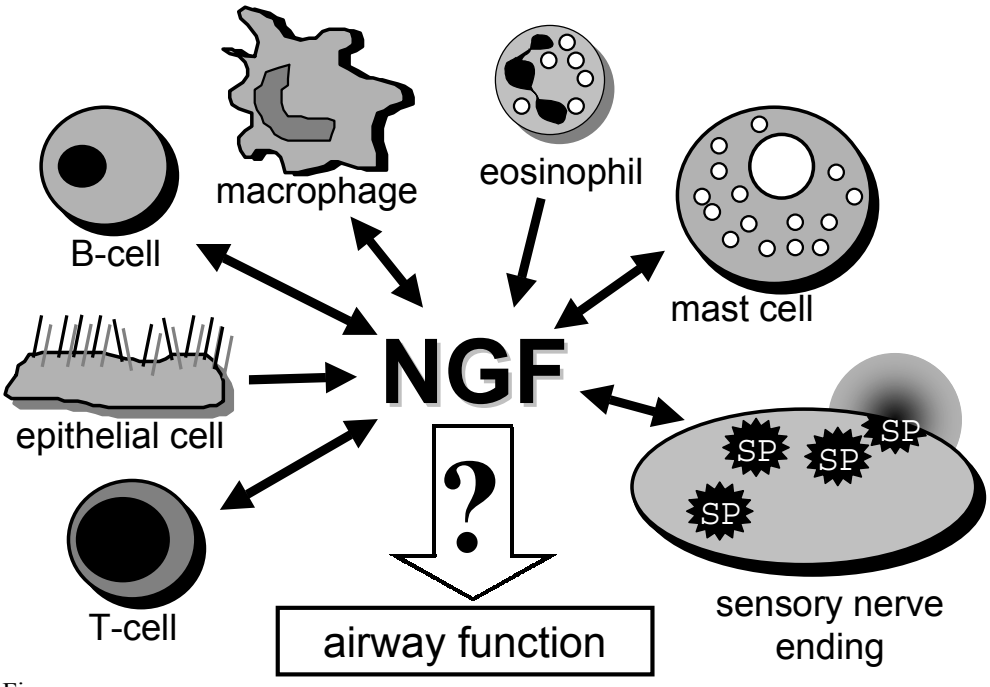
Neurogenic inflammation contributes to pathological phenomena in several diseases. For example, in asthma (13), in inflamed skin (14) and in neuropathic pain (15). Neurogenic inflammation involves a change in function of sensory neurons due to inflammatory mediators, thereby inducing an enhanced release of peptides from the nerves (13). Sensory neurons are characterized by their expression of certain peptides, the tachykinins. The pungent derived from red peppers, capsaicin, induces release of tachykinins. Sensory neurons are sensitive to capsaicin, indicating that these neurons are expressing the vanilloid receptor 1 (VR_1; 16, 17). Sensory nerves containing tachykinins are also referred to as

excitatory non-adrenergic, non-cholinergic (eNANC) nerves. This as opposed to inhibitory NANC (iNANC) nerves, containing vasoactive intestinal peptide and nitric oxide (48).
Several studies have shown a role for neurogenic inflammation in the induction of airway hyperresponsiveness in animal models, in particular for the tachykinins substance P and neurokinin A. Examples are: 13-hydroxyoctadecadienoic acid- (20), IL-5- (21), ozone- (22), citric acid- (23), and toluene diisocyanate-induced (24) airway hyperresponsiveness. Guinea pigs sensitized to and challenged with ovalbumin, as a model for allergic asthma, show an increase in substance P immunoreactive neurons in nodose ganglia (25, 26). Tachykinin levels are elevated in plasma during the exacerbations of asthma and in lavage fluids after allergen challenge (27, 28). Moreover, protective effects on the induction of allergic airway pathology by neurokinin receptor antagonists have been reported (29).
NGF is able to augment neurogenic inflammation (30) and perhaps in this way plays a role in allergic asthma. NGF specifically upregulates synthesis of products of the preprotachykinin gene, which codes for several tachykinins, such as substance P and neurokinin A (31-36). Moreover, NGF changes the properties of sensory nerve endings by inducing a very fast accumulation of second messengers (37) or phosphorylation of key transduction-related proteins or ion channels, thereby sensitizing the peripheral sensory nerve ending (37, 38). Similarly, over-expression of NGF specifically in the airways of mice leads to an enhanced sensory and sympathetic innervation of the airways. These mice were more sensitive to capsaicin, which induced increases in airway resistance (39).

The protein NGF and its receptors
NGF belongs to the family of neurotrophins, which control the survival, differentiation and maintenance of neurons in the peripheral and central nervous systems (40). NGF is a homodimeric molecule (41). Two molecular forms of NGF exist: 7S NGF and 2.5S NGF molecules (42, 43).
Nerve growth factor can interact with two receptors: either the tyrosine kinase receptor A (trkA) or p75. TrkA is a receptor with tyrosine kinase activity that forms a high-affinity binding site for NGF (Kd $\approx 10^{-11}$M, 44). NGF binds to the trkA receptor and the NGF-trkA complex is internalized and retrogradely transported to the nucleus, where mRNA levels for preprotachykinin, the precursor for tachykinins, are affected (34, 35). Alternatively, trkA activation leads, in a tyrosine kinase-dependent manner, to phosphorylation of proteins at the nerve terminal, which can induce changes in the properties of the nerve ending (37, 38).
The receptor p75 can bind several neurotrophic factors with nanomolar affinity: NGF, brain derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3) and NT-4 (reviewed in 61). The p75 low affinity NGF receptor also causes an upregulation in tachykinin content in sensory nerves (45).

NGF and inflammation

Several inflammatory mediators, including interleukin-1, interleukin-4, interleukin-5, tumor necrosis factor α and interferon-γ, induce release of NGF (46, 47). In addition to neurons, also non-neuronal cells such as mast cells (48), fibroblasts (46), T-cells (49, 50), B-cells (51), eosinophils (52) lymphocytes (51) and airway epithelial cells (53) can synthesize NGF (see figure).



Figure

Besides neurons, different types of inflammatory cells, such as macrophages, mast cells, eosinophils, B-cells and T-cells produce and release NGF. Furthermore, airway epithelial cells produce and release NGF. A number of these cells express the high-affinity receptor for NGF, trkA; e.g. sensory neurons containing substance P (SP), T-cells, B-cells, macrophages and mast cells.

Many inflammatory cells express a high affinity NGF receptor: monocytes (30, 38, 54), mast cells (55), basophils (56), macrophages (57), T-cells (50, 58) and B-cells (59, 60). NGF shows various effects in inflammatory models. This could be relevant in relation to allergic asthma (reviewed in 46). Indeed, NGF promotes inflammatory mediator release from basophils (56), mast cells (54, 62), T- and B-

cells (1, 50, 63), eosinophils (52) and macrophages (64). Furthermore, NGF induces antibody synthesis and secretion from B cells (63), induces differentiation of monocytes into macrophages (65) and is an autocrine survival factor that rescues macrophages (57). NGF has several effects on mast cells: it attracts mast cells (66), induces phenotypic switching of the mast cells (67) and changes the expression of cytokines (68).

Role of NGF in inflammatory pain

The role of NGF has been studied extensively in relation to inflammatory pain (69). A local rise in NGF is found in inflammatory pain (69, 70). Furthermore, hyperalgesia can be induced by simply applying NGF locally (71, 72). In inflammatory models, decreasing the amount of available NGF with the use of antibodies reduces inflammatory pain (70, 73-76). Most pain studies suggest that NGF induces pain by an increased release of substance P (15, 77, 78). Furthermore, the number of trkA immunoreactive neurons is enhanced in a model for inflammatory chronic pain (79). Only a few studies suggest a role for NGF in inflammatory pain by affecting immune cell function (73), involving changes in mast cell function (69, 70).

We postulate that similar mechanisms involving NGF in inflammatory pain could play a role in the inflammation in the airways in allergic asthma. The tachykinin substance P induces constriction of the smooth muscle in the airways (6, 80, 81), increase of vascular permeability (14, 80, 82) and have an effect on immune cell function, such as activating and changing the function of mast cells (83-85).

Hypothesis on a role for NGF in the asthmatic disease

We postulate a role for NGF in the induction of allergic asthma. We hypothesize that NGF can affect airway function by changing the properties of the sensory nerves in the airways. In order to reveal the role of NGF in asthma, we first studied the effect of NGF on airway function (chapter 2). Thereafter we performed a more mechanistic approach to analyze whether sensory nerve endings are involved in effects of NGF on airway function (chapter 3). Furthermore, we studied the role of NGF in an allergic model of asthma. In chapter 4 the influence of NGF on acute bronchoconstriction induced by allergen challenge is documented. In chapter 5 this is followed by a study on the influence of NGF on airway hyperresponsiveness, inflammation and increase in substance P containing nerves 24 hrs after allergen challenge. In chapter 6 we speculate on the presumed communication between mast cells and sensory neurons in this respect. In the discussion (chapter 7) we summarize and speculate on mechanisms by which NGF could play a role in the asthmatic disease.

References

1. Levi-Montalcini R., Skaper S. D., Dal Tosco R., Petrelli L., Leon A. Nerve growth factor: from neurotrophin to neurokine. Trends Neurosci. 1996; 19:514-520.
2. Soler M., Imhof E., Perruchoud A. P. Severe acute asthma. Pathophysiology, clinical assessment, and treatment. Respiration 1990; 57:114-121.
3. Pauwels R. The pharmacology of airway hyperresponsiveness and inflammation. Lung 1990; 168:221-229.
4. Hamelmann E., Schwarze J., Takeda K., Oshiba A., Larsen G. L., Irvin C. G., Gelfand E. W. Noninvasive measurement of airway responsiveness in allergic mice using barometric plethysmography. Am. J. Respir. Crit. Care Med. 1997; 156:766-775.
5. Bingham C. O., Austen K. F. Mast-cell responses in the development of asthma. J. Allergy Clin. Immunol. 2000; 105:527-534.
6. Kudlacz E., Shatzer S., Logan D., Olsen K., Knippenberg R., Hsieh L., Esteve H., Maynard G. A role for histamine and substance P in immediate allergic responses in guinea pig airways: characterization of MDL 108,207DA, a dual H1/NK-1 receptor antagonist. Int. Arch. Allergy Immunol. 1998; 115:169-178.
7. Howarth P. H. The airway inflammatory response in allergic asthma and its relationship to clinical disease. Allergy 1995; 50:13-21.
8. Charlesworth E. N. Late-phase inflammation: influence on morbidity. J. Allergy Clin. Immunol. 1996; 98:S291-297.
9. Gauvreau G. M., Watson R. M., O'Byrne P. M. Kinetics of allergen-induced airway eosinophilic cytokine production and airway inflammation. Am. J. Respir. Crit. Care Med. 1999; 160:640-647.
10. Cieslewicz G., Tomkinson A., Adler A., Duez C., Schwarze J., Takeda K., Larson K. A., Lee J. J., Irvin C. G., Gelfand E. W. The late, but not early, asthmatic response is dependent on IL-5 and correlates with eosinophil infiltration. J. Clin. Invest. 1999; 104:301-308.
11. O'Byrne P. M., Gauvreau G. M., Wood L. J. Interaction between haemopoietic regulation and airway inflammation. Clin. Exp. Allergy 1999; 29 Suppl. 2:27-32.
12. Black J. L., Johnson P. R. What determines asthma phenotype? Is it the interaction between allergy and the smooth muscle? Am. J. Respir. Crit. Care Med. 2000; 161:S207-S210.
13. Barnes P. J. Neurogenic inflammation in airways. Int. Arch. Allergy Appl. Immunol. 1991; 94:303-309.

14. Baluk P. Neurogenic inflammation in skin and airways. J. Investig. Dermatol. Symp. Proc. 1997; 2:76-81.
15. Campbell E. A., Gentry C. T., Patel S., Panesar M. S., Walpole C. S., Urban L. Selective neurokinin-1 receptor antagonists are anti-hyperalgesic in a model of neuropathic pain in the guinea-pig. Neuroscience 1998; 87:527-532.
16. Holzer P. Local effector functions of capsaicin-sensitive sensory nerve endings: involvement of tachykinins, calcitonin gene-related peptide and other neuropeptides. Neuroscience 1988; 24:739-768.
17. Kress M., Zeilhofer H. U. Capsaicin, protons and heat: new excitement about nociceptors. Trends Pharmacol. Sci. 1999; 20:112-118.
18. Kesler B. S., Canning B. J. Regulation of baseline cholinergic tone in guinea-pig airway smooth muscle. J. Physiol. (Lond.) 1999; 518:843-855.
19. Belvisi M. G., Miura M., Stretton D., Barnes P. J. Endogenous vasoactive intestinal peptide and nitric oxide modulate cholinergic neurotransmission in guinea-pig trachea. Eur. J. Pharmacol. 1993; 231:97-102.
20. Engels F., Van Houwelingen A. H., Buckley T. L., Van de Velde M. J., Henricks P. A. J., Nijkamp F. P. Airway hyperresponsiveness induced by 13-hydroxyoctadecadienoic acid (13-HODE) is mediated by sensory neuropeptides. Adv. Prostaglandin Thromboxane Leukot. Res. 1995; 23:361-363.
21. Kraneveld A. D., Nijkamp F. P., Van Oosterhout A. J. M. Role for neurokinin-2 receptor in interleukin-5-induced airway hyperresponsiveness but not eosinophilia in guinea pigs. Am. J. Respir. Crit. Care Med. 1997; 156:367-374.
22. Wu Z. X., Morton R. F., Lee L. Y. Role of tachykinins in ozone-induced airway hyperresponsiveness to cigarette smoke in guinea pigs. J. Appl. Physiol. 1997; 83:958-965.
23. Biyah K., Molimard M., Esmonds-Alt X., Advenier C. SR140333 prevents potentiation by citric acid of plasma exudation induced by histamine in airways. Eur. J. Pharmacol. 1996; 308:325-328.
24. Hunter D. D., Satterfield B. E., Huang J., Fedan J. S., Dey R. D. Toluene diisocyanate enhances substance P in sensory neurons innervating the nasal mucosa. Am. J. Respir. Crit. Care Med. 2000; 161:543-549.
25. Fischer A., McGregor G. P., Saria A., Philippin B., Kummer W. Induction of tachykinin gene and peptide expression in guinea pig nodose primary afferent neurons by allergic airway inflammation. J. Clin. Invest. 1996; 9:2284-2291.
26. Undem B. J., Hunter D. D., Liu M., Haak-Frendscho M., Oakragly A., Fischer A. Allergen-induced sensory neuroplasticity in airways. Int. Arch. Allergy Immunol. 1999; 118:150-153.

27. Lundberg J. M. Tachykinins, sensory nerves, and asthma- an overview. Can. J. Physiol. Pharmacol. 1995; 73:908-914.
28. Nieber K., Baumgarten C. R., Rathsack R., Furkert J., Oehme P., Kunkel G. Substance P and beta-endorphin-like immunoreactivity in lavage fluids of subjects with and without allergic asthma. J. Allergy Clin. Immunol. 1992; 90:646-652.
29. Joos G. F., van Schoor J., Kips J. C., Pauwels R. A. The effect of inhaled FK224, a tachykinin NK-1 and NK-2 receptor antagonist, on neurokinin A-induced bronchoconstriction in asthmatics. Am. J. Respir. Crit. Care Med. 1996; 153:1781-1784.
30. Woolf C. J. Phenotypic modification of primary sensory neurons: the role of nerve growth factor in the production of persistant pain. Phil. Trans. R. Soc. Lond. B 1996; 351:441-448.
31. Donnerer J., Schuligoi R., Stein C. Increased content and transport of substance P and calcitonin gene-related peptide in sensory nerves innervating inflamed tissue: evidence for a regulatory function of nerve growth factor in vivo. Neuroscience 1992; 49:693-698.
32. Otten U., Goedert M., Mayer N., Lembeck F. Requirement of nerve growth factor for developing of substance P containing sensory neurons. Nature 1980; 287:158-159.
33. Zhou D., Zheng X., Bai T. R. Both nerve growth factor (NGF) and leukemia inhibitory factor (LIF) augment substance P release in airway explants (Abstract). Am. J. Respir. Crit. Care Med. 2000; 161:A839.
34. Vedder H., Affolter H. U., Otten U. Nerve growth factor (NGF) regulates tachykinin gene expression and biosynthesis in rat sensory neurons during early postnatal development. Neuropeptides 1993; 24:351-357.
35. Lindsay R. M., Harmar A. J. Nerve growth factor regulates expression of neuropeptide genes in adult sensory neurons. Nature 1989; 337:362-364.
36. Grimes M. L., Zhou J., Beattie E. C., Yuen E. C., Hall D. E., Valletta J. S., Topp K. S., LaVail J. H., Bunnett N. W., Mobley W. C. Endocytosis of activated TrkA: Evidence that nerve growth factor induces formation of signalling endosomes. J. Neurosci. 1996; 16:7950-7964.
37. Knipper M., Beck A., Rylett J., Breer H. Neurotrophin induced second messenger responses in rat brain synaptosomes. Neuroreport 1993; 4:483-486.
38. Woolf C. J., Ma Q. P., Allchorne A., Poole S. Peripheral cell types contributing to the hyperalgesic action of nerve growth factor in inflammation. J. Neurosci. 1996; 16:2716-2723.
39. Hoyle G. W., Graham R. M., Finkelstein J. B., Nguyen K. P., Gozal D., Friedman M. Hyperinnervation of the airways in transgenic mice

overexpressing nerve growth factor. Am. J. Respir. Cell Mol. Biol. 1998; 18:149-157.

40. Ibanez C. F. Emerging themes in structural biology of neurotrophic factors. Trends Neurosci. 1998; 21:438-444.
41. Wiesmann C., Ultsch M. H., Bass S. H., de Vos A. M. Crystal structure of nerve growth factor in complex with the ligand- binding domain of the TrkA receptor. Nature 1999; 401:184-188.
42. Shao N., Wang H., Zhou T., Liu C. 7S nerve growth factor has different biological activity from 2.5S nerve growth factor in vitro. Brain Res. 1993; 609:338-340.
43. Silverman R. E., Bradshaw R. A. Nerve growth factor: subunit interactions in the mouse submaxillary gland 7S complex. J. Neurosci. Res. 1982; 8:127-136.
44. Kaplan D. R., Hempstead B. L., Martin-Zanca D., Chao M. V., Parada L. F. The trk proto-oncogene product: a signal transducing receptor for nerve growth factor. Science 1991; 252:554-558.
45. Lee K. F., Li E., Huber L. J., Landis S. C., Sharpe A. H., Chao M. V., Jaenisch R. Targeted mutation of the gene encoding the low affinity NGF receptor p75 leads to deficits in the peripheral sensory nervous system. Cell 1992; 69:737-749.
46. Hattori A., Iwasaki S., Murase K., Tsujimoto M., Sato M., Hayashi K., Kohno M. Tumor necrosis factor is markedly synergistic with interleukin 1 and interferon-gamma in stimulating the production of nerve growth factor in fibroblasts. FEBS Lett. 1994; 340:177-180.
47. Yoshida K., Kakihana M., Chen L. S., Ong M., Baird A., Cage F. H. Cytokine regulation of nerve growth factor-mediated cholinergic neurotrophic activity synthesized by astrocytes and fibroblasts. J. Neurochem. 1992; 59:919-931.
48. Leon A., Buriani A., Dal Toso R., Fabris M., Romanello S., Aloe L., Levi-Montalcini R. Mast cells synthesize, store, and release nerve growth factor. Proc. Natl. Acad. Sci. USA 1994; 91:3739-3743.
49. Mizuma H., Takagi K., Miyake K., Takagi N., Ishida K., Takeo S., Nitta A., Nomoto H., Furukawa Y., Furukawa S. Microsphere embolism-induced elevation of nerve growth factor level and appearance of nerve growth factor immunoreactivity in activated T- lymphocytes in the rat brain. J. Neurosci. Res. 1999; 55:749-761.
50. Lambiase A., Bracci-Laudiero L., Bonini S., Bonini S., Starace G., D'Elios M. M., De Carli M., Aloe L. Human CD4+ T cell clones produce and release nerve growth factor and express high affinity nerve growth receptors. J. Allergy Clin. Immunol. 1997; 100:408-414.

51. Barouch R., Appel E., Kazimirsky G., Braun A., Renz H., Brodie C. Differential regulation of neurotrophin expression by mitogens and neurotransmitters in mouse lymphocytes. J. Neuroimmunol. 2000; 103:112-121.
52. Solomon A., Aloe L., Pe'er J., Frucht-Pery J., Bonini S., Levi-Schaffer F. Nerve growth factor is preformed in and activates human peripheral blood eosinophils. J. Allergy Clin. Immunol. 1998; 102:454-460.
53. Fox A. J., Barnes P. J., Belvisi M. G. Release of nerve growth factor from human airway epithelial cells (Abstract). Am. J. Respir. Crit. Care Med. 1997; 155:A157.
54. Matsuda H., Coughlin M. D., Bienenstock J., Denburg J. A. Nerve growth factor promotes human hemopoietic colony growth and differentiation. Proc. Natl. Acad. Sci. USA 1988; 85:6508-6512.
55. Tam S. Y., Tsai M., Yamaguchi M., Yano K., Butterfield J. H., Galli S. J. Expression of functional TrkA receptor tyrosine kinase in the HMC-1 human mast cell line and in human mast cells. Blood 1997; 90:1807-1820.
56. Burgi B., Otten U. H., Ochensberger B., Rihs S., Heese K., Ehrhard P. B., Ibanez C. F., Dahinden C. A. Basophil priming by neurotrophic factors. Activation through the trk receptor. J. Immunol. 1996; 15:5582-5588.
57. Garaci E., Caroleo M. C., Aloe L., Aquaro S., Piacentini M., Costa N., Amendola A., Micera A., Calio R., Perno C. F., Levi-Montalcini R. Nerve growth factor is an autocrine factor essential for the survival of macrophages infected with HIV. Proc. Natl. Acad. Sci. USA 1999; 96:14013-14018.
58. Ehrhard P. B., Erb P., Graumann U., Otten U. Expression of nerve growth factor and nerve growth factor receptor tyrosine kinase Trk in activated CD4-positive T-cell clones. Proc. Natl. Acad. Sci. USA 1993; 90:10984-10988.
59. Melamed I., Kelleher C. A., Franklin R. A., Brodie C., Hempstead B., Kaplan D., Gelfand E. W. Nerve growth factor signal transduction in human B lymphocytes is mediated by gp140trk. Eur. J. Immunol. 1996; 26:1985-1992.
60. Schenone A., Gill J. S., Zacharias D. A., Windebank A. J. Expression of high- and low-affinity neurotrophin receptors on human transformed B lymphocytes. J. Neuroimmunol. 1996; 64:141-149.
61. Braun A., Lommatzsch M., Renz H. The role of neurotrophins in allergic bronchial asthma. Clin. Exp. Allergy 2000; 30:178-186.
62. Tal M., Liberman R. Local injection of nerve growth factor (NGF) triggers degranulation of mast cells in rat paw. Neurosci. Lett. 1997; 22:129-132.

63. Otten U., Ehrhard P., Peck R. Nerve growth factor induced growth and differentiation of human B lymphocytes. Proc. Natl. Acad. Sci. USA 1989; 86:10059-10063.
64. Susaki Y., Shimizu S., Katakura K., Watanabe N., Kawamoto K., Matsumoto M., Tsudzuki M., Furusaka T., Kitamura Y., Matsuda H. Functional properties of murine macrophages promoted by nerve growth factor. Blood 1996; 88:4630-4637.
65. Ehrhard P. B., Ganter U., Stalder A., Bauer J., Otten U. Expression of functional trk protooncogene in human monocytes. Proc. Natl. Acad. Sci. USA 1993; 90:5423-5427.
66. Sawada J., Itakura A., Tanaka A., Furusaka T., Matsuda H. Nerve growth factor functions as a chemoattractant for mast cells through both mitogen-activated protein kinase and phosphatidylinositol 3-kinase signaling pathways. Blood 2000; 95:2052-2058.
67. Getchell M. L., Kulkarni-Narla A., Takami S., Albers K. M., Getchell T. V. Age-dependent phenotypic switching of mast cells in NGF-transgenic mice. Neuroreport 1995; 6:1261-1266.
68. Marshall J. S., Gomi K., Blennerhassett M. G., Bienenstock J. Nerve growth factor modifies the expression of inflammatory cytokines by mast cells via a prostanoid-dependent mechanism. J. Immunol. 1999; 162:4271-4276.
69. McMahon S. B. NGF as a mediator of inflammatory pain. Phil. Trans. R. Soc. Lond. B 1996; 351:431-440.
70. Lewin G. R., Rueff A., Mendell L. M. Peripheral and central mechanisms of NGF-induced hyperalgesia. Eur. J. Neurosci. 1994; 6:1903-1912.
71. Dyck P. J., Peroutka S., Rask C., Burton E., Baker M. K., Lehman K. A., Gillen D. A., Hokanson J. L., O'Brien P. C. Intradermal recombinant human nerve growth factor induces pressure allodynia and lowered heat-pain treshold in humans. Neurology 1997; 48:501-505.
72. Bergmann I., Reiter R., Toyka K. V., Koltzenburg M. Nerve growth factor evokes hyperalgesia in mice lacking the low- affinity neurotrophin receptor p75. Neurosci. Lett. 1998; 255:87-90.
73. Mendell L. M., Albers K. M., Davis B. M. Neurotrophins, nociceptors, and pain. Microsc. Res. Tech. 1999; 45:252-261.
74. Koltzenburg M., Bennett D. L., Shelton D. L., McMahon S. B. Neutralization of endogenous NGF prevents the sensitization of nociceptors supplying inflamed skin. Eur. J. Neurosci. 1999; 11:1698-1704.
75. Ro L. S., Chen S. T., Tang L. M., Jacobs J. M. Effect of NGF and anti-NGF on neuropathic pain in rats following chronic constriction injury of the sciatic nerve. Pain 1999; 79:265-274.

76. Jaggar S. I., Scott H. C., Rice A. S. Inflammation of the rat urinary bladder is associated with a referred thermal hyperalgesia which is nerve growth factor dependent. Br. J. Anaesth. 1999; 83:442-448.
77. Malcangio M., Ramer M. S., Boucher T. J., McMahon S. B. Intrathecally injected neurotrophins and the release of substance P from the rat isolated spinal cord. Eur. J. Neurosci. 2000; 12:139-144.
78. Dray A., Rang H. The how and why of chronic pain states and the what of new analgesia therapies. Trends Neurosci. 1998; 21:315-317.
79. Pezet S., Ont niente B., Grannec G., Calvino B. Chronic pain is associated with increased TrkA immunoreactivity in spinoreticular neurons. J. Neurosci. 1999; 19:5482-5492.
80. Lundberg J. M., Saria A., Brodin E., Rosell S., Folkers K. A substance P antagonist inhibits vagally induced increase in vascular permeabilty and bronchial smooth muscle contraction in the guinea pig. Proc. Natl. Acad. Sci. USA 1983; 80:1120-1124.
81. Joos G. F., Pauwels R. A., Van der Straeten M. E. The role of neuropeptides as neurotransmitters of non-adrenergic, non- cholinergic nerves in bronchial asthma. Bull. Eur. Physiopathol. Respir. 1988; 23:619-637.
82. Baraniuk J. N., Ali M., Yuta A., Fang S. Y., 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; 160:655-662.
83. Janiszewski J., Bienenstock J., Blennerhassett M. G. Picomolar doses of substance P trigger electrical responses in mast cells without degranulation. Am. J. Physiol. 1994; 276:C138-C145.
84. Mousli M., Hugli T. E., Landry Y., Bronner C. Peptidergic pathway in human skin and rat peritoneal mast cell activation. Immunopharmacol. 1994; 27:1-11.
85. Mousli M., Bronner C., Landry Y., Bockaert J., Rouot B. Direct activation of GTP-binding regulatory proteins (G-proteins) by substance P and compound 48/80. FEBS Lett. 1990; 259:260-262.

Nerve growth factor induces a neurokinin-1 receptor mediated airway hyperresponsiveness in guinea pigs

Annick de Vries, Mark C. Dessing, Ferdi Engels,
Paul A.J. Henricks, and Frans P. Nijkamp

Am. J. Respir. Crit. Care Med. 1999; 159:1541-1544

Abstract

Since asthmatic patients show increased nerve growth factor (NGF) serum levels, we examined the effect of NGF on airway function. Intravenously administered NGF potentiates the histamine-induced bronchoconstriction with a maximum of over 200% in anesthetized spontaneously breathing guinea pigs. Doses of 8 ng and 80 ng NGF per kg body weight induce a significant hyperresponsiveness to histamine. NGF itself does not affect airway reactivity. Airway hyperresponsiveness is observed 30 min and 3 hr after NGF administration, and has disappeared after 24 hr. The neurokinin-1 receptor antagonist SR 140333 completely blocks the NGF-induced hyperresponsiveness, pointing to a role for tachykinins. This is the first report showing a direct relation between peripherally administered NGF and airway hyperresponsiveness. Taking into consideration that plasma NGF levels have been shown to be elevated in asthmatic patients, our result points to an important role for NGF in the pathogenesis of asthma.

Introduction

NGF is a member of the neurotrophin family of proteins that can regulate neuronal development, maintenance, and recovery from injury. NGF has been extensively studied in relation to neurite outgrowth. However, it now becomes clear that NGF also plays a crucial role in inflammation (1).

Inflammation can lead to an enhanced production and release of NGF. Inflammatory mediators, including interleukin-1, interleukin-4, interleukin-5, tumor necrosis factor α, and interferon-γ have been shown to induce the release of NGF (2, 3). In addition to neurons, non-neuronal cells, such as mast cells (4), fibroblasts (2) and epithelial cells (5) are able to synthesize NGF.

NGF affects immune cell activity, as it promotes inflammatory mediator release from basophils (6), mast cells (7, 8), T- and B-cells (9-11) and macrophages (12). NGF is also able to sensitize neurons and induce an enhanced production of Substance P and other tachykinins (13, 14). An enhanced innervation of predominantly sensory nerves, producing Substance P, can be found in the airways of transgenic mice overexpressing NGF in the airways (15). An enhanced expression of tachykinin mRNA in the nodose ganglia in a guinea pig model for asthma has been shown as well (16). Previously, we have shown a role for tachykinins in the development of airway hyperresponsiveness in the guinea pig (17, 18).

Recently, increased levels in serum NGF were found in asthmatic patients (19). Airway inflammation and hyperresponsiveness are characteristic features in the asthmatic disease (20), and NGF seems to be a possible mediator in these events.

Therefore, we investigated whether NGF can induce airway hyperresponsiveness and whether tachykinins are involved.

Methods

Lung function measurement
Male Hartley guinea pigs (400-600 g; Harlan CPB, Zeist, The Netherlands) were anesthetized with urethane (2 g/kg body weight intraperitoneally). Lung function was measured in spontaneously breathing guinea pigs, essentially after Amdur and Mead (21). Air flow and tidal volume were determined by cannulating and connecting the trachea via a Fleisch flow head (Meijnhart, The Netherlands) to a pneumotachograph. A pressure transducer (MP45-2; Validyne Engineering Corp., Northridge, CA) measured the transpulmonary pressure by the difference between the tracheal cannula and an oesophagal cannula. In this way pulmonary resistance (R_L) was determined breath by breath by dividing transpulmonary pressure by airflow at isovolumetric points. R_L values were averaged per three breaths and are presented as actual value. A small polyethylene catheter (PE-50) was placed in the right jugular vein for intravenous administration of different compounds. Animal studies were approved by the Animal Care Committee of the Utrecht University.

Materials
In the present study we used the precursor of NGF, murine NGF 7S (Sigma Chemical Co., St. Louis, MO and Alomone Labs, Jerusalem, Israel) in the guinea pig, as all neurotrophic factors are highly conserved in different species (22). NGF was administered intravenously at doses of 0.8, 8 or 80 ng per kg body weight. NGF was injected 30 min, 3 or 24 hr before starting airway function measurement. The airway resistance was measured in the anesthetized guinea pig upon intravenous administration of increasing doses of histamine. The dose-response curve lasted 30-40 minutes. The neurokinin-1 receptor antagonist SR 140333 (23), 2 μg per kg body weight (kindly provided by Sanofi Recherche, Montpellier, France) was administered intravenously 10 min before the injection of 8 ng NGF per kg body weight. Pilot experiments revealed that 2 μg SR 140333 per kg body weight completely blocked the substance P-induced decrease in blood pressure in the anesthetized guinea pig (data not shown). In control animals, instead of NGF or SR 140333, vehicles were administered; saline containing 0.01 % bovine serum albumine and saline containing 1 % ethanol, respectively.

Statistics
Means and standard error of the mean (SEM) were calculated. P-values were determined using an unpaired Student's t-test for the comparison of two means.

For multiple comparisons of single means an analysis of variance (ANOVA) followed by Bonferroni's test was used. Probability values of $P<0.05$ were considered significantly different.

Results

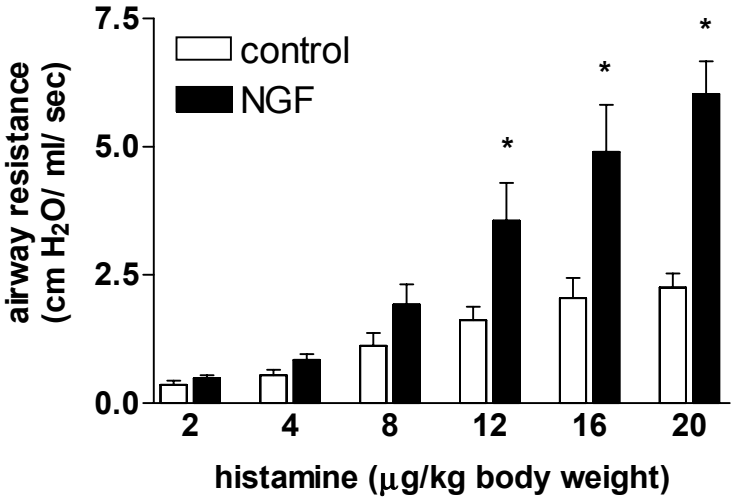


Figure 1. Induction of airway hyperresponsiveness to histamine by NGF. NGF (8 ng per kg body weight) or vehicle was administered 30 min before administration of increasing doses of histamine (intravenously). Bars represent mean ± SEM (n=5 per group). * $p<0.05$ control vs. NGF-treated, Student's t-test.

A strong hyperresponsiveness (an increase in contractile response) to histamine was found 30-70 min after injection of 8 ng NGF per kg body weight (fig. 1). The airway resistance after the highest dose of histamine (20 µg per kg body weight) increased significantly from 2.3 ± 0.3 cm H_2O/ml/sec in the control animals to 6.0 ± 0.6 cm H_2O/ml/sec in the NGF-treated animals ($P<0.01$). NGF did not induce any change in airway resistance by itself (basal resistance 0.10 ± 0.02 cm H_2O/ml/sec in control animals and 0.13 ± 0.01 cm H_2O/ml/sec in NGF (8 ng per kg body weight) animals.

The effect of NGF on airway responsiveness to histamine was dose-dependent (fig. 2). Administration of 0.8 ng NGF was without effect on the histamine-induced increase in airway resistance, whereas 80 ng NGF induced an airway hyperresponsiveness equal to 8 ng NGF.

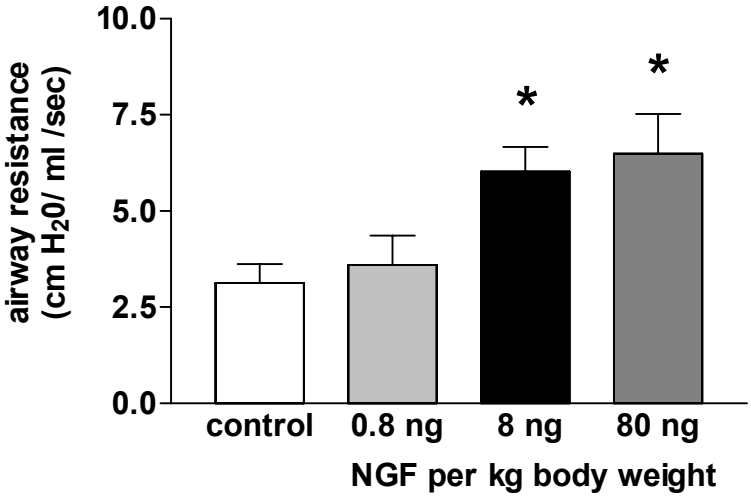


Figure 2. Effect of different doses of NGF on airway responsiveness. Saline and three different doses of NGF, 0.8 ng, 8 ng, or 80 ng NGF per kg body weight, were administered 30 min before administration of increasing doses of histamine. Only the airway resistance to the highest dose of histamine, 20 µg histamine per kg body weight, is depicted. Bars represent mean ± SEM (n=14 for pooled controls, n=4/5 for NGF-treated animals). * P<0.01 control vs. NGF treated, Student's t-test.

A significant increase in airway responsiveness could still be found 3 hr after the injection of NGF (fig. 3). Maximum values were 2.8 ± 0.6 cm H_2O/ml/sec in saline and 5.4 ± 0.4 cm H_2O/ml/sec in NGF-treated animals (P<0.01). Histamine induced increases in airway resistance were no longer different from control animals 24 hr after the intravenous injection of NGF.
The neurokinin-1 receptor antagonist SR 140333 was able to completely inhibit the NGF-induced airway hyperresponsiveness (fig. 4).

Discussion

Asthmatic patients show a highly elevated level of serum NGF (19). This is the first study showing a rather acute increase of airway responsiveness to histamine after a single, peripheral, injection of NGF. Therefore, we postulate a role for NGF in the development of asthma. The induction of airway hyperresponsiveness by NGF was dose-dependent as 8 ng and 80 ng NGF per kg body weight caused a significant increase in airway responsiveness, whereas no effect was found using

the lower dose of 0.8 ng NGF. NGF showed an increase on airway responsiveness within 30 min to 3 hr after the administration, whereas no increase could be determined 24 hr after a single injection of 8 ng NGF.

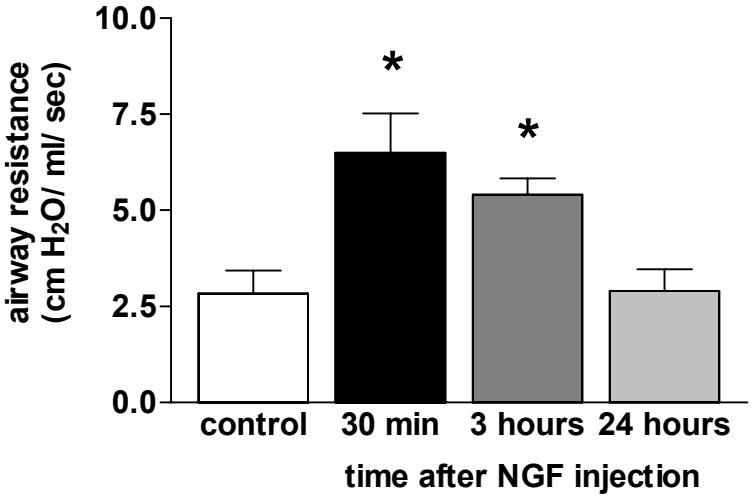


Figure 3. Effect of a single injection of NGF on the airway responsiveness measured at different time points. A single dose of NGF (80 ng per kg body weight) was administered 30 min, 3 hr, or 24 hr before administration of the doses of histamine. Only the airway resistance to the highest dose of histamine, 20 µg histamine per kg body weight, is depicted. Bars represent mean ± SEM (n=15 for pooled controls, n=4/5 for NGF-treated animals). * $P<0.02$ control vs. NGF treated, Student's t-test

NGF is able to augment neurogenic inflammation (24), and this could be one of the mechanisms via which airway hyperresponsiveness was induced. It is known that NGF changes the properties of sensory nerve endings by inducing a very fast accumulation of second messenger in synaptosomes (25), by sensitizing the nerve terminal (24) or by altering neuropeptide levels in sensory nerves (14). Previous studies, in our and other groups, have shown a role for tachykinins in the IL-5 (17), 13-hydroxyoctadecadienoic acid (18), ozone (26), and citric acid (27) induced airway hyperresponsiveness in animal models. Moreover, tachykinin levels are elevated in asthmatics (28), and protective effects of neurokinin receptor antagonists have been reported (29). In this study we show that the NGF-induced airway hyperresponsiveness is mediated via the neurokinin-1 receptor. The preferred ligand for the neurokinin-1 receptor is substance P; however, there is also a relative high affinity for neurokinin A (28).

Two receptors are known for NGF: the low-affinity receptor p75 neurotrophin receptor and the high-affinity tyrosine kinase receptor A (trkA). Upon contact NGF

binds to the trkA receptor and in a tyrosine kinase-dependent manner phosphorylates key transduction-related proteins or ion channels, thereby sensitizing the peripheral sensory nerve ending (25, 30). Alternatively, the NGF-trkA complex is internalized and retrogradely transported to the nucleus, where the preprotachykinin mRNA levels (precursor for substance P and neurokinin A) and protein levels (substance P and neurokinin A) are altered (14, 31). The p75 low-affinity NGF receptor is also shown to be responsible for an upregulation in tachykinin content in sensory nerves (32). Any of these mechanisms could account for an increased release of substance P, presumably from the sensory nerve endings, and thereby induction of airway hyperresponsiveness. The NGF-induced neuronal sensitization could also be indirect, via the release of sensitizing mediators from trkA expressing inflammatory cells, e.g. mast cells and monocytes (7, 24, 30).

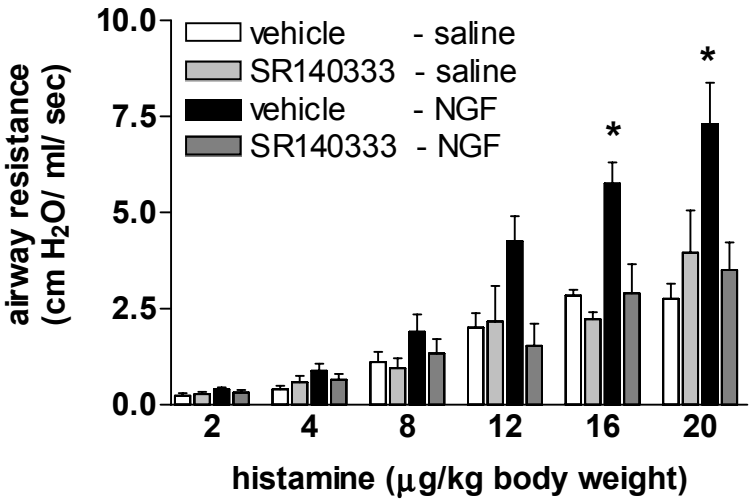


Figure 4. Effect of the neurokinin-1 receptor antagonist SR 140333 on the NGF-induced airway hyperresponsiveness. Vehicle or SR 140333 were administered intravenously 10 min before saline or NGF. Saline or NGF (8 ng per kg body weight) were administered 30 min before administration of increasing doses of histamine. Bars represent mean ± SEM (n=4/5 per group). * $P<0.05$ vehicle-NGF vs. SR 140333-NGF and vs. vehicle-saline, ANOVA followed by Bonferroni's test.

In conclusion, this study shows a clear role for the neurokine NGF in developing airway hyperresponsiveness to histamine. Asthmatic patients show an elevated level of serum NGF (19). This suggests a possible relationship between the high NGF serum levels and the pathological situation in asthmatic patients. In the guinea pig 8 - 80 ng NGF per kg body weight (approximately 25 ml blood in a 500

g weighing guinea pig) induced an airway hyperresponsiveness (fig. 2). The dose range in which NGF caused an airway hyperresponsiveness, was comparable to the serum levels NGF found in asthmatic patients: 150 pg NGF/ml serum (19). In several animal models for pain, neutralizing antibodies against NGF have been proven to attenuate the pathological changes due to inflammation or NGF (19, 33, 34) and even the increased levels of substance P (35). Thus, NGF might be a new target for therapy in asthma.

References

1. Woolf C. J., Safieh-Garabedian B., Ma Q. P., Crilly P., Winter J. Nerve growth factor contributes to the generation of inflammatory sensory hypersensitivity. Neuroscience 1994; 62:327-331.
2. Hattori A., Tanaka E., Murase K., Ishida N., Chatani Y., Tsujimoto M., Hayashi K., Kohno M. Tumor necrosis factor stimulates the synthesis and secretion of biologically active nerve growth factor in non-neuronal cells. J. Biol. Chem. 1993; 286:2577-2582.
3. Yoshida K., Kakihana M., Chen L. S., Ong M., Baird A., Cage F. H. Cytokine regulation of nerve growth factor-mediated cholinergic neurotrophic activity synthesized by astrocytes and fibroblasts. J. Neurochem. 1992; 59:919-931.
4. Leon A., Buriani A., Dal Toso R., Fabris M., Romanello S., Aloe L., Levi-Montalcini R. Mast cells synthesize, store, and release nerve growth factor. Proc. Natl. Acad. Sci. USA 1994; 91:3739-3743.
5. Fox A. J., Barnes P. J., Belvisi M. G. Release of nerve growth factor from human airway epithelial cells (Abstract). Am. J. Respir. Crit. Care Med. 1997; 155:A157.
6. Burgi B., Otten U. H., Ochensberger B., Rihs S., Heese K., Ehrhard P. B., Ibanez C. F., Dahinden C. A. Basophil priming by neurotrophic factors. Activation through the trk receptor. J. Immunol. 1996; 15:5582-5588.
7. Matsuda H., Coughlin M. D., Bienenstock J., Denburg J. A. Nerve growth factor promotes human hemopoietic colony growth and differentiation. Proc. Natl. Acad. Sci. USA 1988; 85:6508-6512.
8. Tal M., Liberman R. Local injection of nerve growth factor (NGF) triggers degranulation of mast cells in rat paw. Neurosci. Lett. 1997; 22:129-132.
9. Lambiase A., Bracci-Laudiero L., Bonini S., Bonini S., Starace G., D'Elios M. M., De Carli M., Aloe L. Human CD4+ T cell clones produce and release nerve growth factor and express high affinity nerve growth receptors. J. Allergy Clin. Immunol. 1997; 100:408-414.

10. Levi-Montalcini R., Skaper S. D., Dal Tosco R., Petrelli L., Leon A. Nerve growth factor: from neurotrophin to neurokine. Trends Neurosci. 1996; 19:514-520.
11. Otten U., Ehrhard P., Peck R. Nerve growth factor induced growth and differentiation of human B lymphocytes. Proc. Natl. Acad. Sci. USA 1989; 86:10059-10063.
12. Susaki Y., Shimizu S., Katakura K., Watanabe N., Kawamoto K., Matsumoto M., Tsudzuki M., Furusaka T., Kitamura Y., Matsuda H. Functional properties of murine macrophages promoted by nerve growth factor. Blood 1996; 88:4630-4637.
13. Grimes M. L., Zhou J., Beattie E. C., Yuen E. C., Hall D. E., Valletta J. S., Topp K. S., LaVail J. H., Bunnett N. W., Mobley W. C. Endocytosis of activated TrkA: Evidence that nerve growth factor induces formation of signalling endosomes. J. Neurosci. 1996; 16:7950-7964.
14. Lindsay R. M., Harmar A. J. Nerve growth factor regulates expression of neuropeptide genes in adult sensory neurons. Nature 1989; 337:362-364.
15. Hoyle G. W., Graham R. M., Finkelstein J. B., Nguyen K. P., Gozal D., Friedman M. Hyperinnervation of the airways in transgenic mice overexpressing nerve growth factor. Am. J. Respir. Cell Mol. Biol. 1998; 18:149-157.
16. Fischer A., McGregor G. P., Saria A., Philippin B., Kummer W. Induction of tachykinin gene and peptide expression in guinea pig nodose primary afferent neurons by allergic airway inflammation. J. Clin. Invest. 1996; 9:2284-2291.
17. Kraneveld A. D., Nijkamp F. P., Van Oosterhout A. J. M. Role for neurokinin-2 receptor in interleukin-5-induced airway hyperresponsiveness but not eosinophilia in guinea pigs. Am. J. Respir. Crit. Care Med. 1997; 156:367-374.
18. Engels F., Van Houwelingen A. H., Buckley T. L., Van de Velde M. J., Henricks P. A. J., Nijkamp F. P. Airway hyperresponsiveness induced by 13-hydroxyoctadecadienoic acid (13-HODE) is mediated by sensory neuropeptides. Adv. Prostaglandin Thromboxane Leukot. Res. 1995; 23:361-363.
19. Bonini S., Lambiase A., Bonini S., Angelucci F., Magrini L., Manni L., Aloe L. Circulating nerve growth factor levels are increased in humans with allergic diseases and asthma. Proc. Natl. Acad. Sci. USA 1996; 93:10955-10960.
20. Barnes P. J. Current issues for establishing inhaled corticosteroids as the antiinflammatory agents of choice in asthma. J. Allergy Clin. Immunol. 1998; 10:S427-S433.

21. Amdur M. O., Mead J. Mechanics of respiration in unanesthetized guinea pigs. Am. J. Physiol. 1958; 192:364-368.
22. Rubin J. S., Bradshaw R. Isolation and partial amino acid sequence analysis of nerve growth factor from the guinea pig prostate. J. Neurosci. Res. 1981; 6:451-464.
23. Esmonds-Alt X., Doutremepuich J. D., Heaulme M., Neliat G., Santucci V., Steinberg R., Vilain P., Bichon D., Ducoux J. P., Proietto V., Van Broeck D., Soubrie P., Le Fur G., Breliere J. C. In vitro and in vivo biological activities of SR140333, a novel potent non-peptide tachykinin NK1 receptor antagonist. Eur. J. Pharmacol. 1993; 250:403-413.
24. Woolf C. J. Phenotypic modification of primary sensory neurons: the role of nerve growth factor in the production of persistant pain. Philos. Trans. R. Soc. Lond. B Biol. Sci. 1996; 351:441-448.
25. Knipper M., Beck A., Rylett J., Breer H. Neurotrophin induced second messenger responses in rat brain synaptosomes. Neuroreport 1993; 4:483-486.
26. Wu Z. X., Morton R. F., Lee L. Y. Role of tachykinins in ozone-induced airway hyperresponsiveness to cigarette smoke in guinea pigs. J. Appl. Physiol. 1997; 83:958-965.
27. Biyah K., Molimard M., Esmonds-Alt X., Advenier C. SR140333 prevents potentiation by citric acid of plasma exudation induced by histamine in airways. Eur. J. Pharmacol. 1996; 308:325-328.
28. Lundberg J. M. Tachykinins, sensory nerves, and asthma- an overview. Can. J. Physiol. Pharmacol. 1995; 73:908-914.
29. Joos G. F., van Schoor J., Kips J. C., Pauwels R. A. The effect of inhaled FK224, a tachykinin NK-1 and NK-2 receptor antagonist, on neurokinin A-induced bronchoconstriction in asthmatics. Am. J. Respir. Crit. Care Med. 1996; 153:1781-1784.
30. Woolf C. J., Ma Q. P., Allchorne A., Poole S. Peripheral cell types contributing to the hyperalgesic action of nerve growth factor in inflammation. J. Neurosci. 1996; 16:2716-2723.
31. Vedder H., Affolter H. U., Otten U. Nerve growth factor (NGF) regulates tachykinin gene expression and biosynthesis in rat sensory neurons during early postnatal development. Neuropeptides 1993; 24:351-357.
32. Lee K. F., Li E., Huber L. J., Landis S. C., Sharpe A. H., Chao M. V., Jaenisch R. Targeted mutation of the gene encoding the low affinity NGF receptor p75 leads to deficits in the peripheral sensory nervous system. Cell 1992; 69:737-749.
33. Ma Q. P., Woolf C. J. The progressive tactile hyperalgesia induced by peripheral inflammation is nerve growth factor dependent. Neuroreport 1997; 8:807-810.

34. McMahon S. B. NGF as a mediator of inflammatory pain. Phil. Trans. R. Soc. Lond. B 1996; 351:431-440.
35. Donnerer J., Schuligoi R., Stein C. Increased content and transport of substance P and calcitonin gene-related peptide in sensory nerves innervating inflamed tissue: evidence for a regulatory function of nerve growth factor in vivo. Neuroscience 1992; 49:693-698.

Nerve growth factor acts via sensory nerve endings in inducing airway hyperresponsiveness in guinea pigs

Annick de Vries, Carolien van Rijnsoever, Ferdi Engels,
Paul A.J. Henricks and Frans P. Nijkamp

3

submitted

Abstract

Administration of nerve growth factor (NGF) induces airway hyperresponsiveness in guinea pigs in vivo, as shown in the previous chapter. A receptor antagonist for the tachykinin neurokinin-1 receptor can prevent this hyperresponsiveness. We further investigated the role of sensory nerves in the NGF-induced airway hyperresponsiveness.
We used isolated tracheal rings from guinea pigs to measure tracheal contractility. In these rings sensory nerve endings are present but lack any contact with their cell bodies. In this in vitro system NGF dose-dependently induced a tracheal hyperresponsiveness to histamine. The neurokinin-1 receptor antagonist SR140333 blocked the induction of tracheal hyperresponsiveness to NGF in this isolated system. This suggested a role for the tachykinin substance P, possibly derived from sensory nerve endings. To investigate the role of sensory nerve endings we used the cannabinoid receptor 1 (CB_1) agonist R-methanandamide to inhibit excitatory events at the nerve terminal. The CB_1 receptor agonist indeed blocked the tracheal hyperresponsiveness to NGF in the isolated system as well as the airway hyperresponsiveness in vivo.
Our results indicate that NGF acts by increasing substance P release from sensory nerve endings, which in turn induces airway hyperresponsiveness in guinea pigs.

Introduction

The neurotrophin NGF is a newly studied mediator in relation to allergic disease such as asthma (1-3). In humans with allergic diseases such as rhinoconjunctivitis, urticaria-angioedema and asthma circulating NGF levels are increased (4). In the study of Bonini and colleagues (4), NGF serum levels were particularly high in patients with allergic asthma. An increase in the neurotrophins NGF, brain-derived neurotrophic factor (BDNF), and neurotrophin-3 (NT-3) in bronchoalveolar lavage fluids after allergen challenge is reported in patients with asthma (2). In patients with allergic rhinitis a very fast increase in NGF in the nasal lavage fluids is already observed 10 min after allergen challenge (5). Furthermore, allergen challenge induced an increase in NGF levels in bronchoalveolar lavage fluid of sensitized mice (3).
Inflammatory mediators, including interleukin-1, interleukin-4, interleukin-5, tumor necrosis factor α, and interferon-γ induce the release of NGF (6, 7). In addition to neurons, also non-neuronal cells such as mast cells (8), fibroblasts (6), T-cells (9), eosinophils (10) lymphocytes (11) and airway epithelial cells (12), synthesize NGF. NGF shows various properties in inflammatory models, which could be interesting in relationship to allergic asthma as well (reviewed in 13).

NGF affects immune cell activity, as it promotes inflammatory mediator release from basophils (14), mast cells (15, 16), T- and B-cells (9, 17, 18) and macrophages (19). Furthermore, NGF induces antibody synthesis and secretion from B cells (18), attracts mast cells (20) and induces differentiation of monocytes into macrophages (21). One study focussed on the role of NGF in the development of allergic asthma. Application of antibodies directed against NGF in a mouse model for allergic asthma can prevent T cells shifting to Th2 cells, a hallmark of allergy (3).
Furthermore, NGF is able to sensitize neurons and induce an enhanced production of substance P and other tachykinins in sensory nerves (22, 23). Tachykinins play a role in neurogenic inflammation and in the development of airway hyperresponsiveness and asthma (24, 25). We showed recently that administration of NGF induced airway hyperresponsiveness in guinea pigs (1). The neurokinin-1 receptor antagonist SR140333 prevented this hyperresponsiveness. This points to a role for substance P, the preferred ligand of the neurokinin-1 receptor (1).
The present study focuses on the role of sensory nerves in NGF-induced airway hyperresponsiveness. For that purpose, we used a tissue preparation containing sensory nerve endings, without any contact with their cell bodies to elucidate the involvement of sensory nerve endings versus the neuronal cell body.

Methods

Materials
We used the precursor of NGF, murine NGF 7S (Alomone Labs, Jerusalem, Israel), dissolved in saline containing 0.01% bovine serum albumine. The neurokinin-1 receptor antagonist SR140333 (26) was kindly provided by Sanofi Recherche (Montpellier, France); it was dissolved in saline containing 1% ethanol. Pilot experiments revealed that SR140333 completely blocked the substance P-induced contraction in the isolated tracheal rings (data not shown). R-methanandamide (Tocris Cookson Ltd., Langford, UK) was used to activate the CB_1 receptor and was dissolved in saline containing 1% ethanol. CB_1 receptor stimulation inhibits neuropeptide release (31).

Animals
Male Hartley guinea pigs (400-600 g; Harlan CPB, Zeist, The Netherlands) were used in all experiments. The Animal Care Committee of the Utrecht University approved the animal studies.

In vitro organ bath studies

To isolate the tracheal rings (3 cartilage segments per ring), the animals were killed by an overdose of pentobarbital (Apharmo, Duiven, The Netherlands) intraperitoneally. Tracheal rings were immediately placed in an isometric organ bath set-up. The baths contained heated (37 °C) Krebs solution (containing: NaCl 6.9 g/l, $NaHCO_3$ 22.1 g/l, glucose monohydrate 1.65 g/l, KCl 0.35 g/l, KH_2PO_4 0.16 g/l, $MgSO_4 \cdot 7H_2O$ 0.29 g/l, $CaCl_2 \cdot 2H_2O$ 0.37 g/l) which was gassed with 95% O_2 / 5% CO_2. The experiment started with four washout periods, lasting 15 min each. During these washouts a tension was applied of 2000 mg, 2000 mg, 4000 mg and 2000 mg, respectively. A histamine concentration response curve (10^{-8} M – 10^{-3} M) was subsequently conducted to measure contractility of the tracheal rings.

NGF was applied at concentrations of 0.2, 2 and 20 ng/ml 30 min before the start of the histamine concentration response curve. SR140333 ($3x10^{-6}$ M) and R-methanandamide ($1x10^{-7}$ M) were applied 10 min before addition of NGF to the organ bath.

In vivo lung function measurement

Guinea pigs were anesthetized with urethane (2 g/kg body weight intraperitoneally). Lung function was measured in spontaneously breathing guinea pigs, essentially after Amdur and Mead (27). Airflow and tidal volume were determined by cannulating and connecting the trachea via a Fleisch flow head (Meijnhart, The Netherlands) to a pneumotachograph. A pressure transducer (MP45-2; Validyne Engineering Corp., Northridge, CA) measured the transpulmonary pressure by the difference between the tracheal cannula and an esophageal cannula. In this way airway resistance (R_L) was determined breath by breath by dividing transpulmonary pressure by airflow at isovolumetric points. R_L values were averaged per three breaths and are presented as actual value. A small polyethylene catheter (PE-50) was placed in the right jugular vein for intravenous administration of R-methanandamide, NGF and histamine.

One dose of NGF was injected 30 min before the start of the lung function measurement, preceded by the administration of R-Methanandamide (0.5 µg/ kg body weight) or its vehicle 10 min earlier. The airway resistance was measured in the anesthetized guinea pig upon intravenous administration of increasing doses of histamine. The dose response curve lasted 30-40 min.

Statistics

Means and standard error of the mean (SEM) were calculated. For concentration or dose response curves a two way ANOVA was performed. Probability values of $P < 0.05$ were considered significantly different.

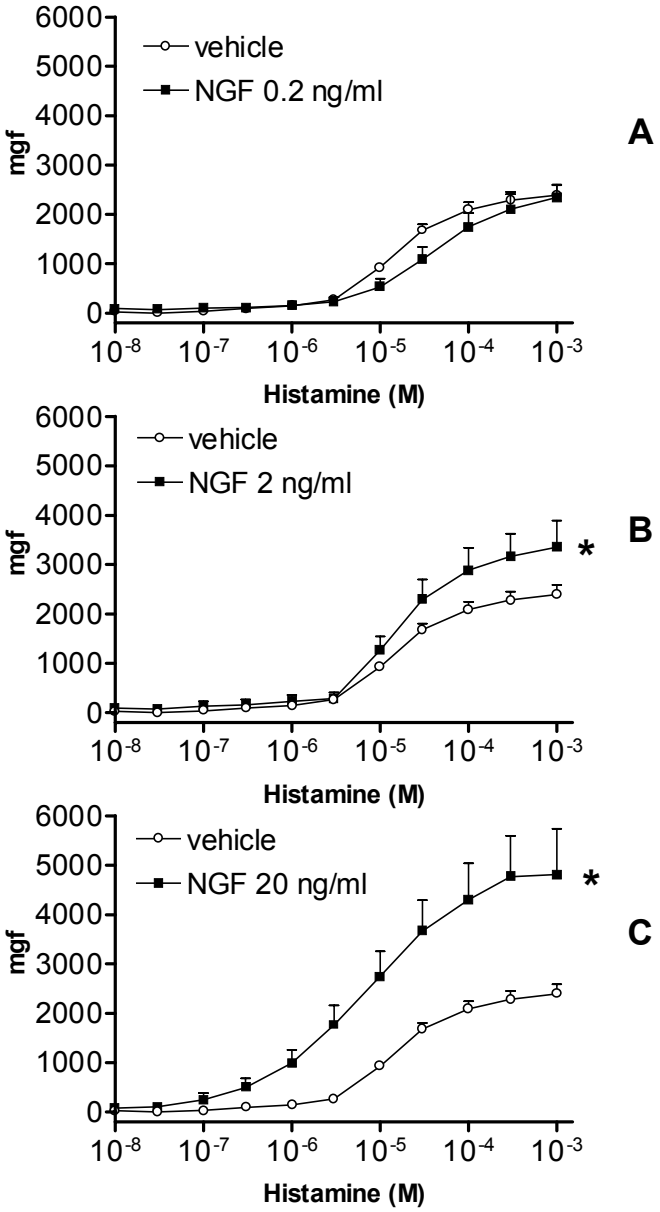


Figure 1. NGF concentration-dependently induces a hyperresponsiveness to histamine in tracheal rings in vitro. A concentration of 0.2 ng/ml NGF (n=9) 30 min before the start of a concentration response curve did not enhance contractility (**A**), whereas concentrations of 2 ng/ml NGF (n=6) (**B**) and a concentration of 20 ng/ml (n=5) (**C**) did induce a tracheal hyperresponsiveness (n=16 for all vehicles). For **B** and **C** * P<0.0001 NGF vs. vehicle.

Results

NGF applied to the organ bath 30 min before the start of a concentration response curve induced a tracheal hyperresponsiveness to histamine (fig. 1). The increase in reactivity was dependent on the concentration of NGF. A concentration of 2 and 20 ng/ml enhanced contractions to histamine ($P<0.0001$), whereas 0.2 ng/ml did not significantly enhance the contractility of the tracheal rings. At the highest concentration of histamine (10^{-3} M) 2 ng/ml NGF increased the contraction from 2391 ± 196 to 3354 ± 543 mgf and 20 ng/ml NGF resulted in an even higher contraction of 4808 ± 930 mgf.

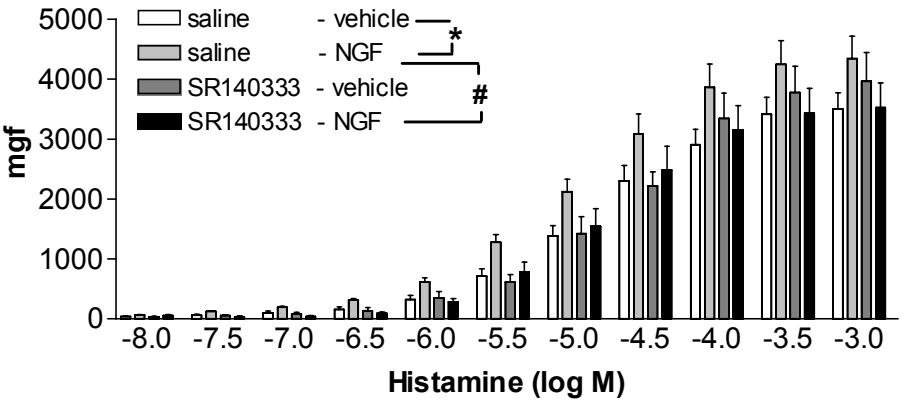


Figure 2. Addition of SR140333 ($3x10^{-6}$ M) 40 min before start of a histamine concentration response curve in the organ bath prevents the hyperresponsiveness of the tracheal rings induced by NGF (2 ng/ml) added 30 min before start of the concentration response curve (n=12). * $P<0.0001$ saline-NGF vs. saline -vehicle; # $P<0.0001$ SR140333 - NGF vs. saline-NGF.

A preincubation of 10 min with the neurokinin-1 receptor antagonist SR140333 inhibited this NGF induced tracheal hyperresponsiveness (fig. 2, $P<0.0001$). R-methanandamide,a CB_1 receptor agonist, blocked the NGF-induced tracheal hyperresponsiveness as well (fig. 3, $P<0.0001$). A 10 min preincubation with R-methanandamide reduced the maximal contraction with NGF from 3058 ± 304 to 2262 ± 163 mgf. SR140333 or R-methanandamide did not affect the contractility of control rings (figs. 2 and 3).

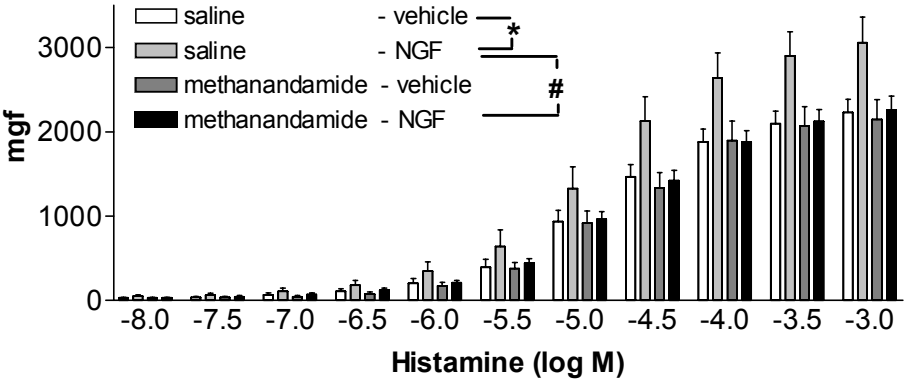


Figure 3. Addition of R-methanandamide ($1x10^{-7}$ M) 40 min before start of a histamine concentration response curve in the organ bath prevents the hyperresponsiveness of the tracheal rings induced by NGF (2 ng/ml) added 30 min before start of the concentration response curve (n=9-16). *P<0.0001 saline-NGF vs. saline-vehicle; #P<0.0001 methanandamide-NGF vs. saline-NGF.

Intravenous administration of NGF induces an airway hyperresponsiveness to histamine in vivo (fig. 4). This airway hyperresponsiveness, observed after administration of 80 ng/kg body weight NGF, was completely prevented by pretreatment with R-methanandamide (fig. 4, P<0.0001). Pretreatment with R-methanandamide reduced NGF-induced airway resistance at the highest dose of histamine from 4.9 ± 0.6 to 2.3 ± 0.3 cm H_2O/ ml / sec. Neither NGF nor R-methanandamide induced a change in basal airway resistance (data not shown).

Discussion

NGF induced an airway hyperresponsiveness to the contractile agonist histamine, as measured in isolated tracheal rings in an organ bath set-up (fig. 1). In this preparation sensory nerve endings lack contact with their cell bodies. Our results imply that contact of the sensory nerve endings with their cell bodies is not necessary for the development of tracheal hyperresponsiveness induced by NGF. Interestingly, the neurokinin-1 receptor antagonist SR140333 blocked NGF-induced tracheal hyperresponsiveness (fig. 2). This points to a role for the preferred ligand of the neurokinin-1 receptor, the tachykinin substance P. Substance P is predominantly released from sensory nerve endings (24). However, cells like monocytes, macrophages, leukocytes and T-cells are also able to synthesize and release substance P (28, 29).

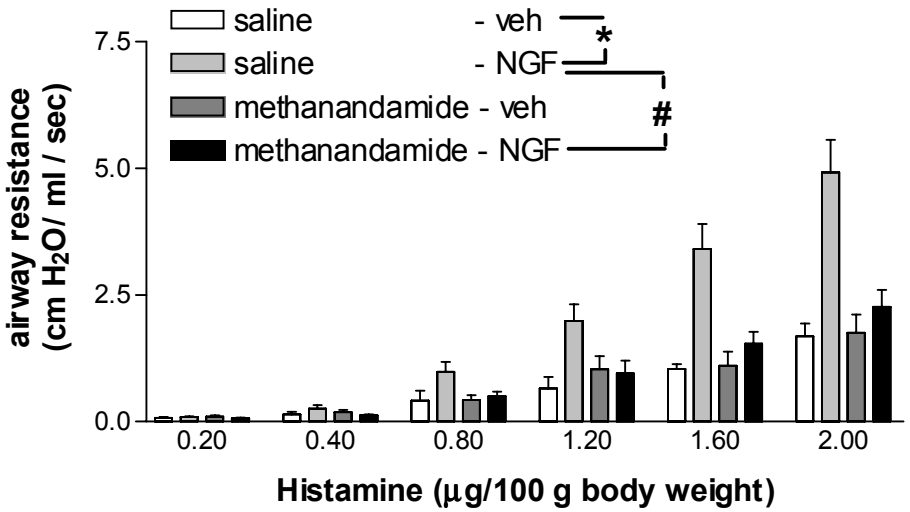


Figure 4. R-methanandamide (0.5 µg/kg body weight) 10 min prior to 80 ng NGF/kg body weight completely prevents the NGF-induced airway hyperresponsiveness (n=5-6). * P<0.0001 saline-NGF vs. saline-vehicle; # P<0.0001 methanandamide-NGF vs. saline-NGF.

A prominent role for the sensory nerve ending in the NGF-induced effects is confirmed by our results with R-methanandamide. This CB_1 receptor agonist (30) also prevented NGF-induced tracheal hyperresponsiveness (fig. 3). CB_1 receptors are almost exclusively present on sensory nerve endings; their stimulation inhibits neuropeptide release (31). One study on CB_1 receptors in the airways shows that alveolar Type II cells also express the CB_1 receptor (32). However, these cells do not produce substance P and therefore a role of these alveolar cells in the induction of airway hyperresponsiveness is unlikely. R-methanandamide also completely prevented the induction of airway hyperresponsiveness by NGF in vivo (fig. 4.). This effect of R-methanandamide in vivo could be via central pathways, affecting responses in the brain stem or central nervous system. However, Richardson and colleagues demonstrated that not the central CB receptors but rather the peripheral CB_1 receptors mediate inhibition of hyperalgesia (33). This study on hyperalgesia suggests, like our study, that the effect of CB_1 receptor is mediated via the inhibition of neurosecretion from sensory nerve endings. Recently, endogenous cannabinoids, like anandamide, have been shown to be full agonists for vanilloid receptor-1, when applied at doses of 10-100 µM (34, 35). Therefore, R-methanandamide may have interacted with the vanilloid receptor-1 in our set-up. However, R-methanandamide is weaker as an activator of the vanilloid receptor-1 than anandamide (1/10 ratio; 36, 37). Therefore it is unlikely that R-

methanandamide (0.1 μM) would act via the vanilloid receptor-1 in the present study.

NGF is known to bind to its high affinity tyrosine kinase receptor A (trkA); thereafter the NGF-trkA complex is internalized and retrogradely transported to the nucleus, where the preprotachykinin mRNA levels (precursor for substance P and neurokinin A) and protein levels (substance P and neurokinin A) are enhanced (23, 38). The p75 low affinity NGF receptor is also involved in the upregulation in tachykinin content in sensory nerves (39). Hoyle and colleagues (40) showed that transgenic mice, over-expressing NGF in the airways, develop a hyperinnervation of the airways and an increased content in substance P. Other studies reported that long term NGF exposure induces upregulation of substance P in sensory nerves, though this was not studied in the airways (e.g. 40, 41). It is generally agreed that protein synthesis occurs in the neuronal cell body, followed by axonal transport to the nerve endings (although very few studies have reported on the role of RNA in axons, 43). Therefore, in the tracheal preparation in our study presented here, an upregulation of substance P content can be excluded, as the nerve endings lacked contact with their cell bodies. Moreover, the administration of NGF can induce an airway hyperresponsiveness in vivo within one hr, a time period we think is too short for protein synthesis, axonal transport and subsequent release of substance P (1). Taken together, this implies there is a clear difference between the long term effects of NGF and the influence of NGF within approximately one hr. Therefore, shortly after NGF application, presumably other mechanisms than substance P upregulation are responsible for the changes induced by NGF.

NGF did not change basal tone in the tracheal rings and application of histamine, as a stimulus, was needed to reveal a tracheal hyperresponsiveness induced by NGF. This implies that NGF did not induce release of contractile mediators by itself. In the present study, either an increase in release of substance P or a change in neurokinin-1 receptor functioning can underlie the changes in contractility. In favor of a change in function of sensory nerve endings, and thereby an increase in substance P release, are the findings with CB_1 receptor agonist. Furthermore, a very recent abstract by Zhou and colleagues reported a long-term (24-48 hrs) increase in substance P release in tracheal explants in response to NGF (44), thereby confirming the role of the nerve ending.

NGF is known to change the properties of sensory nerve endings by inducing a very fast accumulation of second messenger in synaptosomes (45), by sensitizing the nerve terminal (46) by inducing protein kinase C translocation to the membrane (47). Besides this, NGF binding to the TrkA in a tyrosine kinase-dependent manner induces phosphorylation of key transduction-related proteins or ion channels, thereby also sensitizing the peripheral sensory nerve ending (45, 48). NGF increases the response of the vanilloid receptor-1 to capsaicin in 10 min, at the same concentrations (2-100 ng/ml NGF) as used in our study (49). The vanilloid

receptor-1 is the receptor for the perception of heat, protons and for the agonist capsaicin. These stimuli are able to induce release of tachykinins from the sensory nerve endings (34, 35, 49). Since there is presumably no endogenous ligand present in our organ bath for the vanilloid receptor, activation of this receptor in our experiments is not very likely. Taken together, this suggests that more down-stream mechanisms in receptor signaling must be affected. In the same view, the CB_1 receptor agonist inhibiting excitatory processes at the nerve terminal (31) blocks NGF induced tracheal hyperresponsiveness (figs. 3 and 4). Still, the increase in contractility in the tracheal rings due to NGF can also be due to an upregulation in the number or affinity of neurokinin-1 receptors. The NGF-induced neuronal sensitization could be direct, as described above, or indirect, via the release of sensitizing mediators from trkA expressing inflammatory cells, such as mast cells and monocytes (15, 38, 46, 48).
Therefore, we hypothesize a general lowering of the threshold for activation of the sensory nerve endings due to short-term exposure to NGF, which induces a tracheal and airway hyperresponsiveness to histamine.

References

1. de Vries A., Dessing M. C., Engels F., Henricks P. A. J., Nijkamp F. P. Nerve growth factor induces a neurokinin-1 receptor- mediated airway hyperresponsiveness in guinea pigs. Am. J. Respir. Crit. Care Med. 1999; 159:1541-1544.
2. Virchow J. C., Julius P., Lommatzsch M., Luttmann W., Renz H., Braun A. Neurotrophins are increased in bronchoalveolar lavage fluid after segmental allergen provocation. Am. J. Respir. Crit. Care Med. 1998; 158:2002-2005.
3. Braun A., Appel E., Baruch R., Herz U., Botchkarev V., Paus R., Brodie C., Renz H. Role of nerve growth factor in a mouse model of allergic airway inflammation and asthma. Eur. J. Immunol. 1998; 28:3240-3251.
4. Bonini S., Lambiase A., Bonini S., Angelucci F., Magrini L., Manni L., Aloe L. Circulating nerve growth factor levels are increased in humans with allergic diseases and asthma. Proc. Natl. Acad. Sci. USA 1996; 93:10955-10960.
5. Sanico A. M., Stanisz A. M., Gleeson T. D., Bora S., Proud D., Bienenstock J., Koliatsos V. E., Togias A. Nerve growth factor expression and release in allergic inflammatory disease of the upper airways. Am. J. Respir. Crit. Care Med. 2000; 161:1631-1635.
6. Hattori A., Iwasaki S., Murase K., Tsujimoto M., Sato M., Hayashi K., Kohno M. Tumor necrosis factor is markedly synergistic with interleukin 1

and interferon-gamma in stimulating the production of nerve growth factor in fibroblasts. FEBS Lett. 1994; 340:177-180.

7. Yoshida K., Kakihana M., Chen L. S., Ong M., Baird A., Cage F. H. Cytokine regulation of nerve growth factor-mediated cholinergic neurotrophic activity synthesized by astrocytes and fibroblasts. J. Neurochem. 1992; 59:919-931.
8. Leon A., Buriani A., Dal Toso R., Fabris M., Romanello S., Aloe L., Levi-Montalcini R. Mast cells synthesize, store, and release nerve growth factor. Proc. Natl. Acad. Sci. USA 1994; 91:3739-3743.
9. Lambiase A., Bracci-Laudiero L., Bonini S., Bonini S., Starace G., D'Elios M. M., De Carli M., Aloe L. Human CD4+ T cell clones produce and release nerve growth factor and express high affinity nerve growth receptors. J. Allergy Clin. Immunol. 1997; 100:408-414.
10. Solomon A., Aloe L., Pe'er J., Frucht-Pery J., Bonini S., Levi-Schaffer F. Nerve growth factor is preformed in and activates human peripheral blood eosinophils. J. Allergy Clin. Immunol. 1998; 102:454-460.
11. Barouch R., Appel E., Kazimirsky G., Braun A., Renz H., Brodie C. Differential regulation of neurotrophin expression by mitogens and neurotransmitters in mouse lymphocytes. J. Neuroimmunol. 2000; 103:112-121.
12. Fox A. J., Barnes P. J., Belvisi M. G. Release of nerve growth factor from human airway epithelial cells (Abstract). Am. J. Respir. Crit. Care Med. 1998; 155:A157.
13. Braun A., Lommatzsch M., Renz H. The role of neurotrophins in allergic bronchial asthma. Clin. Exp. Allergy 2000; 30:178-186.
14. Burgi B., Otten U. H., Ochensberger B., Rihs S., Heese K., Ehrhard P. B., Ibanez C. F., Dahinden C. A. Basophil priming by neurotrophic factors. Activation through the trk receptor. J. Immunol. 1996; 15:5582-5588.
15. Matsuda H., Coughlin M. D., Bienenstock J., Denburg J. A. Nerve growth factor promotes human hemopoietic colony growth and differentiation. Proc. Natl. Acad. Sci. USA 1988; 85:6508-6512.
16. Tal M., Liberman R. Local injection of nerve growth factor (NGF) triggers degranulation of mast cells in rat paw. Neurosci. Lett. 1997; 22:129-132.
17. Levi-Montalcini R., Skaper S. D., Dal Tosco R., Petrelli L., Leon A. Nerve growth factor: from neurotrophin to neurokine. Trends Neurosci. 1996; 19:514-520.
18. Otten U., Ehrhard P., Peck R. Nerve growth factor induced growth and differentiation of human B lymphocytes. Proc. Natl. Acad. Sci. USA 1989; 86:10059-10063.
19. Susaki Y., Shimizu S., Katakura K., Watanabe N., Kawamoto K., Matsumoto M., Tsudzuki M., Furusaka T., Kitamura Y., Matsuda H.

Functional properties of murine macrophages promoted by nerve growth factor. Blood 1996; 88:4630-4637.

20. Sawada J., Itakura A., Tanaka A., Furusaka T., Matsuda H. Nerve growth factor functions as a chemoattractant for mast cells through both mitogen-activated protein kinase and phosphatidylinositol 3-kinase signaling pathways. Blood 2000; 95:2052-2058.
21. Ehrhard P. B., Ganter U., Stalder A., Bauer J., Otten U. Expression of functional trk protooncogene in human monocytes. Proc. Natl. Acad. Sci. USA 1993; 90:5423-5427.
22. Grimes M. L., Zhou J., Beattie E. C., Yuen E. C., Hall D. E., Valletta J. S., Topp K. S., LaVail J. H., Bunnett N. W., Mobley W. C. Endocytosis of activated TrkA: Evidence that nerve growth factor induces formation of signalling endosomes. J. Neurosci. 1996; 16:7950-7964.
23. Lindsay R. M., Harmar A. J. Nerve growth factor regulates expression of neuropeptide genes in adult sensory neurons. Nature 1989; 337:362-364.
24. Lundberg J. M. Tachykinins, sensory nerves, and asthma- an overview. Can. J. Physiol. Pharmacol. 1995; 73:908-914.
25. Biyah K., Molimard M., Esmonds-Alt X., Advenier C. SR140333 prevents potentiation by citric acid of plasma exudation induced by histamine in airways. Eur. J. Pharmacol. 1996; 308:325-328.
26. Esmonds-Alt X., Doutremepuich J. D., Heaulme M., Neliat G., Steinberg R., Vilain P., Bichon D., Ducoux J. P., Proietto V., Van Broeck D., Soubrie P., Le Fur G., Breliere J. C. In vitro and in vivo biological activities of SR140333, a novel potent non-peptide tachykinin NK_1 receptor antagonist. Eur. J. Pharmacol. 1993; 250:403-413.
27. Amdur M. O., Mead J. Mechanics of respiration in unanesthetized guinea pigs. Am. J. Physiol. 1958; 192:364-368.
28. Ho W. Z., Lai J. P., Zhu X. H., Uvaydova M., Douglas S. D. Human monocytes and macrophages express substance P and Neurokinin-1 receptor. J. Immunol. 1997; 159:5654-5660.
29. De Giorgio R., Tazzari P. L., Barbara G., Stanghellini V., Corinaldesi R. Detection of substance P immunoreactivity in human peripheral leukocytes. J. Neuroimmunol. 1998; 82:175-181.
30. Abadji V., Lin S., Taha G., Griffin G., Stevenson L. A., Pertwee R. G., Makriyannis A. (R)-methanandamide: a chiral novel anandamide possessing higher potency and metabolic stability. J. Med. Chem. 1994; 37:1889-1893.
31. Hohmann A. G., Herkenham M. Localization of central cannabinoid CB1 receptor messenger RNA in neuronal subpopulations of rat dorsal root ganglia: a double-label in situ hybridization study. Neurosci. 1999; 90:923-931.

32. Rice W., Shannon J. M., Burton F., Fiedeldey D. Expression of a brain-type cannabinoid receptor (CB1) in alveolar Type II cells in the lung: regulation by hydrocortisone. Eur. J. Pharmacol. 1997; 327:227-232.
33. Richardson J. D., Kilo S., Hargreaves K. M. Cannabinoids reduce hyperalgesia and inflammation via interaction with peripheral CB1 receptors. Pain 1998; 75:111-119.
34. Szolcsanyi J. Are cannabinoids endogenous ligands for the VR1 capsaicin receptor? Trends Pharmacol. Sci. 2000; 21:41-42.
35. Smart D., Gunthorpe M. J., Jerman J. C., Nasir S., Gray J., Muir A. I., Chambers J. K., Randall A. D., Davis J. B. The endogenous lipid anandamide is a full agonist at the human vanilloid receptor (hVR1). Br. J. Pharmacol. 2000; 129:227-230.
36. Smart D., Jerman J. C. Anandamide: an endogenous activator of the vanilloid receptor. Trends Pharmacol. Sci. 2000; 21:134.
37. Zygmunt P. M., Petersson J., Andersson D. A., Chuang H., Sorgard M., Di Marzo V., Julius D., Hogestatt E. D. Vanilloid receptors on sensory nerves mediate the vasodilator action of anandamide. Nature 1999; 400:452-457.
38. Vedder H., Affolter H. U., Otten U. Nerve growth factor (NGF) regulates tachykinin gene expression and biosynthesis in rat sensory neurons during early postnatal development. Neuropeptides 1993; 24:351-357.
39. Lee K. F., Li E., Huber L. J., Landis S. C., Sharpe A. H., Chao M. V., Jaenisch R. Targeted mutation of the gene encoding the low affinity NGF receptor p75 leads to deficits in the peripheral sensory nervous system. Cell 1992; 69:737-749.
40. Hoyle G. W., Graham R. M., Finkelstein J. B., Nguyen K. P., Gozal D., Friedman M. Hyperinnervation of the airways in transgenic mice overexpressing nerve growth factor. Am. J. Respir. Cell Mol. Biol. 1998; 18:149-157.
41. Miller M. S., Buck S. H., Sipes I. G., Yamamura H. I., Burks T. F. Regulation of substance P by nerve growth factor: disruption by capsaicin. Brain Res. 1982; 250:193-196.
42. Donnerer J., Schuligoi R., Stein C. Increased content and transport of substance P and calcitonin gene-related peptide in sensory nerves innervating inflamed tissue: evidence for a regulatory function of nerve growth factor in vivo. Neuroscience 1992; 49:693-698.
43. Van Minnen J. RNA in the axonal domain: a new dimension in neuronal functioning? Histochem. J. 1994; 26:377-391.
44. Zhou D., Zheng X., Bai T. R. Both nerve growth factor (NGF) and leukemia inhibitory factor (LIF) augment substance P release in airway explants (Abstract). Am. J. Respir. Crit. Care Med. 2000; 161:A839.

45. Knipper M., Beck A., Rylett J., Breer H. Neurotrophin induced second messenger responses in rat brain synaptosomes. Neuroreport 1993; 4:483-486.
46. Woolf C. J. Phenotypic modification of primary sensory neurons: the role of nerve growth factor in the production of persistant pain. Phil. Trans. R. Soc. Lond. B 1996; 351:441-448.
47. Dupont J. L., Janoshazi A., Bellahcene M., Mykita S., de Barry J. Reversible protein kinase C activation in PC12 cells: effect of NGF treatment. Eur. J. Neurosci. 2000; 12:215-226.
48. Woolf C. J., Ma Q. P., Allchorne A., Poole S. Peripheral cell types contributing to the hyperalgesic action of nerve growth factor in inflammation. J. Neurosci. 1996; 16:2716-2723.
49. Shu X., Mendell L. M. Nerve growth factor acutely sensitizes the response of adult rat sensory neurons to capsaicin. Neurosci. Lett. 1999; 274:159-162.

Antibodies directed against nerve growth factor inhibit the acute bronchoconstriction induced by allergen challenge in sensitized guinea pigs

Annick de Vries, Ferdi Engels, Paul A.J. Henricks, Thea Leusink- Muis, Axel Fischer[1], and Frans P. Nijkamp.

[1] Division of Allergology, Charité Virchow Campus, Biomedical Research Center, Berlin, Germany

4

submitted

Abstract

Intravenous administration of nerve growth factor (NGF) to guinea pigs results in airway hyperresponsiveness within 1 hr, as we have demonstrated before. In the present study we documented the involvement of NGF in the acute allergic airway response.
Guinea pigs that are sensitized to ovalbumin show an acute bronchoconstriction directly after challenge with ovalbumin. Intra-tracheal application of 10 μg antibodies directed against NGF (anti-NGF) one hr before the challenge reduces the acute severe bronchoconstriction to approximately 40% and the sustained bronchoconstriction to approximately 20% of the reaction in controls. This shows a high potency of anti-NGF in diminishing the direct bronchoconstriction.
Inhibition of the tyrosine kinases of the tyrosine kinase receptor A, the high-affinity receptor for NGF, has no effect on the bronchoconstriction. Therefore we postulate that the p75, the low-affinity receptor for neurotrophins, is responsible for the acute bronchoconstriction.
Our findings suggest a role for NGF in the induction of the acute asthmatic reaction. These findings offer a new potential therapeutic strategy for the treatment of asthma.

Introduction

NGF is a member of the neurotrophin family of proteins that regulate neuronal development, maintenance, and recovery from injury. NGF has been extensively studied in relation to neurite outgrowth. However, NGF can also play a crucial role in inflammation (1).
Inflammation leads to an enhanced production and release of NGF. Inflammatory mediators, including interleukin-1, interleukin-4, interleukin-5, tumor necrosis factor α, and interferon-γ have been shown to induce the release of NGF (2, 3). In addition to neurons, non-neuronal cells such as mast cells (4), fibroblasts (2), T-cells (5) and epithelial cells (6) are able to synthesize NGF.
NGF affects immune cell activity, as it promotes inflammatory mediator release from basophils (7), mast cells (8, 9), T- and B-cells (5, 10, 11) and macrophages (12). NGF is also able to sensitize neurons and induce an enhanced production of substance P and other tachykinins in sensory nerves (13, 14). Tachykinins play a role in neurogenic inflammation and in the development of airway hyperresponsiveness and asthma (15, 16). Furthermore, we showed that a neurokinin-1 receptor antagonist blocked NGF induced airway hyperresponsiveness, indicating a role for the neurokinin-1 receptor ligand substance P (17).

Interestingly, circulating NGF levels are increased in humans with allergic diseases (rhinoconjunctivitis, urticaria-angioedema, asthma, (18). In the study of Bonini and colleagues, NGF serum levels were particularly high in patients with allergic asthma (18). Also, allergen challenge induces an increase in NGF levels in bronchoalveolar lavage fluid of sensitized mice (19). Moreover, allergen provocation induces an increase in NGF levels in nasal lavage fluid in patients with allergic rhinitis (20).
NGF can interact with two receptors: either the low-affinity receptor p75, which can also bind other neurotrophic factors such as brain derived neurotrophic factor and neurotrophin 3, or the tyrosine kinase receptor A (trkA). Of these two receptors, only the trkA is linked to tyrosine kinases for its signal transduction. So far, no data are available on the receptors for NGF and their function in the airways.
In a previous study we have demonstrated that intravenous administration of NGF to guinea pigs results in airway hyperresponsiveness, indicating a role for NGF in the development of one of the symptoms of the asthmatic disease (17). These findings prompted us to further investigate the role of NGF in allergic asthma. We tested the effects of an antibody directed against NGF and an inhibitor of the tyrosine kinases of the trkA in the acute ovalbumin-induced bronchoconstriction in guinea pigs.

Materials and methods

Ovalbumin sensitization
Male Hartley guinea pigs (weighing approximately 400 g; Harlan CPB, Zeist, The Netherlands) were sensitized to ovalbumin, grade V (SIGMA, St. Louis, MO) by injections of a gel containing 20 μg ovalbumin/ml, and 200 mg $Al(OH)_3$/ml as adjuvant. Six injections were placed: one injection of 0.5 ml intraperitoneally, one of 0.1 ml nuchally, two injections of 0.1 ml axillarly, and two of 0.1 ml inguinally. Control animals were not sensitized and received instead a vehicle gel, not containing ovalbumin.

Measurement of acute bronchoconstriction
Fourteen days after the sensitization procedure, the guinea pigs were individually placed in a box to measure airway function before and after ovalbumin challenge. Each challenge consisted of a 2 sec nebulization of 0.01% ovalbumin in sterile saline (Ultra-Neb 2000™, Devilbiss, Sommerset, PA). All animals received the ovalbumin challenge.
The airway function of the unrestrained guinea pigs was measured using whole body plethysmography (BUXCO, Sharon, CT) as previously described by

Hamelmann and colleagues (21). As parameter of airway function the enhanced pause (Penh) was determined. The Penh closely reflects the changes in pulmonary resistance during bronchoconstriction (21). As suggested by Drazen and colleagues (22), we inspected the barometric plethysmograph pressure tracings and assured ourselves that the pressure variations corresponded to those from which the Penh parameter was derived. After 5 min of measuring base line airway function, challenge with ovalbumine was performed.

Application of anti-NGF and tyrosine kinase inhibitor
Treatment with anti-NGF or tyrosine kinase inhibitor took place 1 hr before ovalbumin challenge, similar to Fryer and colleagues (23). The guinea pigs were treated intra-tracheally with 10 μg anti-NGF dissolved in 50 μl saline (anti human β-NGF, R & D Systems, Minneapolis, MI) or with 10 μg IgG isotype control antibody (IgG goat, SIGMA). The polyclonal antibody directed against human β-NGF was raised in the goat and purified (IgG fraction) by affinity chromatography. A dose of 0.1 μg/ml neutralizes 5 ng/ml NGF. For treatment with tyrphostin AG879 (Calbiochem Novabiochem, Läufelfingen, Switzerland), 200 μl of 0.2 mM tyrphostin AG879 was applied intra-tracheally. Control animals recieved the vehicle: saline containing 2% DMSO. Tyrphostin AG879 is an inhibitor specific for the tyrosine kinases on the trkA (24).
In order to apply the solutions intra-tracheally, the guinea pigs were anesthetized with halothane (2% halothane, 0.4 l/min O_2, 2 l/min N_2O, approximately 15-30 sec). The animals all regained consciousness within 30 sec after this procedure.

Animal studies were approved by the Animal Care Committee of the Utrecht University.

Statistics
Means and standard error of the mean (SEM) were calculated. P-values were determined using an unpaired two-tailed Student's t-test for the comparison of two means. Probability values of $P<0.05$ were considered significantly different.

Results

Ovalbumin challenge in sensitized guinea pigs resulted in an increase in Penh that started about 3 min after the ovalbumin challenge and which resulted in a maximal Penh between 5 and 10 min. after the challenge. The maximal Penh rose above values of 20, which is comparable to the bronchoconstriction induced by more than 20 μg/kg body weight histamine intravenously and corresponds to a severe bronchoconstriction.

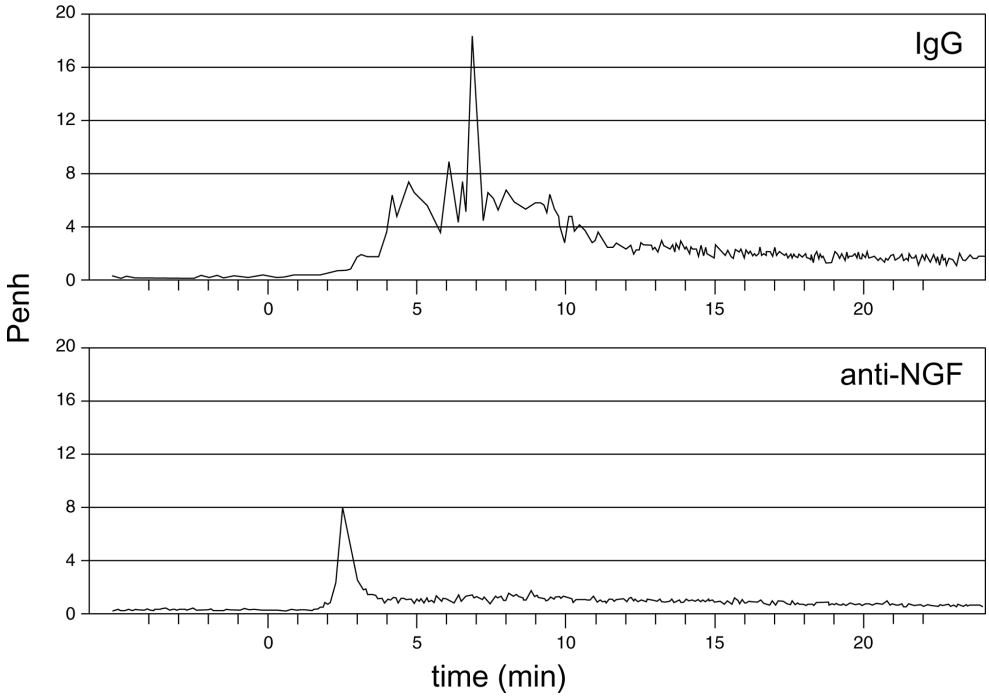


Figure 1. Typical Penh traces in time after allergen challenge. Sensitized guinea pigs were challenged with ovalbumin at time=0. Animals had been were treated with IgG control antibody (upper panel) or with anti-NGF (lower panel).

Anti-NGF prevented the severe and sustained bronchoconstriction induced by the ovalbumin challenge (fig. 1). Treatment with anti-NGF showed a rapid decline to a low and stable Penh after reaching no peak at all or a very low peak Penh, whereas in the control antibody treated guinea pigs the peak Penh was much higher and decreased more slowly (fig. 1). The area under the curve was diminished greatly by the anti-NGF treatment. In the typical curves (fig. 1), the area under the curve measured form 0 to 20 min after allergen challenge declined from 33.4 to 11.7 arbitrary values. Measurement of the area under the curve for the whole group was not possible, as 2 of the control animals showed an extreme bronchoconstriction and had to be taken out of the equipment. This indicates that the inhibitory effect of anti-NGF is even more pronounced since none of the animals treated with anti-NGF showed such a severe bronchoconstriction.

Peak Penh was significantly diminished by application of anti-NGF (fig. 2). The peak for non-sensitized animals was 0.34 ± 0.04 (n=4), whereas ovalbumin challenge in ovalbumin-sensitized and control antibody treated animals lead to an increase to 14.60 ± 3.30 (n=4). Anti-NGF reduced the peak to 5.98 ± 1.92 (n=5, $P<0.03$ anti-NGF vs. control antibody). Treatment with anti-NGF diminished

bronchoconstriction as compared to control antibody treatment, not only when comparing peak bronchoconstriction, but also when comparing the sustained bronchoconstriction at 5, 10 and 15 min after the allergen challenge (fig. 2).

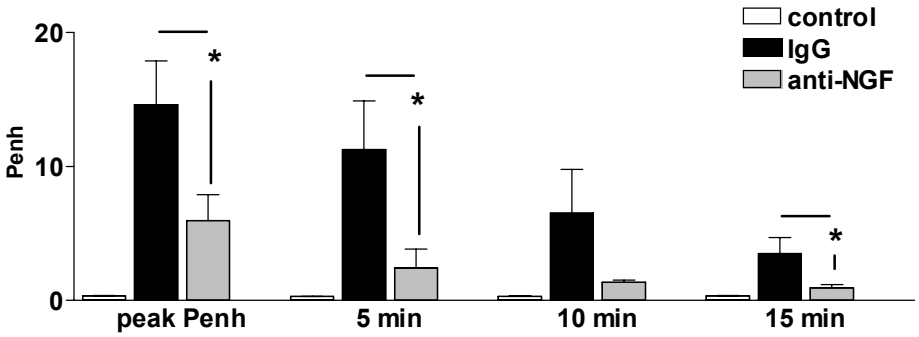


Figure 2. Treatment with anti-NGF one hr before ovalbumin challenge reduces the acute bronchoconstriction. Bronchoconstriction is shown at peak, 5 min, 10 min, and 15 min after ovalbumin challenge. Controls are non-sensitized animals (open bars), IgG and anti-NGF are guinea pigs sensitized to ovalbumin, treated with either the control IgG antibody (solid bars) or anti-NGF (grey bars), respectively (n=4/5). Bronchoconstriction is expressed as Penh. * P< 0.03, anti-NGF vs. IgG, Student's t-test.

Pre-treatment with the tyrosine kinase inhibitor tyrphostin AG879 did not affect the acute bronchoconstriction induced by allergen challenge, making involvement of this messenger in this process rather unlikely (fig. 3).

Discussion

Antibodies directed against NGF inhibited the duration of the severe and sustained bronchoconstriction to allergen challenge (fig. 1 and 2). The Penh value was largely reduced as well at several time points after the ovalbumin challenge (fig. 3). This inhibitory effect indicates a beneficial effect of anti-NGF on the duration and magnitude of the severe bronchoconstriction.
Locally applied anti-NGF one hr before allergen challenge markedly reduced the bronchoconstriction; this strongly suggests that a rapid local release of NGF took place. Increased levels of NGF have been reported in relation to allergic responses several times. In patients suffering from allergic asthma an enhancement in NGF levels in serum (18) as well as in bronchoalveolar lavage is reported (25, 26). Allergen challenge in patients with allergic rhinitis leads to an acute increase in NGF in nasal lavage fluid (27). The NGF increase is also seen in an animal model: in sensitized mice, levels of NGF are enhanced in the bronchoalveolar lavage 24

hrs after allergen challenge (19). However, before challenge NGF levels in sensitized mice are equal to control animals (19). This implies, that allergen challenge is necessary for NGF upregulation. The increase in NGF serum levels in patients suffering from allergic asthma could be due to a constant exposure to allergens in their environment. As allergen challenge is presumably necessary for NGF increases (19), we suggest that NGF is released immediately after allergen challenge. Supporting this view, is the strong inhibitory effect of anti-NGF applied only one hr before allergen challenge reported here. Furthermore, a large variety of cells that are able to release NGF, such as eosinophils, airway epithelium, mast cells, basophils, T-cells and fibroblasts (2, 4-7, 28) are present in the airways, and these cells could be the source of NGF.

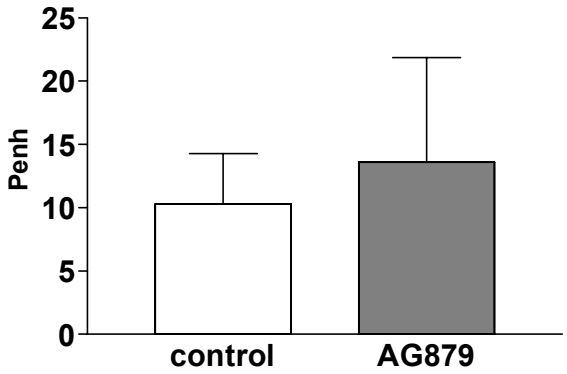


Figure 3. Treatment with tyrphostin AG879 one hr before ovalbumin challenge does not reduce the acute bronchoconstriction. The peak bronchoconstriction is shown after ovalbumin challenge. All guinea pigs are sensitized to ovalbumin and are challenged with ovalbumin. 'Control' refers to animals which are treated with vehicle whereas 'AG879' refers to animals treated with tyrphostin AG879 (n=5 for both groups). Bronchoconstriction is expressed as Penh.

In our previous study, we showed that intravenously administered NGF induces an airway hyperresponsiveness within 1 hr (17). Probably substance P plays a role in NGF-induced airway hyperresponsiveness, since a neurokinin-1 receptor antagonist was able to prevent airway hyperresponsiveness (17). Few studies have been published on the involvement of tachykinins in acute allergic responses in the airways. Antigen-induced microvascular leakage revealed a role for substance P in the early allergic response (29). Recently it was shown that toluene diisocyanate induces a rapid increase of substance P; 1 hr after inhalation of toluene diisocyanate the number of substance P immuno-reactive nerve fibres was already increased (30). We postulate that stimulaton of the sensory neurons by NGF could modulate the allergen-induced bronchoconstriction in our present study.

Confirming this idea is the enhancing effect of NGF on the release of tachykinins from sensory nerve endings by capsaicin (31).
We further demonstrated that inhibition of the tyrosine kinases of the trkA did not influence the acute bronchoconstriction induced by allergen challenge (fig. 3). NGF can bind to either the trkA, the high-affinity receptor for NGF, which is specific for NGF, or to the p75, the low-affinity receptor for NGF and other neurotrophic factors (32). We hypothesize that the p75 mediates the effects of NGF in the development of the acute allergic response. So far, no studies report on p75 or trkA function in the airways.
In addition to an acute allergic reaction and airway hyperresponsiveness, allergic asthma causes airway inflammation (33). In other models, the involvement of p75 has been revealed in relation to inflammation. Inflammation induced an up-regulation of p75 in inflamed tissues, suggesting that NGF, or other neurotrophic factors such as brain derived neurotrophic factor or neurotrophin 3, participate in NGF-induced hyperalgesia via p75 (34).
NGF is involved in airway hyperresponsiveness, a characteristic feature of asthma (17). In our present study we show that NGF also plays a crucial role the cascade of events leading to an acute bronchoconstriction due to allergen challenge, possibly via the p75, a receptor for neurotrophic factors. This indicates that NGF is involved in the early events of an acute allergic reaction. Therefore, we hypothesize a regulatory role for NGF in both acute allergic responses as well as in the induction of airway hyperresponsiveness. This offers a new potential approach for the treatment of asthma.

References

1. Woolf C. J., Safieh-Garabedian B., Ma Q. P., Crilly P., Winter J. Nerve growth factor contributes to the generation of inflammatory sensory hypersensitivity. Neuroscience 1994; 62:327-331.
2. Hattori A., Iwasaki S., Murase K., Tsujimoto M., Sato M., Hayashi K., Kohno M. Tumor necrosis factor is markedly synergistic with interleukin 1 and interferon-gamma in stimulating the production of nerve growth factor in fibroblasts. FEBS Lett. 1994; 340:177-180.
3. Yoshida K., Kakihana M., Chen L. S., Ong M., Baird A., Cage F. H. Cytokine regulation of nerve growth factor-mediated cholinergic neurotrophic activity synthesized by astrocytes and fibroblasts. J. Neurochem. 1992; 59:919-931.
4. Leon A., Buriani A., Dal Toso R., Fabris M., Romanello S., Aloe L., Levi-Montalcini R. Mast cells synthesize, store, and release nerve growth factor. Proc. Natl. Acad. Sci. USA 1994; 91:3739-3743.

5. Lambiase A., Bracci-Laudiero L., Bonini S., Bonini S., Starace G., D'Elios M. M., De Carli M., Aloe L. Human CD4+ T cell clones produce and release nerve growth factor and express high affinity nerve growth receptors. J. Allergy Clin. Immunol. 1997; 100:408-414.
6. Fox A. J., Barnes P. J., Belvisi M. G. Release of nerve growth factor from human airway epithelial cells (Abstract). Am. J. Respir. Crit. Care Med. 1997; 155:A157.
7. Burgi B., Otten U. H., Ochensberger B., Rihs S., Heese K., Ehrhard P. B., Ibanez C. F., Dahinden C. A. Basophil priming by neurotrophic factors. Activation through the trk receptor. J. Immunol. 1996; 15:5582-5588.
8. Matsuda H., Coughlin M. D., Bienenstock J., Denburg J. A. Nerve growth factor promotes human hemopoietic colony growth and differentiation. Proc. Natl. Acad. Sci. USA 1988; 85:6508-6512.
9. Tal M., Liberman R. Local injection of nerve growth factor (NGF) triggers degranulation of mast cells in rat paw. Neurosci. Lett. 1997; 22:129-132.
10. Levi-Montalcini R., Skaper S. D., Dal Tosco R., Petrelli L., Leon A. Nerve growth factor: from neurotrophin to neurokine. Trends Neurosci. 1996; 19:514-520.
11. Otten U., Ehrhard P., Peck R. Nerve growth factor induced growth and differentiation of human B lymphocytes. Proc. Natl. Acad. Sci. USA 1989; 86:10059-10063.
12. Susaki Y., Shimizu S., Katakura K., Watanabe N., Kawamoto K., Matsumoto M., Tsudzuki M., Furusaka T., Kitamura Y., Matsuda H. Functional properties of murine macrophages promoted by nerve growth factor. Blood 1996; 88:4630-4637.
13. Grimes M. L., Zhou J., Beattie E. C., Yuen E. C., Hall D. E., Valletta J. S., Topp K. S., LaVail J. H., Bunnett N. W., Mobley W. C. Endocytosis of activated TrkA: Evidence that nerve growth factor induces formation of signalling endosomes. J. Neurosci. 1996; 16:7950-7964.
14. Lindsay R. M., Harmar A. J. Nerve growth factor regulates expression of neuropeptide genes in adult sensory neurons. Nature 1989; 337:362-364.
15. Lundberg J. M. Tachykinins, sensory nerves, and asthma- an overview. Can. J. Physiol. Pharmacol. 1995; 73:908-914.
16. Biyah K., Molimard M., Esmonds-Alt X., Advenier C. SR140333 prevents potentiation by citric acid of plasma exudation induced by histamine in airways. Eur. J. Pharmacol. 1996; 308:325-328.
17. de Vries A., Dessing M. C., Engels F., Henricks P. A. J., Nijkamp F. P. Nerve growth factor induces a neurokinin-1 receptor- mediated airway hyperresponsiveness in guinea pigs. Am. J. Respir. Crit. Care Med. 1999; 159:1541-1544.

18. Bonini S., Lambiase A., Bonini S., Angelucci F., Magrini L., Manni L., Aloe L. Circulating nerve growth factor levels are increased in humans with allergic diseases and asthma. Proc. Natl. Acad. Sci. USA 1996; 93:10955-10960.
19. Braun A., Appel E., Baruch R., Herz U., Botchkarev V., Paus R., Brodie C., Renz H. Role of nerve growth factor in a mouse model of allergic airway inflammation and asthma. Eur. J. Immunol. 1998; 28:3240-3251.
20. Sanico A. M., Koliatsos V. E., Stanisz A. M., Bienenstock J., Togias A. Neural hyperresponsiveness and nerve growth factor in allergic rhinitis. Int. Arch. Allergy Immunol. 1999; 118:154-158.
21. Hamelmann E., Schwarze J., Takeda K., Oshiba A., Larsen G. L., Irvin C. G., Gelfand E. W. Noninvasive measurement of airway responsiveness in allergic mice using barometric plethysmography. Am. J. Respir. Crit. Care Med. 1997; 156:766-775.
22. Drazen J. M., Finn P. W., De Sanctis G. T. Mouse models of airway responsiveness: physiological basis of observed outcomes and analysis of selected examples using these outcome indicators. Annu. Rev. Physiol. 1999; 61:593-625.
23. Fryer A. D., Costello R. W., Yost B. L., Lobb R. R., Tedder T. F., Steeber D. A., Bochner B. S. Antibody to VLA-4, but not to L-selectin, protects neuronal M2 muscarinic receptors in antigen-challenged guinea pig airways. J. Clin. Invest. 1997; 99:2036-2044.
24. Ohmichi M., Pang L., Ribon V., Gazit A., Levitzki A., Saltiel A. R. The tyrosine kinase inhibitor tyrphostin blocks the cellular actions of nerve growth factor. Biochem. 1993; 32:4650-4658.
25. Virchow J. C., Julius P., Lommatzsch M., Luttmann W., Renz H., Braun A. Neurotrophins are increased in bronchoalveolar lavage fluid after segmental allergen provocation. Am. J. Respir. Crit. Care Med. 1998; 158:2002-2005.
26. Undem B. J., Hunter D. D., Liu M., Haak-Frendscho M., Oakragly A., Fischer A. Allergen-induced sensory neuroplasticity in airways. Int. Arch. Allergy Immunol. 1999; 118:150-153.
27. Sanico A. M., Stanisz A. M., Gleeson T. D., Bora S., Proud D., Bienenstock J., Koliatsos V. E., Togias A. Nerve growth factor expression and release in allergic inflammatory disease of the upper airways. Am. J. Respir. Crit. Care Med. 2000; 161:1631-1635.
28. Bonini S., Lambiase A., Levi-Schaffer F., Aloe L. Nerve growth factor: an important molecule in allergic inflammation and tissue remodelling. Int. Arch. Allergy Immunol. 1999; 118:159-162.
29. Kudlacz E., Shatzer S., Logan D., Olsen K., Knippenberg R., Hsieh L., Esteve H., Maynard G. A role for histamine and substance P in immediate

allergic responses in guinea pig airways: characterization of MDL 108,207DA, a dual H1/NK-1 receptor antagonist. Int. Arch. Allergy Immunol. 1998; 115:169-178.

30. Hunter D. D., Satterfield B. E., Huang J., Fedan J. S., Dey R. D. Toluene diisocyanate enhances substance P in sensory neurons innervating the nasal mucosa. Am. J. Respir. Crit. Care Med. 2000; 161:543-549.
31. Shu X. Q., Mendell L. M. Neurotrophins and hyperalgesia. Proc. Natl. Acad. Sci. U S A 1999; 96:7693-7696.
32. Ibanez C. F. Emerging themes in structural biology of neurotrophic factors. Trends Neurosci. 1998; 21:438-444.
33. Pauwels R. The pharmacology of airway hyperresponsiveness and inflammation. Lung 1990; 168:221-229.
34. Wakabayashi Y., Maeda T., Kwok Y. N. Increase of p75 immunoreactivity in rat urinary bladder following inflammation. Neuroreport 1996; 7:1141-1144.

Nerve growth factor plays a role in the induction of airway hyperresponsiveness in an animal model for allergic asthma: a role for substance P?

Annick de Vries, Axel Fischer[1], Ferdi Engels, Paul A.J. Henricks, Thea Leusink-Muis, Silke Wiegant[2], Petra Hartmann[2], Mark C. Dessing, Robert ten Broeke and Frans P. Nijkamp.

[1] Division of Allergology, Charité Virchow Campus, Biomedical Research Center, Berlin, Germany;
[2] Institute for Anatomy and Cellbiology, Justus-Liebig University, Giessen, Germany

submitted

Abstract

We studied the involvement of NGF and its high-affinity receptor in the development of airway hyperresponsiveness and pulmonary inflammation in a model for allergic asthma. Ovalbumin challenge induced an airway hyperresponsiveness after 24 hrs in ovalbumin-sensitized guinea pigs. This coincided with an increase in the amount of NGF in bronchoalveolar lavage fluid. Simultaneously, an increase in the percentage of substance P immunoreactive neurons in the nodose ganglia and an increase in the amount of substance P in lung tissue were found. We used tyrosine kinase inhibitors to block the signal transduction of the high-affinity NGF receptor, tyrosine kinase A (trkA). Treatment with specific tyrosine kinase inhibitors (K252a or tyrphostin AG879) inhibited the development of airway hyperresponsiveness and prevented the increase in substance P in the nodose ganglia and lung tissue completely. Similar treatment with antibodies directed against NGF (anti-NGF) did not reduce the airway hyperresponsiveness and partially inhibited increases in both the percentage of substance P immunoreactive neurons and the amount of substance P in lung tissue. Neither treatment with K252a, tyrphostin AG879 or anti-NGF changed the influx of inflammatory cells in the bronchoalveolar lavage fluid due to allergen challenge. We conclude that high-affinity receptor for NGF, trkA, plays a role in the induction of airway hyperresponsiveness in our model for allergic asthma. Furthermore, substance P derived from sensory nerves seems to mediate the NGF-induced effects.

Introduction

Nerve growth factor (NGF) is a member of the neurotrophin family of proteins that can regulate neuronal development, maintenance, and recovery from injury. NGF has been extensively studied in relation to neurite outgrowth. However, it now becomes clear that NGF also plays a crucial role in inflammation (1).

Inflammation can lead to an enhanced production and release of NGF. Inflammatory mediators, including interleukin-1, interleukin-4, interleukin-5, tumor necrosis factor α, and interferon-γ have been shown to induce the release of NGF (2, 3). In addition to neurons, non-neuronal cells, such as mast cells (4), fibroblasts (2), T-cells (5), eosinophils (6), lymphocytes (7), macrophages (8, 9) and airway epithelial cells (10) are able to synthesize NGF.

An increase in circulating NGF levels is found in humans with allergic diseases like rhinoconjunctivitis, urticaria-angioedema and asthma (11). In the study of Bonini and colleagues (11), NGF serum levels are particularly high in patients with allergic asthma. Allergen provocation causes an increase in NGF levels also in

nasal lavage fluid of patients with allergic rhinitis (12). Furthermore, allergen challenge induces an increase in NGF levels in bronchoalveolar lavage fluid of sensitized mice (13).
NGF affects immune cell activity, as it promotes inflammatory mediator release from basophils (14), mast cells (15, 16), T- and B-cells (5, 13, 14) and macrophages (19). NGF is also able to affect neuronal function and to induce an enhanced production of Substance P and other tachykinins (16,17). Tachykinins play a role in neurogenic inflammation and in the development of airway hyperresponsiveness and asthma (22, 23). In a previous study we have demonstrated that administration of NGF to guinea pigs results in airway hyperresponsiveness, which is a hallmark of the asthmatic constitution (24).
Taken together, this indicates that NGF may play a role in allergic reactions in the airways. This prompted us to investigate whether NGF is indeed involved in the development of airway hyperresponsiveness and pulmonary inflammation in allergic asthma. NGF can interact with two receptors: either the trkA that binds NGF with high affinity, or the low-affinity receptor p75, which can also bind brain derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3) and NT-4. Of these receptors, only trkA uses tyrosine kinases for its signal transduction (reviewed in 21). We tested the effects of an antibody against NGF and specific tyrosine kinase inhibitors on ovalbumin-induced airway hyperresponsiveness and influx of inflammatory cells into the lungs in sensitized guinea pigs. Simultaneously, we measured levels of NGF in bronchoalveolar lavage fluid and determined the number of substance P containing nerves in the nodose ganglia as well as the amount of substance P in the lung tissue.

Materials and Methods

Animal model
Male Duncan Hartley guinea pigs (weighing approximately 400 g; Harlan CPB, Zeist, The Netherlands) were sensitized to ovalbumin, grade V (SIGMA, St. Louis, MO) by injections of a gel containing 20 µg ovalbumin/ml, and 200 mg $Al(OH)_3$/ml as adjuvant. Six injections were placed: one injection of 0.5 ml intra-peritoneally, one of 0.1 ml nuchally, two injections of 0.1 ml axillarly, and two of 0.1 ml inguinally.
Fourteen days after the sensitization procedure, the guinea pigs were challenged with ovalbumin. The animals received an intra-peritoneal injection with the histamine H_1 receptor antagonist, pyrilamine (10 mg/kg body weight; SIGMA), 30 min before allergen challenge to prevent asphyxsal collapse. Each challenge consisted of a nebulization of 0.01% ovalbumin in sterile saline (Ultra-Neb 2000™, Devilbiss, Sommerset, PA) for 1 min in a box in which the animals had been

placed individually. The animals were observed closely and we assured ourselves that the guinea pig did respond to the challenge. If not, an extra challenge that lasted for 30 sec – 1 min was given.
Control animals were either not sensitized and received a vehicle gel, not containing ovalbumin, and subsequently challenged with ovalbumin, or were sensitized with ovalbumin and subsequently received nebulized saline instead of an ovalbumin challenge.
For the measurements of NGF in the bronchoalveolar lavage fluid and substance P in neurons and lung tissue, a separate group of female guinea pigs (200-250 g body weight, Charles River Wiga, Kreislegg, Germany) with an alternative sensitization procedure was used. These guinea pigs were sensitized by an injection of 1 ml containing 10 mg ovalbumin and 50 mg $Al(OH)_3$ intraperitoneally at days 1, 14, and 28. Control animals received the same injections without ovalbumin. At day 35, sensitized and control animals were exposed to the allergen by nebulization of 10 ml ovalbumin solution (0.1%) for 60 min in a chamber (pari-boy type 37.00, pari-Werk GmbH, Starnberg, Germany; ref. 26). Data obtained from this model for allergic asthma are presented in figure 4 only. The changes in substance P were similar in this guinea pig model for allergic asthma when compared to the other model described in this materials and methods section (see results section).

Treatment with antibodies and tyrosine kinase inhibitors
One hr before and six hrs after the ovalbumin challenge, the ovalbumin-sensitized guinea pigs were treated with either antibodies directed against NGF or tyrosine kinase inhibitors or their vehicles. The control animals were treated with 100 µl saline.
For anti-NGF antibodies, either rabbit anti mouse (SIGMA; 4.7 mg protein/100 µl) or goat anti human (R & D Systems, Minneapolis, MI); 10 µg protein/50 µl) NGF antibodies were applied intra-nasally or intra-tracheally. The treatment with anti-NGF effectively diminished the acute bronchoconstriction after allergen challenge (see chapter 4). Rabbit serum (SIGMA) or goat IgG (SIGMA) were used as controls, respectively.
Two different tyrosine kinase inhibitors were used, K252a (Calbiochem Novabiochem, Läufelfingen, Switzerland) and tyrphostin AG879 (Calbiochem Novabiochem). Both are specific inhibitors of the tyrosine kinases on the trkA (22, 23). Both were dissolved in saline containing 2% DMSO. We applied 100 µl of 0.1 mM K252a or 200 µl of 0.2 mM tyrphostin AG879 intra-nasally, whereas saline containing 2% DMSO was used as a control.
In order to apply the solutions intra-nasally or intra-tracheally, the guinea pigs were anesthetized using halothane (2% halothane, 0.4 l O_2/min, 2 l N_2O/min, approximately 15-30 sec). The animals all regained consciousness within 30 sec after this procedure.

Lung function measurement
The airway resistance was measured 24 hrs after the ovalbumin challenge in the anesthetized guinea pigs upon intravenous administration of increasing doses of histamine (OPG, Zeist The Netherlands) or methacholine (Janssen Chimica, Beerse, Belgium). For this purpose, guinea pigs were anesthetized with urethane (2 g/kg body weight intraperitoneally). Lung function was measured in spontaneously breathing guinea pigs, essentially after Amdur and Mead (29). Air flow and tidal volume were determined by cannulating and connecting the trachea via a Fleisch flow head (Meijnhart, The Netherlands) to a pneumotachograph. A pressure transducer (MP45-2; Validyne Engineering Corp., Northridge, CA) measured the transpulmonary pressure by the difference between the tracheal cannula and an esophageal cannula. In this way, airway resistance was determined breath by breath by dividing transpulmonary pressure by air flow at isovolumetric points. Airway resistance values were averaged per three breaths and are presented as actual value. A small polyethylene catheter (PE-50) was placed in the right jugular vein for intravenous administration of histamine or methacholine.

Bronchoalveolar lavage
After completion of the dose-response curve, the lungs were lavaged three times through the tracheal cannula with 10 ml aliquots warmed (37 ºC) saline. Total amount of bronchoalveolar lavage cells was counted using a Burker-Türk chamber. For differential cell counts, cytospin preparations were stained with Diff-Quick (Merz & Dade, Dudingen, Switzerland). Cells were identified into mononuclear cells, lymphocytes, neutrophils and eosinophils by standard morphology.
NGF was measured in bronchoalveolar lavage fluid obtained 24 hrs after allergen challenge from animals treated according to the alternative sensitization protocol using a commercial ELISA kit from R&D Systems.

Immunohistochemistry
Nodose ganglia were obtained from the guinea pigs 24 hrs after allergen challenge directly after finishing lung function measurement, or just 24 hrs after allergen challenge when the alternative sensitization protocol was used . Zamboni-fixed sensory ganglia were rinsed in 0.1 M phosphate buffer and cryoprotected with 18 % sucrose in phosphate buffer overnight. Sections of the sensory ganglia were cut on a cryostat (500 OM Microm, Waldorf, Germany) at 10 µm and air dried for 30 min. Sections were incubated with a blocking solution containing 1% bovine serum albumin and 10% normal swine serum in phosphate buffer for 60 min, followed by an overnight incubation with a monoclonal rat antibody against substance P (1:1000, Boehringer Ingelheim, Ingelheim, Germany). After washing in phosphate buffered saline, a biotinylated anti-rat-immunoglobulin from sheep (Amersham, Uppsala, Sweden, dilution 1:50) was applied for 1 hr. After several washes in

phosphate-buffered saline, sections were incubated with a mixture of streptavidin-Texas-Red-conjugate (Amersham, dilution 1:50). Slides were washed thoroughly in phosphate-buffered saline and covered with coverslips with buffered glycerol (pH 8.6). For microscopic evaluation, slides were coded and randomly two sections were chosen and in each section 100 neurons were examined for their substance P immunoreactivity.

Radioimmunoassay
Lung samples were obtained at the same moment from the same animals as sensory ganglia. Samples of peripheral lung were snap frozen in liquid nitrogen. Substance P radioimmunoassay was performed as described previously by McGregor and colleagues (30).

Statistics
Means and standard error of the mean (SEM) were calculated. P-values were determined using a two-tailed unpaired Student's t-test for the comparison of two means, unless stated otherwise.

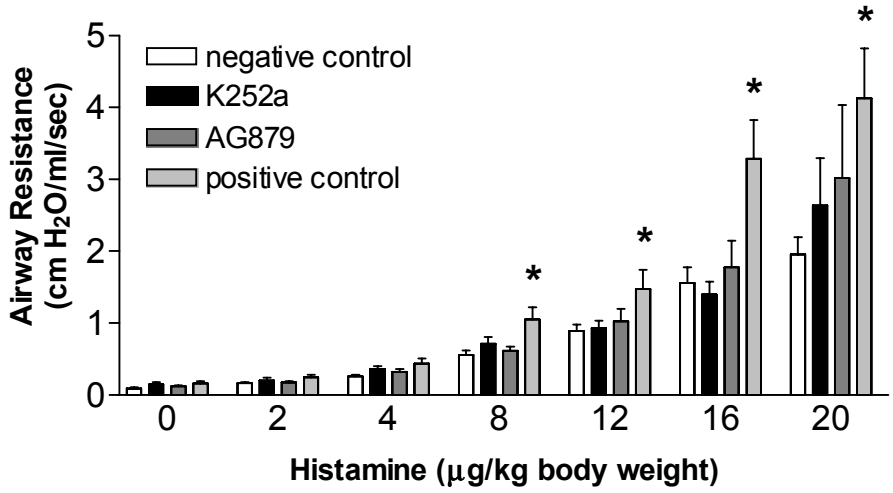


Figure 1. Effect of tyrosine kinase inhibitors K252a and tyrphostin AG879, administered one hr before and six hrs after allergen challenge, on airway hyperresponsiveness 24 hrs after allergen challenge. Airway responsiveness was measured in vivo in guinea pigs upon administration of increasing doses of histamine. Negative control = non-sensitized and ovalbumin challenged, vehicle treated; K252a and AG879 = ovalbumin-sensitized and challenged, K252a or tyrphostin AG879 treated; positive control = ovalbumin-sensitized and challenged; n=8-11. *P<0.05 positive vs. negative control.

Results

Lung function

Airway hyperresponsiveness to histamine was found 24 hrs after the ovalbumin challenge in ovalbumin-sensitized guinea pigs as compared to non-sensitized animals. The ovalbumin-sensitized animals showed an increased responsiveness at the histamine dose of 8 μg/kg and all higher doses ($p<0.05$, fig. 1). Treatment with K252a or tyrphostin AG879 could prevent the hyperresponsiveness to histamine after ovalbumin challenge. Thus, the animals treated with these tyrosine kinase inhibitors did not show a significant increase in airway responsiveness at any concentration of histamine (fig. 1). Treatment with antibodies directed against NGF (anti-NGF) did not reduce the hyperresponsiveness to histamine (positive control airway resistance at highest dose of histamine 4.85 ± 1.44, vs. anti-NGF 3.99 ± 1.34 cm H_2O/ml/sec, both groups n=6, $P>0.05$).

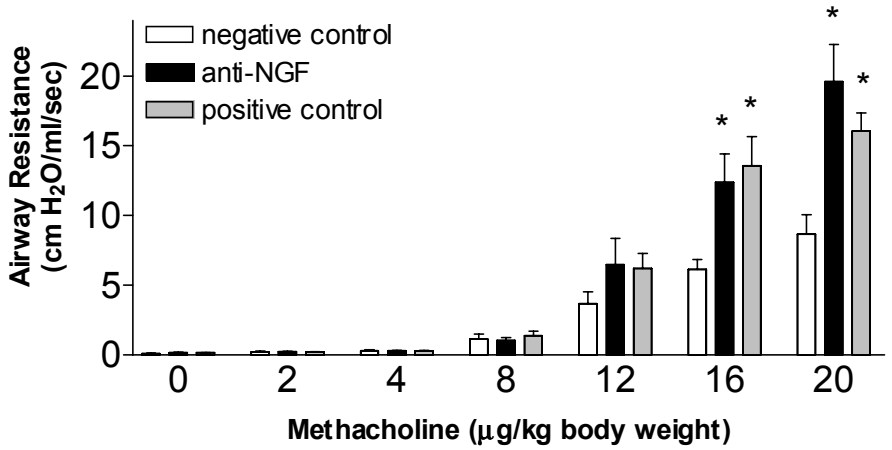


Figure 2. Effect of anti-NGF, administered one hr before and six hrs after allergen challenge, on airway hyperresponsiveness 24 hrs after allergen challenge. Airway responsiveness was measured in vivo in guinea pigs upon administration of increasing doses of methacholine. Negative control = ovalbumin-sensitized and vehicle challenged, control antibody treated; anti-NGF = ovalbumin-sensitized and challenged, anti-NGF treated; positive control = ovalbumin-sensitized and challenged; n=5-6 per group. * $P<0.02$ anti-NGF and positive control vs. negative control.

In our ovalbumin animal model, an airway hyperresponsiveness is not only observed for histamine, but also for other contractile agonists; this resemblesthe asthmatic disease. We therefore tested airway responsiveness to the cholinergic

receptor agonist methacholine as well. Ovalbumin induced an increased airway responsiveness to methacholine in ovalbumin-sensitized animals 24 hrs after ovalbumin challenge as compared to vehicle challenge (fig. 2). The airway resistance at a methacholine dose of 20 μg/kg body weight increased from 8.64 ± 1.43 (vehicle challenged) to 16.05 ± 1.31 cm H_2O/ml/sec (ovalbumin challenged, control antibody treated; $P<0.02$). Treatment with anti-NGF did not reduce this hyperresponsiveness either (airway resistance was 19.61 ± 2.68 cm H_2O/ml/sec at a methacholine dose of 20 μg/kg body weight).

Table 1. Number of cells in bronchoalveolar lavage fluid

	ovalbumin sensitization	**ovalbumin challenge**	**total cell count in bronchoalveolar lavage fluid**
negative control	-	+	19.4 ± 3.4
positive control (control antibody IgG)	+	+	49.5 ± 6.9 *
anti-NGF	+	+	73.2 ± 10.2 *
positive control (vehicle)	+	+	65.3 ± 19.3 *
K252a	+	+	44.9 ± 4.1 *
AG879	+	+	46.6 ± 11.7 *

* = $P<0.03$ vs. negative control

Bronchoalveolar lavage

The airway hyperresponsiveness coincided with an influx of cells in the bronchoalveolar lavage fluid (table 1). The percentage of eosinophils increased in ovalbumin-sensitized animals as compared to non-sensitized animals due to allergen challenge (from 11% ± 3.2 in non-sensitized to 43.6% ± 6.0 in sensitized animals, $P<0.001$, fig. 3). Neither the total amount of cells in the bronchoalveolar lavage fluid nor the percentage of eosinophils was affected by treatment with anti-NGF, K252a or tyrphostin AG879 (table 1 and fig. 3). The percentages of lymphocytes, neutrophils and macrophages were not affected by treatment with anti-NGF, K252a or tyrphostin AG879 (data not shown).

NGF levels were enhanced in bronchoalveolar lavage fluid in sensitized animals as compared to non-sensitized animals 24 hrs after allergen challenge (fig. 4 A). NGF levels increased from 22.6 ± 2.6 to 34.5 ± 6.0 pmol/ml ($P<0.03$ one-tailed Student's t-test).

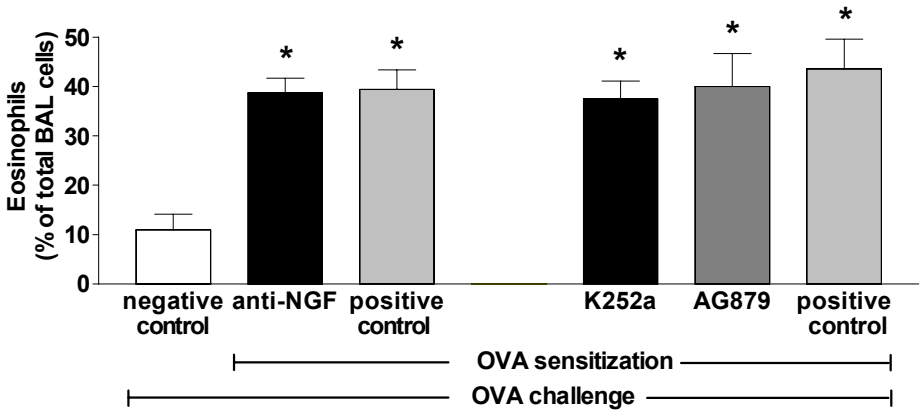


Figure 3. The percentage of eosinophils of the bronchoalveolar lavage (BAL) cells did not change 24 hrs after allergen challenge by anti-NGF or tyrosine kinase inhibitor treatment. Treatment with either respective vehicles (positive control), anti-NGF, K252a or tyrphostin AG879 took place one hr before and six hrs after allergen challenge. * = P<0.001 all groups vs. negative control.

Substance P

The number of substance P immunoreactive neurons was counted in the ganglia of the same animals that were used to determine NGF levels in bronchoalveolar lavage fluid. The percentage of substance P immunoreactive neurons in the nodose ganglia was augmented in sensitized animals as compared to non-sensitized animals 24 hrs after allergen challenge: from 22 ± 2 to 43 ± 4 % (P<0.0003, fig. 4 B). At the same time, the substance P content of lung tissue was increased as well: from 216 ± 17 to 472 ± 41 pmol/g wet weight (P<0.0002, fig. 4 C).

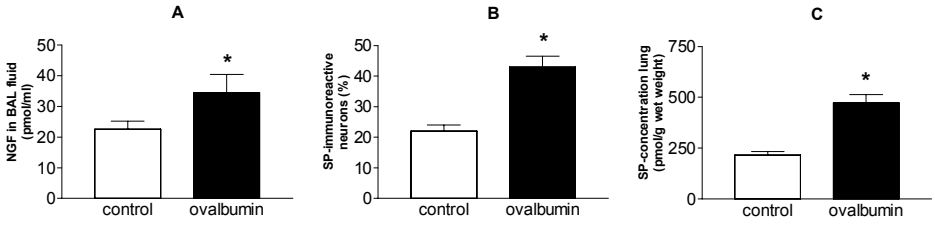


Figure 4. (A) The amount of NGF was elevated in bronchoalveolar lavage fluid (* P<0.03, one-tailed Students t-test), and (B) the number of substance P immunoreactive neurons in nodose ganglia was increased (* P<0.0003), as well as (C) the amount of substance P in lung tissue (* P<0.0002) 24 hrs after ovalbumin challenge in ovalbumin-sensitized animals (ovalbumin, n=3) as compared to non-sensitized animals (control, n=5).

A similar increase, as present in guinea pigs sensitized following the alternative sensitization procedure, was found in the guinea pigs, which were used for lung function measurements. The percentage substance P immunoreactive neurons in the nodose ganglia increased from 24.6 ± 1.8 to 44.2 ± 2.6 % and 46.0 ± 2.5 ($P<0.01$) for antibody and vehicle positive controls, respectively (fig. 5). The substance P content in the lung increased from 250 ± 33 to 519 ± 38 and 484 ± 12 pmol/g wet weight ($P<0.04$) for antibody and vehicle positive controls, respectively (fig. 6).

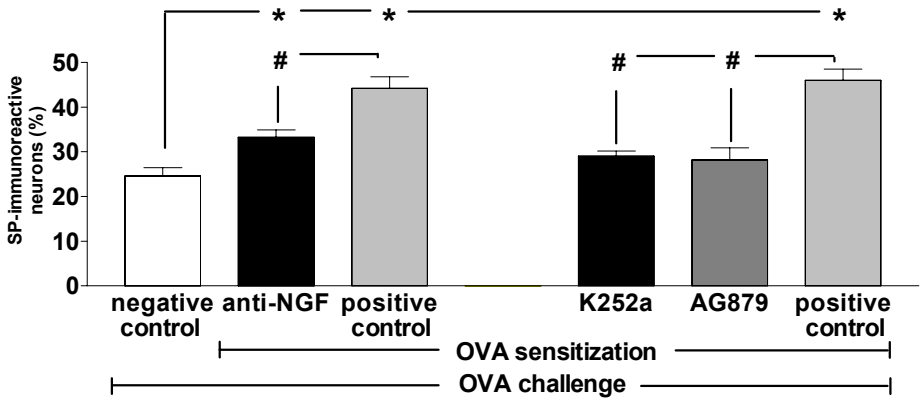


Figure 5. The percentage of substance P immunoreactive neurons in nodose ganglia increased after allergen challenge and this increase was partially inhibited by anti-NGF and completely blocked by tyrosine kinase inhibitor treatment. Treatment with either control serum and anti-NGF or vehicles, K252a and tyrphostin AG879 took place one hr before and six hrs after allergen challenge. All groups n=4-5. * $P<0.01$ anti-NGF and positive controls vs. negative controls, # $P<0.01$ anti-NGF, K252a and AG879 vs. respective positive control.

The guinea pigs that were treated with the tyrosine kinase inhibitors, revealed a complete inhibition of the increase in the percentage of substance P immunoreactive neurons: from 46.0 ± 2.5 to 29.0 ±1.1 for K252a and to 28.2 ± 2.7 % for tyrphostin AG879 ($P<0.01$, fig. 5). A similar decrease was seen when measuring substance P content in the lung tissue: from 484 ± 12 to 262 ± 74 for K252a and to 292 ±43 pmol/g wet weight for tyrphostin AG879 ($P<0.02$, fig. 6). Treatment with anti-NGF induced a decline but did not completely prevented the increase in the percentage of substance P immunoreactive neurons or the amount of substance P in lung tissues of sensitized animals as compared to the non-sensitized animals (figs. 5 and 6). The precentage of substance P immunoreactive neurons was down-regulated from 44.2 ± 2.6 to 33.3 ± 1.7 % by treatment with anti-NGF

(P<0.01), and the substance P content in lung tissue was decreased from 519 ± 38 to 360 ± 32 pmol/g wet weight (P<0.02). However, when compared to the negative controls, the number of substance P immunoreactive neurons was still increased (P<0.01) as was the substance P content (P<0.04).

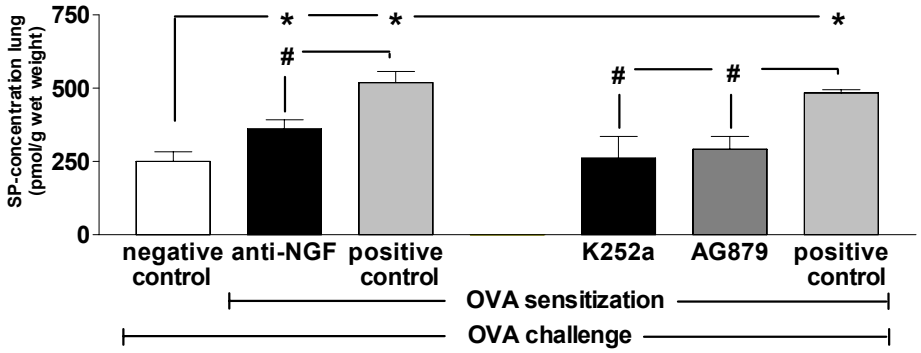


Figure 6. Substance P concentration in the lung tissues increased after allergen challenge. This increase was partially reduced by anti-NGF treatment. It was completely prevented by tyrosine kinase inhibitor treatment. Treatment with either control serum or anti-NGF or vehicles, K252a and tyrphostin AG879 took place one hr before and six hrs after allergen challenge. All groups n=5 * P<0.004 anti-NGF and positive controls vs. negative control, # P<0.02 anti-NGF, K252a and AG879 vs. respective positive control.

Discussion

Guinea pigs sensitized to ovalbumin showed an airway hyperresponsiveness to histamine and methacholine 24 hrs after allergen challenge (figs. 1 and 2). Specific inhibition of the trkA tyrosine kinases by the inhibitors K252a and tyrphostin AG879 inhibited the development of airway hyperresponsiveness. Apparently, the inhibition of the down-stream signaling of the trkA receptor did effectively prevent induction of airway hyperresponsiveness. The importance of the trkA in long-term inflammatory processes is confirmed by the enhanced numbers of trkA immunoreactive neurons in a model for inflammatory chronic pain (31). We conclude that the trkA receptor is involved in the development of airway hyperresponsiveness in our model for allergic asthma.

The cellular influx in the airways in this allergic asthma model was not affected by interfering with trkA. Therefore, the possible involvement of NGF in the airway function of allergic guinea pigs can be envisioned as being distal to inflammatory cell influx into the airways.

NGF is the neurotrophic factor with the highest binding affinity for trkA (25). Interestingly, the development of airway hyperresponsiveness in this model of

allergic asthma coincided with an increase in NGF levels in bronchoalveolar lavage fluid 24 hrs after allergen challenge (fig. 4 A). Possibly, NGF originates from the infiltrating inflammatory cells. Indeed, NGF formation has been demonstrated in various immune organs including the spleen, lymph nodes, and thymus, and immune cells such as mast cells, eosinophils, and B- and T-cells (32). Increases in the levels of NGF in the airways have been reported before in sensitized mice 24 hrs after allergen challenge (13) and in allergic asthmatic patients after allergen challenge (33). It is unknown which cells are the major source for NGF in the airways. Sanico and colleagues recently showed a rapid increase in NGF in nasal lavage fluids shortly after allergen provocation in patients with allergic rhinitis (12). The authors suggest mast cells as most likely source of NGF.

As shown before, guinea pigs sensitized to and challenged with ovalbumin display an increase in substance P immunoreactive neurons in the nodose ganglia, however, not in jugular or dorsal root ganglia (26, 34). In our present study, we found a similar increase was found in substance P immunoreactive neurons in the nodose ganglia (fig. 4 B) as well as an increase in substance P content in the lung tissue (fig. 4 C). There was a correlation between the increase of NGF in bronchoalveolar lavage fluid and the increase of substance P in lung tissue (r^2=0.625, P<0.02, n=8). Therefore, it is likely that NGF is the important mediator for the induction of increased levels of in substance P. This is confirmed by Hunter and colleagues, who showed that tracheal application of NGF can increase the percentage of substance P immunoreactive neurons in nodose ganglia (35). Substance P containing fibers in guinea pig airways are C-fibers, whereas neurons with Aδ-fibers are normally not containing substance P. NGF induced the formation of substance P in nodose neurons with Aδ-fibers. The increase is similar to the increase in substance P in nodose ganglion neurons with Aδ-fibers in allergic models (26, 34, 35).

Interestingly, the tyrosine kinase inhibitors inhibited the increases in substance P immunoreactive neurons and substance P content in the lung tissue (fig. 5 and 6). Furthermore, increases in substance P together with induction of airway hyperresponsiveness have been reported before; for instance in models using toluene diisocyanate (36), ozone (37) or citric acid (23) to induce changes in airway function. Furthermore, our group has shown a role for tachykinins in the IL-5 (38) and 13-hydroxyoctadecadienoic acid (39) induced airway hyperresponsiveness in animal models. Moreover, tachykinin levels are elevated in patients with asthma (22), and protective effects of neurokinin receptor antagonists have been reported (40). Anti-NGF did not completely prevent substance P increases in this model for allergic asthma. This may be the reason that airway hyperresponsiveness was not prevented by anti-NGF treatment.

NGF acts on a variety of cells of the immune system, including mast cells, eosinophils, and B- and T-lymphocytes (41). Inhibiting trkA signaling did not affect the number of cells; however, it could have affected the activity of the cells in the airways and thus lowering the pathologic effects of the allergic inflammation. For instance, mast cells function could have been altered as mast cells express trkA (42) and NGF has been shown to induce phenotypic switching of the mast cells (43) and change the cytokines that are expressed (44). NGF can activate eosinophils (6) as well as T-cells (5) via trkA. Therefore the tyrosine kinase inhibitors could have affected their activation state. Furthermore, there is a role for NGF as an autocrine survival factor that rescues macrophages (9).

We hypothesized a beneficial effect on allergic airway pathology of antibodies directed against NGF for several reasons. Firstly, NGF is upregulated after allergen challenge in our guinea pig model (fig. 4 A) as well as in mice (13), as in patients with allergic asthma or allergic rhinitis (12, 13, 33). Furthermore, inhibiting trkA prevented the induction of airway hyperresponsiveness, and NGF is the preferred ligand for trkA. In line with this is our previous finding that administration of NGF results in an airway hyperresponsiveness (24). However, using antibodies against NGF did not inhibit the development of airway hyperresponsiveness. In our experimental conditions, the same amount of anti-NGF effectively diminished *acute* bronchoconstriction, after allergen challenge (chapter 4); this implies that the amount of anti-NGF is sufficient to block effects mediated by NGF in the guinea pig airways. The fact that anti-NGF did not reduce airway hyperresponsiveness in our guinea pig model is in contrast with the findings of Braun and colleagues, who did demonstrate a reduction in airway hyperresponsiveness by treatment with antibodies directed against NGF (13). The latter attenuation by anti-NGF of airway responsiveness was obtained in mice; therefore species difference may account for the different effects of anti-NGF.

Interestingly, the increased percentage of substance P immunoreactive neurons and the increase in substance P content in the lung tissue were down-regulated by anti-NGF. However, the inhibition was not complete: there was still a significant rise both in the percentage of immunoreactive neurons for substance P as well as in the amount of substance P in the lung tissue (figs. 5 and 6). These data suggest that an increase in substance P levels changed the airway responsiveness; indeed, the tyrosine kinase inhibitors blocked the increase of substance P (figs. 5 and 6) and of airway hyperresponsiveness (fig. 1). This again points to a causal relationship between increases in substance P and the development of airway hyperresponsiveness.

The partial inhibition by anti-NGF of increases of substance P and the lack of effect of anti-NGF on ovalbumin-induced airway hyperresponsiveness could be explained by the action of NT-3. NT-3 is elevated in bronchoalveolar lavage fluid after allergen challenge in patients with allergic asthma (33). NT-3 can also bind

trkA, although with lower affinity than NGF (45). NT-3 could activate trkA and thereby cause airway hyperresponsiveness without influence of antibodies directed against NGF. Furthermore, NT-3 can induce substance P release (46).
NGF induces airway hyperresponsiveness, as we showed before (24). IN the present study, we showed that the high affinity receptor for NGF, trkA, is involved in the development of airway hyperresponsiveness and in the concomitant increases in substance P 24 hrs after allergen challenge. Antibodies directed against NGF did not prevent the induction of airway hyperresponsiveness after allergen challenge; possibly because anti-NGF did not completely block substance P increases.
We conclude that NGF plays a role in the induction of airway hyperresponsiveness, in our model for allergic asthma through its high affinity receptor trkA. Furthermore, substance P derived from sensory nerves might underlie the NGF-induced effects. This is in accordance with our previous data that showed that NGF induces airway hyperresponsiveness via the neurokinin-1 receptor. Presumably, NGF is not the only neurotrophin underlying airway hyperresponsiveness.

References

1. Woolf C. J., Safieh-Garabedian B., Ma Q. P., Crilly P., Winter J. Nerve growth factor contributes to the generation of inflammatory sensory hypersensitivity. Neuroscience 1994; 62:327-331.
2. Hattori A., Iwasaki S., Murase K., Tsujimoto M., Sato M., Hayashi K., Kohno M. Tumor necrosis factor is markedly synergistic with interleukin 1 and interferon-gamma in stimulating the production of nerve growth factor in fibroblasts. FEBS Lett. 1994; 340:177-180.
3. Yoshida K., Kakihana M., Chen L. S., Ong M., Baird A., Cage F. H. Cytokine regulation of nerve growth factor-mediated cholinergic neurotrophic activity synthesized by astrocytes and fibroblasts. J. Neurochem. 1992; 59:919-931.
4. Leon A., Buriani A., Dal Toso R., Fabris M., Romanello S., Aloe L., Levi-Montalcini R. Mast cells synthesize, store, and release nerve growth factor. Proc. Natl. Acad. Sci. USA 1994; 91:3739-3743.
5. Lambiase A., Bracci-Laudiero L., Bonini S., Bonini S., Starace G., D'Elios M. M., De Carli M., Aloe L. Human CD4+ T cell clones produce and release nerve growth factor and express high affinity nerve growth receptors. J. Allergy Clin. Immunol. 1997; 100:408-414.

6. Solomon A., Aloe L., Pe'er J., Frucht-Pery J., Bonini S., Levi-Schaffer F. Nerve growth factor is preformed in and activates human peripheral blood eosinophils. J. Allergy Clin. Immunol. 1998; 102:454-460.
7. Barouch R., Appel E., Kazimirsky G., Braun A., Renz H., Brodie C. Differential regulation of neurotrophin expression by mitogens and neurotransmitters in mouse lymphocytes. J. Neuroimmunol. 2000; 103:112-121.
8. Akiyama S., Hikawa N., Kusakabe T., Kawakami T., Kobayashi H., Takenaka T., Tomita T. Expression of neurotrophins and their receptors in alveolar macrophage (AM) and the lung (Abstract). Am. J. Resp. Crit. Care Med. 1998; 157:A439
9. Garaci E., Caroleo M. C., Aloe L., Aquaro S., Piacentini M., Costa N., Amendola A., Micera A., Calio R., Perno C. F., Levi-Montalcini R. Nerve growth factor is an autocrine factor essential for the survival of macrophages infected with HIV. Proc. Natl. Acad. Sci. USA 1999; 96:14013-14018.
10. Fox A. J., Barnes P. J., Belvisi M. G. Release of nerve growth factor from human airway epithelial cells (Abstract). Am. J. Respir. Crit. Care Med. 1997; 155:A157.
11. Bonini S., Lambiase A., Bonini S., Angelucci F., Magrini L., Manni L., Aloe L. Circulating nerve growth factor levels are increased in humans with allergic diseases and asthma. Proc. Natl. Acad. Sci. USA 1996; 93:10955-10960.
12. Sanico A. M., Stanisz A. M., Gleeson T. D., Bora S., Proud D., Bienenstock J., Koliatsos V. E., Togias A. Nerve growth factor expression and release in allergic inflammatory disease of the upper airways. Am. J. Respir. Crit. Care Med. 2000; 161:1631-1635.
13. Braun A., Appel E., Baruch R., Herz U., Botchkarev V., Paus R., Brodie C., Renz H. Role of nerve growth factor in a mouse model of allergic airway inflammation and asthma. Eur. J. Immunol. 1998; 28:3240-3251.
14. Burgi B., Otten U. H., Ochensberger B., Rihs S., Heese K., Ehrhard P. B., Ibanez C. F., Dahinden C. A. Basophil priming by neurotrophic factors. Activation through the trk receptor. J. Immunol. 1996; 15:5582-5588.
15. Matsuda H., Coughlin M. D., Bienenstock J., Denburg J. A. Nerve growth factor promotes human hemopoietic colony growth and differentiation. Proc. Natl. Acad. Sci. USA 1988; 85:6508-6512.
16. Tal M., Liberman R. Local injection of nerve growth factor (NGF) triggers degranulation of mast cells in rat paw. Neurosci. Lett. 1997; 22:129-132.
17. Levi-Montalcini R., Skaper S. D., Dal Tosco R., Petrelli L., Leon A. Nerve growth factor: from neurotrophin to neurokine. Trends Neurosci. 1996; 19:514-520.

18. Otten U., Ehrhard P., Peck R. Nerve growth factor induced growth and differentiation of human B lymphocytes. Proc. Natl. Acad. Sci. USA 1989; 86:10059-10063.
19. Susaki Y., Shimizu S., Katakura K., Watanabe N., Kawamoto K., Matsumoto M., Tsudzuki M., Furusaka T., Kitamura Y., Matsuda H. Functional properties of murine macrophages promoted by nerve growth factor. Blood 1996; 88:4630-4637.
20. Grimes M. L., Zhou J., Beattie E. C., Yuen E. C., Hall D. E., Valletta J. S., Topp K. S., LaVail J. H., Bunnett N. W., Mobley W. C. Endocytosis of activated TrkA: Evidence that nerve growth factor induces formation of signalling endosomes. J. Neurosci. 1996; 16:7950-7964.
21. Lindsay R. M., Harmar A. J. Nerve growth factor regulates expression of neuropeptide genes in adult sensory neurons. Nature 1989; 337:362-364.
22. Lundberg J. M. Tachykinins, sensory nerves, and asthma- an overview. Can. J. Physiol. Pharmacol. 1995; 73:908-914.
23. Biyah K., Molimard M., Esmonds-Alt X., Advenier C. SR140333 prevents potentiation by citric acid of plasma exudation induced by histamine in airways. Eur. J. Pharmacol. 1996; 308:325-328.
24. de Vries A., Dessing M. C., Engels F., Henricks P. A. J., Nijkamp F. P. Nerve growth factor induces a neurokinin-1 receptor- mediated airway hyperresponsiveness in guinea pigs. Am. J. Respir. Crit. Care Med. 1999; 159:1541-1544.
25. Ibanez C. F. Emerging themes in structural biology of neurotrophic factors. Trends Neurosci. 1998; 21:438-444.
26. Fischer A., McGregor G. P., Saria A., Philippin B., Kummer W. Induction of tachykinin gene and peptide expression in guinea pig nodose primary afferent neurons by allergic airway inflammation. J. Clin. Invest. 1996; 9:2284-2291.
27. Koizumi S., Contreras M. L., Matsuda Y., Hama T., Lazarovici P., Guroff G. K-252a: a specific inhibitor of the action of nerve growth factor on PC 12 cells. J. Neurosci. 1988; 8:715-721.
28. Ohmichi M., Pang L., Ribon V., Gazit A., Levitzki A., Saltiel A. R. The tyrosine kinase inhibitor tyrphostin blocks the cellular actions of nerve growth factor. Biochem. 1993; 32:4650-4658.
29. Amdur M. O., Mead J. Mechanics of respiration in unanesthetized guinea pigs. Am. J. Physiol. 1958; 192:364-368.
30. McGregor G. P., Hartel R., Haberberger R., Kummer W., Voigt K. Preprotachykinin-A gene expression occurs transiently in the developing rat endocrine pancreas and can be regulated in RINm5F cells. Endocrinology 1995; 136:2538-2546.

31. Pezet S., Ont niente B., Grannec G., Calvino B. Chronic pain is associated with increased TrkA immunoreactivity in spinoreticular neurons. J. Neurosci. 1999; 19:5482-5492.
32. Aloe L., Bracci-Laudiero L., Bonini S., Manni L. The expanding role of nerve growth factor: from neurotrophic activity to immunologic diseases. Allergy 1997; 52:883-894.
33. Virchow J. C., Julius P., Lommatzsch M., Luttmann W., Renz H., Braun A. Neurotrophins are increased in bronchoalveolar lavage fluid after segmental allergen provocation. Am. J. Respir. Crit. Care Med. 1998; 158:2002-2005.
34. Undem B. J., Hunter D. D., Liu M., Haak-Frendscho M., Oakragly A., Fischer A. Allergen-induced sensory neuroplasticity in airways. Int. Arch. Allergy Immunol. 1999; 118:150-153.
35. Hunter D. D., Myers A. C., Undem B. J. Nerve growth factor-induced phenotypic switch in guinea pig airway sensory neurons. Am. J. Respir. Crit. Care Med. 2000; 161:1985-1990.
36. Hunter D. D., Satterfield B. E., Huang J., Fedan J. S., Dey R. D. Toluene diisocyanate enhances substance P in sensory neurons innervating the nasal mucosa. Am. J. Respir. Crit. Care Med. 2000; 161:543-549.
37. Wu Z. X., Morton R. F., Lee L. Y. Role of tachykinins in ozone-induced airway hyperresponsiveness to cigarette smoke in guinea pigs. J. Appl. Physiol. 1997; 83:958-965.
38. Kraneveld A. D., Nijkamp F. P., Van Oosterhout A. J. M. Role for neurokinin-2 receptor in interleukin-5-induced airway hyperresponsiveness but not eosinophilia in guinea pigs. Am. J. Respir. Crit. Care Med. 1997; 156:367-374.
39. Engels F., Van Houwelingen A. H., Buckley T. L., Van de Velde M. J., Henricks P. A. J., Nijkamp F. P. Airway hyperresponsiveness induced by 13-hydroxyoctadecadienoic acid (13-HODE) is mediated by sensory neuropeptides. Adv. Prostaglandin Thromboxane Leukot. Res. 1995; 23:361-363.
40. Joos G. F., van Schoor J., Kips J. C., Pauwels R. A. The effect of inhaled FK224, a tachykinin NK-1 and NK-2 receptor antagonist, on neurokinin A-induced bronchoconstriction in asthmatics. Am. J. Respir. Crit. Care Med. 1996; 153:1781-1784.
41. Aloe L., Simone M. D., Properzi F. Nerve growth factor: a neurotrophin with activity on cells of the immune system. Microsc. Res. Tech. 1999; 45:285-291.
42. Tam S. Y., Tsai M., Yamaguchi M., Yano K., Butterfield J. H., Galli S. J. Expression of functional TrkA receptor tyrosine kinase in the HMC-1 human mast cell line and in human mast cells. Blood 1997; 90:1807-1820.

43. Getchell M. L., Kulkarni-Narla A., Takami S., Albers K. M., Getchell T. V. Age-dependent phenotypic switching of mast cells in NGF-transgenic mice. Neuroreport 1995; 6:1261-1266.
44. Marshall J. S., Gomi K., Blennerhassett M. G., Bienenstock J. Nerve growth factor modifies the expression of inflammatory cytokines by mast cells via a prostanoid-dependent mechanism. J. Immunol. 1999; 162:4271-4276.
45. Clary D. O., Reichardt L. F. An alternatively spliced form of the nerve growth factor receptor TrkA confers an enhanced response to neurotrophin 3. Proc. Natl. Acad. Sci. USA 1994; 91:11133-11137.
46. Malcangio M., Ramer M. S., Boucher T. J., McMahon S. B. Intrathecally injected neurotrophins and the release of substance P from the rat isolated spinal cord. Eur. J. Neurosci. 2000; 12:139-144.

Co-culturing of primary mast cells and primary sensory neurons induces small alterations in the function of both cell types

Annick de Vries, Paula M. Mommersteeg, Ferdi Engels,
Paul A.J. Henricks and Frans P. Nijkamp

Abstract

We presume nerve growth factor (NGF) released by mast cells underlies functional changes in sensory nerves in our animals models for asthma, since mast cells are found in close proximity of sensory nerves. In this study we used primary cultures of both cell types to investigate possible functional interactions. Sensory neurons were derived from embryonic dorsal root ganglia and mast cells were cultured from bone marrow-derived cells. We observed adherence of the mast cells to the neurites, whereas normally these mast cells are in suspension during culture. No functional gap junctions were found between mast cells and neurons. The presence and stimulation of mast cells induced small enhancements in the expression of preprotachykinin (PPT) mRNA, the precursor for tachykinins such as substance P and neurokinin A, in the dorsal root ganglion neurons. No expression of PPT mRNA was found in mast cells, suggesting that the mast cells do not synthesize PPT and, subsequently, no tachykinins. Presence of the dorsal root ganglion neurons generated minor alterations in the capability of the mast cells to degranulate.
We conclude that mast cells adhere to neurons and small functional changes in mast cells and neurons due to co-culturing and stimulation were found. Excreted factors could underlie interaction between these two types of cells.

Introduction

Mast cells are found in close proximity to neurites (1, 2). These neurites are mostly of sensory origin (1, 3, 4). Sensory neurons express several peptides, the tachykinins, and are sensitive to capsaicin (5, 6). Sensory neurons are involved in neurogenic inflammation, for example in the asthmatic disease (7), neuropathic pain (8) and inflamed skin (9). Neurogenic inflammation involves changes in function of the sensory neurons due to inflammatory mediators which results in an enhanced release of tachykinins from the nerves (7). The role of mast cells in hypersensitivity reactions is well known, by its activation with allergen and IgE (10). However, mast cells may play a role in the phenomenon of neurogenic inflammation as well by affecting nerve function (2, 4, 11). Indeed, mediators released from mast cells have been suggested to affect neuronal function. These mediators include arachidonic acid metabolites, serotonin, nitric oxide, nerve growth factor and histamine (4, 12, 13). Additionally, mediators released by nerves, such as acetylcholine, NGF, substance P and ATP affect mast cell function (4, 12). Therefore, functional interaction between mast cells and sensory neurons seems very likely.

In most in vitro studies, single mediators, released by either neurons or mast cells, change the properties of either mast cells (13, 14) or neurons (15, 16). In vivo studies revealed possible interactions between both cell types (17). In mouse skin preparations the number of close contacts between mast cells and neurons changed depending on environmental changes (1). This indicates that mast cell-neuron contacts might have physiological relevance. Furthermore, allergen provocation in the airways, presumably merely activating mast cells, changes neuronal function in the airways (18). Mucosal mast cells are activated and secrete mast cell proteases by Pavlovian conditioning (19). This indicates that neuronal activation can directly stimulate mast cells. Inhibiting the release of mediators from either neurons or mast cell in vivo can prevent activation of the other (3, 20). For instance, in vivo depletion of neuropeptides with capsaicin reduced IgE-mediated inflammatory response; this indicates that neurons are involved in mast cell-mediated inflammation (21).
Very few studies report on co-cultures of mast cells and neurons. Such studies are either using two cell lines (22-24), or one cell line and primary cultured neurons (22-25). They evidence communication between neurons and mast cells, but do not document how this affects function and properties of either cell. In our present study, we examined the effect of co-culturing primary derived-mast cells and sensory neurons on functional properties of the two types of cells.

Materials and methods

Dorsal root ganglia culture
Dorsal root ganglia were removed aseptically from 16 day-old embryos of Wistar rats (GDL, Utrecht, the Netherlands) and pooled in ice-cold L-15 medium (GIBCO). Dorsal root ganglion neurons were cultured essentially as previously described by Dijkhuizen and colleagues (26). Dorsal root ganglia were cervical and thoracic of origin, which form a large percentage of NGF sensitive neurons (27, 28). These tachykinin containing nerves partly innervate the airways (29). The dorsal root ganglia were transferred to DMEM/F-12 (mixture 3:1) medium supplemented with 200 µg/ml transferrin, 200 µM putrescine, 40 nM progresterone, 60 nM selenium, 10 µg/ml insulin, and 0.01% albumine (all GIBCO). A mitotic inhibitor, $5x10^{-4}$ M cytosine-β-D-arabinofuranoside (Sigma) prevented was added to the medium mixture, to prevent non-neuronal cells from growing. This concentration of cytosine-β-D-arabinofuranoside prevents division of non-neuronal cells without influencing the growth of the dorsal root ganglion neurons (23, 30).
Single dorsal root ganglia were cultured in either six wells–plates (Costar) or in smaller culture slides (Falcon, micronic, 8 chamber polystyrene vessel). Plates

were coated with polyornithin (Sigma) and laminin (Boehringer Mannheim). Cultures were grown at 37ºC in 5% CO_2 humidified atmosphere.

Bone marrow-derived mast cells (BMMC)
The bone marrow of eight- to 10 weeks old BALB/c mice (GDL) was aseptically flushed from femurs into complete RPMI. The complete RPMI contained 4 mM L-glutamine, 50 µM mercaptoethanol, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 µg/ml streptomycin and 0.1 mM non-essential amino acids (GIBCO). The cell suspension was washed twice in complete RPMI. Fetal calf serum (10% vol/vol) and pokeweed-stimulated spleen supernatant (20% vol/vol) were added to the medium. Spleen single cell suspensions were activated with pokeweed at a cell density of $2x10^6$ cells/ml and the supernatant harvested after 6-7 days. Bone marrow-derived cells were cultured in 75-cm² tissue culture flasks at a density of $2x10^5$ cells/ml. Medium was refreshed every week to achieve a final concentration of $1x10^5$ cells/ml (essentially after Karimi and colleagues; 31, 32). After three weeks of culture the cells developed into an essentially pure population of mucosal type mast cells (31, 32).

Co-culture of mast cells and dorsal root ganglia
Bone marrow-derived mast cells were added to the dorsal root ganglion cultures at the 2nd day of the dorsal root ganglion-culture. Cells were cultured in either modified DMEM/F12 with different concentrations of NGF or a combination of DMEM/F12 and complete RPMI. Different medium mixtures of DMEM/F12 and complete RPMI had no effect on PPT mRNA expression in the dorsal root ganglion-culture. Therefore we used a medium consisting of half chemically defined medium and half mast cell medium (co-culture medium) in all co-cultures.

ß-hexosaminidase release
Beta-hexosaminidase release was used as parameter for mast cell degranulation (31). Cells were washed in Tyrode buffer (supplemented with 2.38% HEPES, 0.1% BSA at pH 7.2). Mast cells were added at 40.000 cells per well in a 96 flat bottom wells plate (Costar). To investigate mast cell activation upon FCεRI crosslinking, cells were incubated with anti-dinitrophenyl (DNP) IgE ($H1DNP_\varepsilon$ 26.82 hybridoma, a kind gift from J. Riviera, NIH, USA; 20% vol/vol) at 37 °C for 1.5 hours. After washing, cells were activated by adding multivalent antigen DNP-HSA (Sigma) at concentrations between 1-30 ng/ml and incubated for 30 min at 37ºC. To obtain total β-hexosaminidase content, cells in control wells were lysed using 1% triton X-100. Supernatants were analyzed for β-hexosaminidase activity by incubation with 80 µM 4-methylumbelliferyl-N-actetyl-β-D-glucosaminide (Sigma) in citrate buffer (0.1 M, pH 4.5) for 60 min at 37ºC. Subsequently, the

reaction was stopped by addition of 100 µl glycine buffer (0.2 M glycine, 0.2 M NaCl, pH 10.7) and fluorescence was measured at λ_{ex}: 360/ λ_{em}: 460 using a Millipore Cytofluor 2350 microplate reader. Degranulation was calculated as the amount of β-hexosaminidase activity present in the supernatant as a percentage of the total β-hexosaminidase activity.
For the β-hexosaminidase experiments involving co-cultures, dorsal root ganglion neurons were grown in the 96 wells plates. Single cell suspensions of dorsal root ganglia were cultured in order to obtain an equal number of neurons in each well. To derive single cells suspensions, dorsal root ganglia were incubated with 2% collagenase type I (Sigma) for 30 min at 37°C in 5% CO_2 humidified atmosphere. Dorsal root ganglia were transferred into fresh medium, and triturated 10 times through a flame-polished glass Pasteur pipet (modified from Lindsay; 33).

Gap junction probes
To investigate the presence of functional gap junctions, either neurons or mast cells were loaded with a fluorescent probe. Lucifer yellow was injected into a neuronal cell body of a co-culture in which mast cells and dorsal root ganglion neurons were present for 3 or 4 days. Cultures were viewed microscopically with a BX50wi fluorescence microscope (Olympus) using a water immersion objective (F1) and using an excitation cube U-mwb ($\lambda_{ex} < 450$, $\lambda_{em} > 515$). Alternatively, mast cells were loaded with the fluorescent probe 4-(and-5)-carboxyfluorescein diacetate (carboxyfluorescein). Cells were washed twice with RPMI and incubated at 37ºC with 10 mM carboxyfluorescein. Subsequently, the cells were washed and added to the dorsal root ganglion-culture at day 3 in culture. Cells were fixed with 1% paraformaldehyde after two washes with PBS (37ºC) at 10 min, 1 hr and 2 hrs after addition. Mounting medium (Vectashield Mounting medium, Molecular Probes) was applied and cultures were covered with cover slides for microscopical examination. A Nikon Eclipse E600 with Nomarski optics and epifluorescence (filters λ_{ex}: 465-495/ λ_{em}: 515-555) was used and images were recorded digitally using an Apogee KX2 camera (Lambert Instruments B.V.).

RNA isolation and RT-PCR
Dorsal root ganglia were collected by scraping the cells off the plates, whereas bone marrow-derived cells were spun down. Cells were rinsed with PBS and RNA was isolated from these cells with a total RNA isolation system-kit (Promega). We used oligo-dT of different lengths for the reverse transcriptase (RT) reaction (10 min at 22ºC, 45 min at 42ºC, 5 min at 99ºC; all products Perkin Elmer). Amplitaq gold (Perkin Elmer) was used for the PCR which consisted of 30-40 cycles (95 ºC/60 ºC/72 ºC each one min). Rat preprotachykinin (PPT) primers were: sense 5'-AGA GGA AAT CGG TGC CAA CG-3' and antisense 5'-TTC GTA GTT CTG

CAT TGC GC-3' with 4 different product lengths; β-PPT (321 bp), γ-PPT (276bp), α-PPT (267bp) and δ-PPT (220bp; 30). All splice variants can give rise to substance P; β-PPT and γ-PPT can code for neurokinin A, whilst neurokinin K can only be translated from β-PPT and neurokinin-γ merely from γ-PPT. These primers were also suitable to detect mice PPT, though mice only have three splice variants instead of four. Rat GAPDH primers: sense 5'-CAC GGC AAG TTC AAC GGC AC-3' and antisense 5'-TCT GAG TGG CAG TGA TGG CA-3' (all Pharmacia Biotech).

Immunocytochemistry
Dorsal root ganglion-cultures were fixed at four days in culture with 4% paraformaldehyde in PBS and permeabilized with 100% methanol (–20 ºC for 10 min). The peroxidase reactivity was blocked for 10 min with 0.1% NaN_3 and 0.3% H_2O_2 in 50 mM Tris-buffer. This was followed by 3 washes with 50 mM Tris-buffer (pH 7.6), and subsequently samples were washed for 15 min in 50 mM Tris, 135 mM NaCl at pH 7.5, with 1% horse serum (TBS-HS). Samples were incubated with the anti-neurofilament antibody RT 97 (Boehringer Mannheim) at a concentration of 0.5 µg/ ml in TBS-HS for 1 hr, at room temperature. Isotype control plates were incubated with mouse anti-human thyroglobulin (Sigma, 0.5 µg/ml). The negative control consisted of TBS-HS. Cultures were washed with TBS-HS and incubated with horseradish peroxidase (HRP) conjugated rabbit anti-mouse antibody (Dako, 1:200 in TBS-HS) for 30 min. This was followed by three washes with TBS-HS and three washes with 50 mM Tris-buffer, pH 7.6 and the cultures were stained with 60% 3,3'–diaminobenzidine tetrahydrochloride (in 50 mM Tris-buffer, pH 7.6, with 0.2% $(NH_4)_2SO_4$, $NiSO_4$ and 0.02 % H_2O_2) for maximally 30 min at room temperature. The culture slides were washed with 50 mM Tris-buffer. Subsequently, mast cells were stained with toluidine blue (0.5% toluidine blue in glacial acetic acid, pH 2.7, Sigma) for 2 min. Slides were washed with water, and air-dried slides were embedded in DPEX. Cultures were examined microscopically using a normal light microscope and images digitized using a video camera.

Results and discussion

Co-culture of bone marrow-derived mast cells and sensory neurons
Primary mast cell cultures normally grow in suspension. These mast cells did not adhere to the poly-ornithin/laminin coated wells. When co-cultured with dorsal root ganglia, mast cells specifically adhered to the neurites (fig. 1). Another study showed close contacts between mast cells and neurites when co-culturing sympathetic neurons and RBL-2H3 cells, a rat basophilic leukemia cell line, which

is a model for mucosal mast cells (22). However, our present study is, to our knowledge, the first report showing binding of mast cells to sensory neurons in a primary culture.

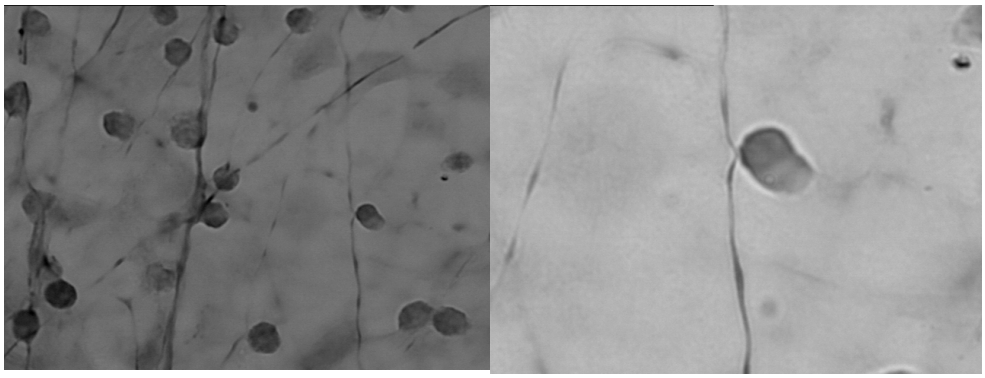


Figure 1. Typical example of bone marrow-derived mast cells attached to neurites in dorsal root ganglion-culture. Neurites are immunostained for neurofilament and mast cells are visualized using toluidine blue. Right panel is detail of left panel.

Numerous studies show a functional and morphological relationship in vivo between mast cells and capsaicin-sensitive sensory nerves in pig skin (35), murine skin (1), rat intestine (17), guinea pig ileum (36), rat dura mater (3, 20) and rat and swine airway mucosa (35, 37).
Communication between cells which are in close contact often occurs via gap junctions. Furthermore, both neurons and mast cells express similar connexins (the proteins forming gap junctions) namely the connexins 32 and 43 (38-40). In order to reveal formation of gap junctions between mast cells and neurites, we applied specific probes: either Lucifer yellow, a gap junction permeant tracer (41, 42), or carboxyfluorescein, which is specific for detecting gap junctions (43, 44). The dorsal root ganglia were cultured and at day 2 co-cultured with mast cells for three days. Lucifer yellow was injected into the cell body of a neuron, and this dye was clearly transported throughout the neurites. No fluorescence could be detected in mast cells (data not shown). This indicated that there is no intercellular transport from neurons to mast cells via gap junctions. In a separate set of experiments, mast cells were labeled with carboxyfluorescein and added to a four-day dorsal root ganglion-culture and fixed at 10 min, 1 hr and 2 hrs after addition of the mast cells. No fluorescent dye could be detected in the neurites (fig. 2). This also indicated that there were no functional gap junctions between mast cells and neurons.
As we did detect contacts between mast cells and neurites (fig. 1), but no functional gap junctions (fig. 2), obviously, gap junctions are not necessary for communication between mast cells and neurons. Instead, these cells might

mutually affect each other by their respective mediators in a paracrine fashion (4, 12, 13). This has been suggested before, as soluble factors from RBL-2H3 cells stimulated cooperatively the neurite outgrowth of sympathetic neurons (45).

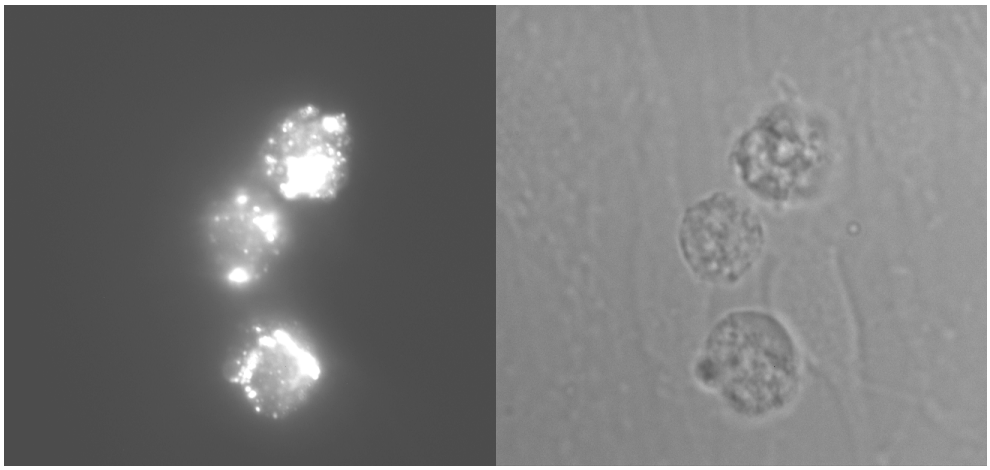


Figure 2. Fluorescent image (left) and Komarski transformed light microscopic image (right) of carboxyfluorescein-loaded mast cells. The carboxyfluorescein was loaded in the mast cells, and mast cells were co-cultured with dorsal root ganglion neurons for 2 hrs. Fluorescence was not visible in the neurites (left panel), which were present in close proximity the mast cells, as can be seen in the right panel, indicating there are no gap junctions formed.

Change in sensory nerves

In order to investigate whether the mast cells were able to change functional properties of the neurons, the expression of PPT, the precursor for tachykinins, mRNA was determined using a RT-PCR. Naive dorsal root ganglia dissected from the embryos already expressed PPT mRNA. Increasing concentrations of NGF in the culture medium induced enhanced expression of PPT mRNA in the monoculture of these neurons. We found a clear increase in the expression of PPT between 0 and 10 ng/ml NGF, whereas we did not find differences with higher concentrations (10-200 ng/ml) of NGF. Our findings are in accordance with the study of Lindsay and others, which also showed an increase in PPT expression with NGF concentrations between 0 and 25 ng/ml (33). Since NGF can also be derived from satellite cells, Schwann cells or fibroblasts present in the culture (46-48), actual concentrations of NGF in culture could be higher than the exogenously added NGF. Therefore, we cultured the dorsal root ganglia at 5 ng/ml NGF to avoid a maximum increase in PPT mRNA. We did not culture the dorsal root ganglia without NGF, thereby preventing upregulation by the very low concentration of 5 ng/ml NGF itself, as neurons would otherwise not survive (49,

50). Withdrawal of a neurotrophic factor would induce a down-regulation in the number of neurons and therefore PPT expression as well (49).

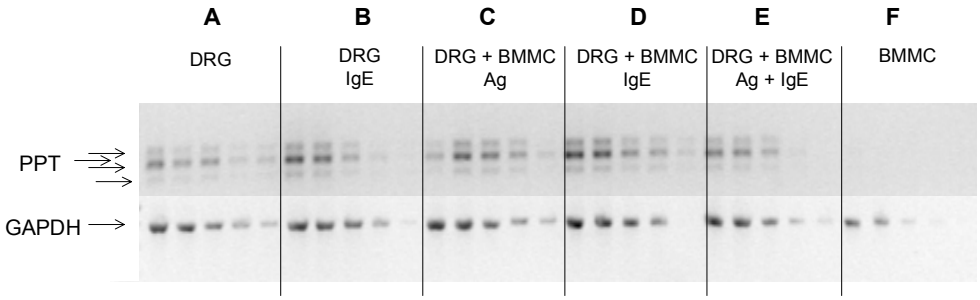


Figure 3. PPT mRNA expression of dorsal root ganglion-cultures (DRG) and/or mast cells (BMMC) in the absence or presence of antigen (Ag) and/or IgE. GAPDH was used as a housekeeping gene. Sequential dilutions of cDNA were used for the PCR; dilutions were the same in all conditions for PPT and GAPDH amplification, starting at an equal amount of RNA in the RT reaction. The four arrows before PPT bands point to four different splice variants of this precursor.

Dorsal root ganglion-cultures were maintained with 5 ng/ml NGF for 1 day. On day 2 the mast cells were added and co-cultured with the dorsal root ganglia for another 3 days. PPT mRNA expression of different cultures is shown in fig. 3. All panels showed highly comparable GAPDH bands, which made it possible to compare DNA bands for PPT directly. Four splice variants of PPT were observed (30).

The primary cultures of mast cells showed no evidence for the production of any substance P, as we detected no PPT mRNA in the mast cell culture (panel F, fig. 3). To our knowledge no other studies describe any data on substance P synthesis in mast cells. Interestingly, other hemapoietic cells like monocytes, macrophages, leukocytes and T-cells are able to synthesize and release substance P (51-53). Of course, merely no detection of PPT mRNA by using a RT-PCR can not exclude production of substance P at the protein level.

PPT mRNA amplification of dorsal root ganglia cultured without mast cells is shown in panel A of fig. 3. Co-culturing of dorsal root ganglia with the primary mast cells affected the PPT expression. The addition of unstimulated mast cells to the dorsal root ganglion-culture, either with addition of only antigen (panel C, fig. 3) or only IgE (panel D, fig. 3) to the culture, enhanced PPT expression slightly. PPT bands showed a higher density on the gel and PPT bands are clearly visible at the highest dilution. The presence of stimulated mast cells to the dorsal root ganglion-culture (panel E, fig. 3) did not further affect levels of PPT mRNA.

All changes described so far are slight changes in PPT mRNA expression. We did not detect any PPT mRNA in mast cells; therefore we exclude that increases in

PPT mRNA in the sensory neurons are due to PPT mRNA present in mast cells. As a negative control for co-culturing with mast cells, dorsal root ganglion neurons were incubated with IgE alone and this did not upregulate PPT expression (panel B, fig.3).
Mediators, such as histamine and arachidonic acid metabolites released from activated mast cells can affect neuronal function (4, 12). Another mediator released from mast cells is NGF, which is able to upregulate PPT expression (54, 55). In our present study here, we found small changes in neuronal function. It could be that changes were actually larger, but could not be detected due to technical limitations. Indeed, in our experiments, we used a semi-quantitative RT-PCR in which samples are related to the amount of the housekeeping enzyme GAPDH. As not all mast cells are washed-off before RNA isolation, due to adherence of part of the population of mast cells to the dorsal root ganglia, this could have induced an increase in the amount of housekeeping gene when isolating total RNA. This could have resulted in an underestimation of expression of PPT mRNA.

Change in mast cells
Calcium mobilization occurred in mast cells when co-cultured neurons were activated with bradykinin (24). In our study, we examined the function of the mast cells by stimulation with IgE and antigen, and changes in function due to the presence of the neurons. Merely very small enhancements in ß-hexosaminidase release by the mast cells were detected as a result of co-culturing with the dorsal root ganglion neurons (fig. 4).
Previous findings in neuron-mast cell co-cultures showed a communication between the cells, but no change in the function of neurons or mast cells (22, 24).We expected more pronounced changes in our co-culture system of bone marrow-derived mast cells and sensory neurons, as different mediators released from each of these cells have pronounced effects in a mono-culture of either mast cells or sensory neurons. Mediators released from activated mast cells, e.g. histamine and arachidonic acid metabolites (4, 12, 16) can alter neuronal function. Another mediator released from mast cells, NGF can induce substance P synthesis in sensory neurons (52, 53, 57). We and others showed that substance P enhances mast cell mediator release or stimulates the mast cell (13, 32, 57, 58). Direct mast cell-neurite communication occurred via substance P in a co-culture of mast cells and neurons (24). In our present study, we co-cultured neurons with mucosal mast cells. Cultured mucosal mast cells increase responsiveness to substance P only after addition of stem cell factor and interleukin-4 to the culture medium (32). This could be the reason that we found only small changes in mast cell function, as we did not add interleukin-4 or stem cell factor to the mast cell cultures. However, whether or not substance P could induce a change in responsiveness to FcεR1 crosslinking has not been studied (32).

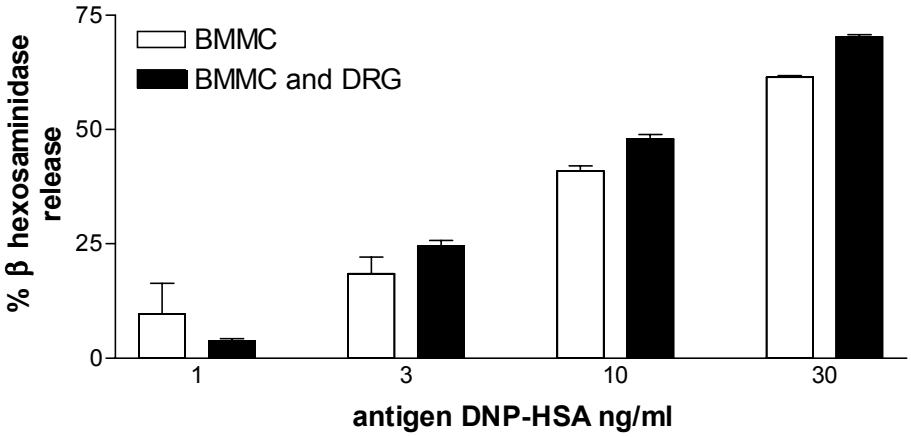


Figure 4. IgE-mediated ß-hexosaminidase release as percentage of total cellular content of ß-hexosaminidase from bone marrow-derived mast cells. Release is slightly enhanced by co-culturing the mast cells with dorsal root ganglion neurons for 3 days. Mast cells were cultured on poly-ornithine and lamin-coated wells with or without dorsal root ganglion neurons. In control experiments, neurons were treated as the mast cells (incubated with IgE and the antigen DNP-HSA) and showed a small aspecific ß-hexosaminidase release, which was subtracted from the data obtained with the mast cells co-cultured with neurons. (n=3).

Data on exogenously applied mediators indicate that the levels of mediators released from either mast cells or neurons in our experiments were probably not sufficient to induce large changes in the other cell type. Indeed, only a high concentrations of exogenous, but not endogenous, substance P are able to release histamine from isolated human skin fragments (59). In contrast, one study showed that activation of mast cells in co-culture with sympathetic neurons altered neuronal physiology (60). In this study, rat peritoneal mast cells were activated with anti-IgE in co-culture with neurons derived from mouse superior cervical ganglia. Mast cell activation caused depolarization of the neurons and decreased their membrane resistance (60).
In conclusion, our present study shows that mast cells adhere to neurites but that only small functional changes could be detected when co-culturing these two types of cells.

Acknowledgements
Authors thank Paul A. Dijkhuizen for his assistance starting the dorsal root ganglion-cultures, Jeroen van Bergenhenegouwen for his assistance with the RT-PCR, Janine Schuurman and René Houtman for culturing of the mast cells, Mieke

Struik for her assistance with the lucifer yellow injections and Frank A.M. Redegeld for his helpful comments.

References

1. Botchkarev V. A., Eichmuller S., Peters E. M., Pietsch P., Johansson O., Maurer M., Paus R. A simple immunofluorescence technique for simultaneous visualization of mast cells and nerve fibers reveals selectivity and hair cycle - dependent changes in mast cell - nerve fiber contacts in murine skin. Arch. Dermatol. Res. 1997; 289:292-302.
2. Bienenstock J., Macqueen G., Sestini P., Marshall J. S. Mast cell/nerve interactions in vitro an in vivo. Am. Rev. Respir. Dis. 1991; 143:S55-S58.
3. Dimitriadou V., Rouleau A., Trung Tuong M. D., Newlands G. J., Miller H. R., Luffau G., Schwartz J. C., Garbarg M. Functional relationships between sensory nerve fibers and mast cells of dura mater in normal and inflammatory conditions. Neuroscience 1997; 77:829-839.
4. Purcell W. M., Atterwill C. K. Mast cells in neuroimmune function: neurotoxicological and neuropharmacological perspectives. Neurochem. Res. 1995; 20:521-532.
5. Holzer P. Local effector functions of capsaicin-sensitive sensory nerve endings: involvement of tachykinins, calcitonin gene-related peptide and other neuropeptides. Neuroscience 1988; 24:739-768.
6. Kress M., Zeilhofer H. U. Capsaicin, protons and heat: new excitement about nociceptors. Trends Pharmacol. Sci. 1999; 20:112-118.
7. Barnes P. J. Neurogenic inflammation in airways. Int. Arch. Allergy Appl. Immunol. 1991; 94:303-309.
8. Campbell E. A., Gentry C. T., Patel S., Panesar M. S., Walpole C. S., Urban L. Selective neurokinin-1 receptor antagonists are anti-hyperalgesic in a model of neuropathic pain in the guinea-pig. Neuroscience 1998; 87:527-532.
9. Baluk P. Neurogenic inflammation in skin and airways. J. Investig. Dermatol. Symp. Proc. 1997; 2:76-81.
10. Adorini L., Arai K., Capra J. D., Ishizaka K., Smitt-Verhulst A.-M., Waksman B. H. Human Basophils and Mast Cells: Biological Aspects. In: Marone G, ed. Chemical Immunology. Basel: Karger, 1995; 208-235.
11. Levi-Montalcini R., Skaper S. D., Dal Tosco R., Petrelli L., Leon A. Nerve growth factor: from neurotrophin to neurokine. Trends Neurosci. 1996; 19:514-520.

12. Silver R., Silverman A.-J., Vitkovic L., Lederhendler I. I. Mast cells in the brain: evidence and functional significance. Trends Neurosci. 1996; 19:25-31.
13. Janiszewski J., Bienenstock J., Blennerhassett M. G. Picomolar doses of substance P trigger electrical responses in mast cells without degranulation. Am. J. Physiol. 1994; 276:C138-C145.
14. Marshall J. S., Gomi K., Blennerhassett M. G., Bienenstock J. Nerve growth factor modifies the expression of inflammatory cytokines by mast cells via a prostanoid-dependent mechanism. J. Immunol. 1999; 162:4271-4276.
15. Nicol G. D., Lopshire J. C., Pafford C. M. Tumor necrosis factor enhances the capsaicin sensitivity of rat sensory neurons. J. Neurosci. 1997; 17:975-982.
16. Carlson N. G., Wieggel W. A., Chen J., Bacchi A., Rogers S. W., Gahring L. C. Inflammatory cytokines IL-1 alpha, IL-1 beta, IL-6, and TNF-alpha impart neuroprotection to an excitotoxin through distinct pathways. J. Immunol. 1999; 163:3963-3968.
17. Bienenstock J., Tomioka M., Matsuda H., Stead R. H., Quinonez G., Simon G. T., Coughlin M. D., Denburg J. A. The role of mast cells in inflammatory processes: evidence for nerve/mast cell interactions. Int. Arch. Allergy Appl. Immunol. 1987; 82:238-243.
18. Undem B. J., Riccio M. M., Weinreich D., Ellis J. L., Myers A. C. Neurophysiology of mast cell-nerve interactions in the airways. Int. Arch. Allergy Immunol. 1995; 107:199-201.
19. MacQueen G., Marshall J., Perdue M., Siegel S., Bienenstock J. Pavlovian concitioning of rat mucosal mast cells to secrete rat mast cell protease II. Science 1989; 243:83-85.
20. Rozniecki J. J., Dimitriadou V., Lambracht-Hall M., Pang X., Theoharides T. C. Morphological and functional demonstration of rat dura mater mast cell-neuron interactions in vitro and in vivo. Brain Res. 1999; 849:1-15.
21. Miller G. W., Liuzzi F. J., Ratzlaff R. E. Involvement of an axonal reflex in IgE-mediated inflammation in mouse skin. J. Neuroimmunol. 1995; 57:137-141.
22. Blennerhassett M. G., Tomioka M., Bienenstock J. Formation of contacts between mast cells and sympathetic neurons in vitro. Cell. Tissue Res. 1991; 265:121-128.
23. Blennerhassett M. G., Janiszewski J., Bienenstock J. Sympathetic nerve contact alters membrane resistance of cells of the RBL-2H3 mucosal mast cell line. Am. J. Respir. Cell Mol. Biol. 1992; 6:504-509.

24. Suzuki R., Furuno T., McKay D. M., Wolvers D., Teshima R., Nakanishi M., Bienenstock J. Direct neurite-mast cell communication in vitro occurs via the neuropeptide substance P. J. Immunol. 1999; 163:2410-2415.
25. Blennerhassett M. G., Bienenstock J. Apparent innervation of rat basophilic leukaemia (RBL-2H3) cells by sympathetic neurons in vitro. Neurosci. Lett. 1990; 120:50-54.
26. Dijkhuizen P. A., Hermens W. T. J. M. C., Teunis M. A. T., Verhaagen J. Adenoviral vector-directed expression of neurotrophin-3 in rat dorsal root ganglion explants results in a robust neurite outgrowth response. J. Neurobiol. 1997; 33:172-184.
27. Skaper S. D., Selak I., Varon S. Molecular requirements for survival of cultured avian and rodent dorsal root ganglionic neurons responding to different trophic factors. J. Neurosci. Res. 1982; 8:251-261.
28. Murray S. S., Bartlett P. F., Cheema S. S. Differential loss of spinal sensory but not motor neurons in the p75NTR knockout mouse. Neurosci. Lett. 1999; 267:45-48.
29. Fischer A., McGregor G. P., Saria A., Philippin B., Kummer W. Induction of tachykinin gene and peptide expression in guinea pig nodose primary afferent neurons by allergic airway inflammation. J. Clin. Invest. 1996; 9:2284-2291.
30. Nicholas R. S., Winter J., Wren P., Bergmann R., Woolf C. J. Peripheral inflammation increases the capsaicin sensitivity of dorsal root ganglion neurons in a nerve growth factor-dependent manner. Neuroscience 1999; 91:1425-1433.
31. Karimi K., Redegeld F. A., Heijdra B., Nijkamp F. P. Stem cell factor and interleukin-4 induce murine bone marrow cells to develop into mast cells with connective tissue type characteristics in vitro. Exp. Hematol. 1999; 27:654-662.
32. Karimi K., Redegeld F. A., Blom R., Nijkamp F. P. Stem cell factor and interleukin-4 increase responsiveness of mast cells to substance P. Exp. Hematol. 2000; 28:626-634.
33. Lindsay R. M., Harmar A. J. Nerve growth factor regulates expression of neuropeptide genes in adult sensory neurons. Nature 1989; 337:362-364.
34. Crofford L. J., Sano H., Karalis K., Webster E. L., Goldmuntz E. A., Chrousos G. P., Wilder R. L. Local secretion of corticotropin-releasing hormone in the joints of Lewis rats with inflammatory arthritis. J. Clin. Invest. 1992; 90:2555-2564.
35. Alving K., Sundstrom C., Matran R., Panula P., Hokfelt T., Lundberg J. M. Association between histamine-containing mast cells and sensory nerves in the skin and airways of control and capsaicin-treated pigs. Cell Tissue Res. 1991; 264:529-538.

36. Atwood L., James C., Morris G. P., Vanner S. Cellular pathways of mast cell- and capsaicin-sensitive nerve-evoked ileal submucosal arteriolar dilations. Am. J. Physiol. 1998; 275:G1063-1072.
37. Bienenstock J., Perdue M., Blennerhassett M., Stead R., Kakuta N., Sestini P., Vancheri C., Marshall J. Inflammatory cells and the epithelium. Mast cell/nerve interactions in the lung in vitro and in vivo. Am. Rev. Respir. Dis. 1988; 138:S31-34.
38. Dowling-Warriner C. V., Trosko J. E. Induction of gap junctional intercellular communication, connexin43 expression, and subsequent differentiation in human fetal neuronal cells by stimulation of the cyclic AMP pathway. Neuroscience 2000; 95:859-868.
39. Vliagoftis H., Hutson A. M., Mahmudi-Azer S., Kim H., Rumsaeng V., Oh C. K., Moqbel R., Metcalfe D. D. Mast cells express connexins on their cytoplasmic membrane. J. Allergy Clin. Immunol. 1999; 103:656-662.
40. Yamamoto T., Hertzberg E. L., Nagy J. I. Epitopes of gap junctional proteins localized to neuronal subsurface cisterns. Brain Res. 1990; 527:135-119.
41. Kwak B. R., van Veen T. A., Analbers L. J., Jongsma H. J. TPA increases conductance but decreases permeability in neonatal rat cardiomyocyte gap junction channels. Exp. Cell. Res. 1995; 220:456-463.
42. Chaytor A. T., Martin P. E., Evans W. H., Randall M. D., Griffith T. M. The endothelial component of cannabinoid-induced relaxation in rabbit mesenteric artery depends on gap junctional communication. J. Physiol. (Lond.) 1999; 520:539-550.
43. Revel J. P., Yancey S. B., Meyer D. J., Nicholson B. Cell junctions and intercellular communication. In Vitro 1980; 16:1010-1017.
44. Wade M. H., Trosko J. E., Schindler M. A fluorescence photobleaching assay of gap junction-mediated communication between human cells. Science 1986; 232:525-528.
45. Suzuki M., Furuno T., Teshima R., Sawada J., Nakanishi M. Soluble factors from rat basophilic leukemia (RBL-2H3) cells stimulated cooperatively the neurite outgrowth of PC12 cells. Biol. Pharm. Bull. 1998; 21:1267-1270.
46. Turnley A. M., Bartlett P. F. Nerve growth factor modulates myelin-associated glycoprotein binding to sensory neurons. Int. J. Dev. Neurosci. 1999; 17:109-119.
47. Zhou X. F., Deng Y. S., Chie E., Xue Q., Zhong J. H., McLachlan E. M., Rush R. A., Xian C. J. Satellite-cell-derived nerve growth factor and neurotrophin-3 are involved in noradrenergic sprouting in the dorsal root ganglia following peripheral nerve injury in the rat. Eur. J. Neurosci. 1999; 11:1711-1722.

48. Murase K., Murakami Y., Takayanagi K., Furukawa Y., Hayashi K. Human fibroblast cells synthesize and secrete nerve growth factor in culture. Biochem. Biophys. Res. Comm. 1992; 184:373-379.
49. Eichler M. E., Rich K. M. Death of sensory ganglion neurons after acute withdrawal of nerve growth factor in dissociated cell cultures. Brain Res. 1989; 482:340-346.
50. Tong J. X., Eichler M. E., Rich K. M. Intracellular calcium levels influence apoptosis in mature sensory neurons after trophic factor deprivation. Exp. Neurol. 1996; 138:45-52.
51. Ho W. Z., Lai J. P., Zhu X. H., Uvaydova M., Douglas S. D. Human monocytes and macrophages express substance P and neurokinin-1 receptor. J. Immunol. 1997; 159:5654-5660.
52. Killingsworth C. R., Shore S. A., Alessandrini F., Dey R. D., Paulauskis J. D. Rat alveolar macrophages express preprotachykinin gene-1 mRNA encoding tachykinins. Am. J. Physiol. 1997; 273:L1073-L1081.
53. De Giorgio R., Tazzari P. L., Barbara G., Stanghellini V., Corinaldesi R. Detection of substance P immunoreactivity in human peripheral leukocytes. J. Neuroimmunol. 1998; 82:175-181.
54. Miller M. S., Buck S. H., Sipes I. G., Yamamura H. I., Burks T. F. Regulation of substance P by nerve growth factor: disruption by capsaicin. Brain Res. 1982; 250:193-196.
55. Donnerer J., Schuligoi R., Stein C. Increased content and transport of substance P and calcitonin gene-related peptide in sensory nerves innervating inflamed tissue: evidence for a regulatory function of nerve growth factor in vivo. Neuroscience 1992; 49:693-698.
56. Vedder H., Affolter H. U., Otten U. Nerve growth factor (NGF) regulates tachykinin gene expression and biosynthesis in rat sensory neurons during early postnatal development. Neuropeptides 1993; 24:351-357.
57. Mousli M., Bronner C., Landry Y., Bockaert J., Rouot B. Direct activation of GTP-binding regulatory proteins (G-proteins) by substance P and compound 48/80. FEBS Lett. 1990; 259:260-262.
58. Janiszewski J., Bienenstock J., Blennerhassett M. G. Substance P induces whole cell current transients in RBL-2H3 cells. Am. J. Physiol. 1992; 263:C736-742.
59. Tausk F., Undem B. Exogenous but not endogenous substance P releases histamine from isolated human skin fragments. Neuropeptides 1995; 29:351-355.
60. Janiszewski J., Bienenstock J., Blennerhassett M. G. Activation of rat peritoneal mast cells in coculture with sympathetic neurons alters neuronal physiology. Brain Behav. Immun. 1990; 4:139-150.

General discussion

Nerve growth factor: possible mechanisms of action in allergic asthma

Neurotrophins, in particular nerve growth factor (NGF), are recently discovered players in the asthmatic disease (1-5). Our data presented in this thesis confirm a role for NGF in the pathologic effects and altered airway function in a guinea pig model for allergic asthma. In this chapter, we will discuss possible mechanisms involved in the effects of NGF on airway function and pathology.

1. Release of NGF

Patients with allergic asthma show increases in serum levels of NGF (2). We showed in chapter 5 that in a guinea pig model for allergic asthma, there is a rise in NGF in bronchoalveolar lavage fluid 24 hrs after allergen challenge. This increase in NGF in bronchoalveolar lavage fluid after allergen provocation is found as well in mice (3), and in man (1). We are not aware of a study showing which cells are responsible for the increase in levels of NGF. In the airways, a variety of cells are present during allergic inflammation that can synthesize NGF: mast cells (6, 7), fibroblasts (8), T- and B-cells (9, 10), eosinophils (11) lymphocytes (10) and airway epithelial cells (12; see top fig. 1). NGF levels are increased shortly, within 10 min, after allergen provocation in patients suffering from allergic rhinitis (13). The authors of this paper suggest that mast cells are a very likely source for NGF; indeed mast cells release mediators within minutes after nasal provocation with allergen (13). Any of the other cell types could be responsible for long-term increases in NGF as suggested by Virchow and colleagues (1).

2. NGF and immune cells

Several studies provide evidence that NGF may play a role in neuroimmune interactions (4). Immune cells synthesize and release NGF and NGF has several effects on these cells (fig. 1).

2.1. Mast cells

Mast cells synthesize, store and release NGF (6, 7). Antigen-induced increass in vagal sensory and parasympathetic activity may be a consequence of the allergic reaction in the airways, presumably through mast cell activation (14, 15). The factor released from the mast cells to induce changes in neuronal function could be NGF (16). Indeed, factors released by mast cells are shown to induce neurite outgrowth, which could almost completely be inhibited by antibodies directed against NGF (17). Activation of rat peritoneal mast cells in co-culture with sympathetic neurons alters neuronal physiology by inducing depolarization and decreasing membrane resistance (18).

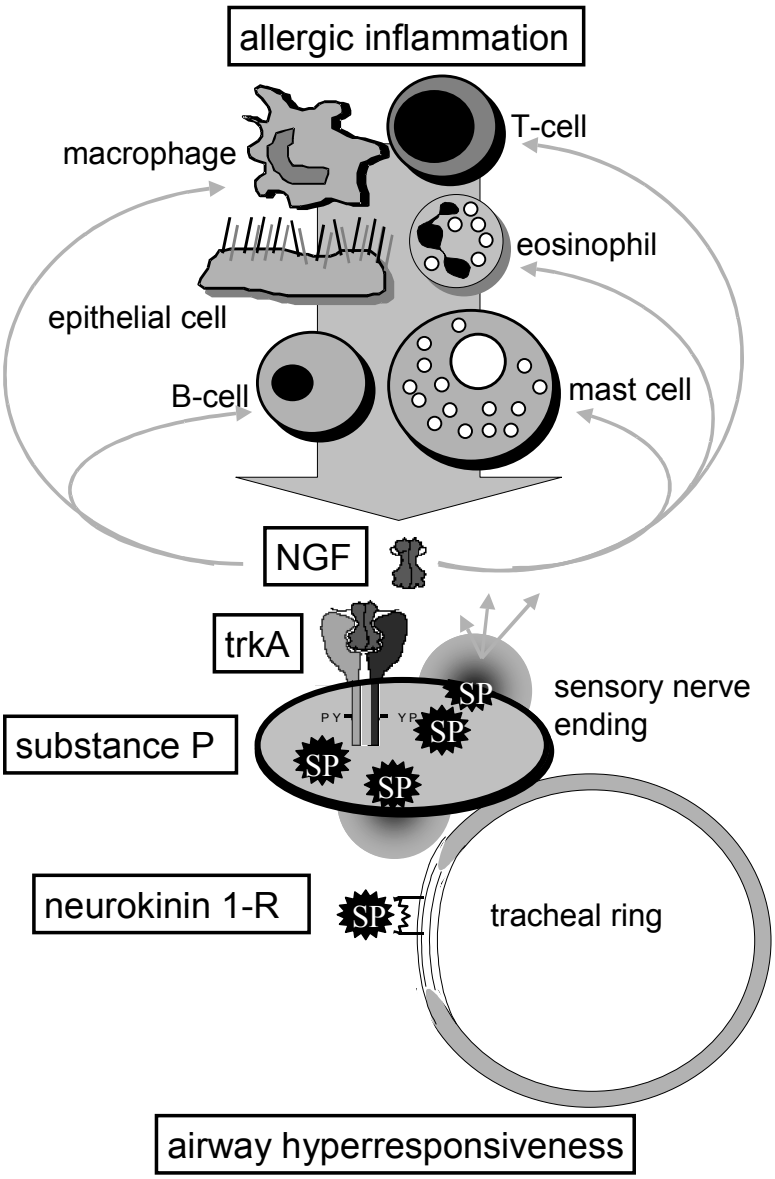


Figure 1. Proposed model for the effects of NGF on airway function. Macrophages, epithelial cells, B- and T-cells, eosinophils and mast cells can release NGF in an inflammatory situation (6, 10, 12, 48). NGF levels are enhanced in the airways (1, 3, 13 and chapter 5) leading to a change of sensory nerve function and substance P release (chapters 2, 3 and 5). This induces activation of neurokinin-1 receptors leading to airway hyperresponsiveness.

In several animal models for allergic asthma substance P synthesis is increased (18-20 and chapter 5). The increase in substance P could be mimicked by administration of NGF (22). It could be hypothesized that mast cells are the major source for NGF in allergic models. This would imply that mast cell activation could induce NGF release and thereby induce substance P increases. We tried to reveal whether mast cell activation induced a change in neuronal preprotachykinin (PPT), the precursor for tachykinins, mRNA. Minor changes were induced in neuronal expression of PPT mRNA by mast cell co-culture and activation (chapter 6). Whether or not this is a valid reflection of an allergic situation remains a matter of debate. Further experiments are needed to elucidate whether or not mast cells or other cells are the major source for NGF in an allergic situation.
A close relationship exists between the increased NGF plasma values and the number of infiltrating mast cells in vernal keratoconjunctivitis (4). This is confirmed by a recent study revealing NGF as a chemoattractant for mast cells (23). Several studies report effects of NGF on mast cells. A number of studies reveal that NGF can induce differentiation of mast cells and the closely related basophils (14, 24-28). Furthermore, NGF induces changes in expression of inflammatory mediators: interleukin-6 (IL-6) and PGE_2 are induced whereas tumor necrosis factor α (TNFα) is inhibited (29). Furthermore, NGF induces mast cell degranulation in vivo (30) and prevents apoptosis of mast cells (31). Inducing differentiation, increasing inflammatory cytokines and prevention of apoptosis of mast cells can all increase the airway pathology due to allergic inflammation. Presumably, actions of NGF are mediated by the trkA which is expressed on mast cells (7, 31-33). Taken together, it is very likely that NGF affects mast cells (fig. 1).

2.2. T- and B-lymphocytes

Many studies that suggest effects of NGF on T-cells, and vice versa, could be of great interest in allergic pathologies. Allergy is characterized by T helper (Th) cells that are of Th2 type, producing Th2 type cytokines (34, 35). Human CD4+ T cell clones (preferentially of activated Th2 type) produce and release NGF, and express high-affinity NGF receptors (9, 36, 37). Furthermore, Th2 type cytokines can induce release of NGF: IL-4 (38) and IL-10 (39) induce release of NGF from astrocytes. In contrast, the Th1-derived cytokines interferon-γ and IL-2 do not induce NGF synthesis (39). This suggests T-cells can be a likely source of NGF in an allergic condition besides mast cells. Likewise, in a model for multiple sclerosis (an autoimmune disease) NGF promotes induction of Th2 type cytokines and prevents the synthesis of Th1 type cytokines (40). NGF induces differentiation of eosinophils, basophils and mast cells, which is suggested to involve T-cell activation and release of T-cell mediators (24). In a murine model for allergic

asthma, a shift from Th1 to Th2 type T-cells was prevented by lowering the amount of available NGF by the use of antibodies directed against NGF (3).
Apart from T-lymphocytes, B-cells synthesize NGF as well. B-cells increase NGF expression upon stimulation with lipopolysaccharide (LPS) and substance P (10, 37). NGF affects B cells presumably via trkA that is phosphorylated upon stimulation with NGF (41). No p75 mRNA has been detected in B cells (42). NGF induces growth and differentiation of B cells and induces antibody synthesis and secretion from B cells (43).
This indicates that NGF could play a role in the induction of Th2 lymphocytes as well as differentiation of B lymphocytes; both phenomena important in the induction of allergic asthma (fig. 1).

2.3. Eosinophils

Eosinophils produce NGF as well (11). As an eosinophil influx into the airways is reported often in patients with allergic asthma (e.g. 44, 45) and in animal models for allergic asthma (e.g. 46 and chapter 5), eosinophils can therefore be a potential source of NGF in allergic asthma.
Furthermore, NGF induces differentiation of eosinophils (24). NGF can act on human peripheral blood eosinophils to preferentially release inflammatory mediators (4, 11, 47). NGF enhances survival and cytotoxic activity of human eosinophils and acts as a chemoattractant (47; fig. 1). This could imply that antibodies directed against NGF or tyrosine kinase inhibitors for trkA would reduce eosinophilic infiltration in bronchoalveolar lavage fluid in our model for allergic asthma. However, we did not find any change in eosinophil numbers after inhibition of NGF or blocking the phosphorylation of trkA (chapter 5). Most likely, other chemoattractants for eosinophils overruled the lack of chemoattraction induced by NGF. It would be interesting to reveal whether eosinophils release less inflammatory mediators when NGF function is blocked.

2.4. Macrophages

Macrophages are a source of NGF (48) and NGF acts as an autocrine factor. NGF acts on macrophages presumably via activation of trkA, and enhances release of IL-1β (49) and induces an increase of Fcγ-receptors (50). It is suggested that differentiation of monocytes into macrophages is dependent upon trk expression (51). This implies that neurotrophins are involved in the differentiation of monocytes into macrophages. Furthermore, NGF is essential for the survival of macrophages, which are infected with HIV (48). In addition, macrophages regulate synthesis of NGF in Schwann cells and fibroblast-like cells via IL-1β (52). Taken together, NGF induces macrophage differentiation, survival and IL-1β release, and macrophages themselves increase release of NGF by other cell types via IL-1β.

This implies that macrophages are involved in a worsening of NGF-mediated pathological effects at several levels (fig. 1).

3. NGF and sensory nerves

In chapter 2 we studied whether increases in NGF could be responsible for changes in airway function. We showed that application of NGF could induce airway hyperresponsiveness in vivo. Furthermore, we demonstrated that the induction of airway hyperresponsiveness was mediated by sensory nerve endings (chapters 2 and 3). The tachykinin substance P, presumably released from the sensory nerves, is a key player in this respect (chapters 2, 3 and 5; fig. 1).

3.1. Sensory nerve endings

In tracheal rings NGF was able to induce tracheal hyperresponsiveness, which was mediated by neurokinin-1 receptors (chapter 3). Presumably, NGF did not change substance P synthesis, as in the tracheal rings sensory nerve endings lacked contact with their cell bodies. Furthermore, the effects of NGF were established within 1 hr, which makes protein synthesis unlikely (chapters 2 and 3). A very recent study by Zhou and colleagues showed an increase in substance P release 24 and 48 hrs after application of 5 ng/ml NGF in tracheal explants (53). In the preparation of Zhou and colleagues, an increase in substance P formation can also be excluded as sensory nerve endings lacked contact with their cell bodies (53). We hypothesize that substance P release is not significantly increased 30 min after addition of NGF in tracheal explants, as we did not measure a change in basal contraction, but instead that substance P release is increased upon stimulation with histamine.

NGF can change the properties of sensory nerve endings, without affecting protein synthesis, in various ways. Indeed, NGF can induce a fast accumulation of second messengers (54), protein kinase C translocation to the membrane (55) or phosphorylation of key transduction-related proteins or ion channels (54, 56, 57). Further evidence for a role of sensory nerve endings in induction of NGF-induced tracheal hyperresponsiveness is provided by the use of a cannabinoid-1 (CB_1) receptor agonist, which inhibits excitatory processes at the nerve terminal (58). The CB_1 receptor agonist blocked NGF-induced tracheal hyperresponsiveness (chapter 3).

The NGF-induced neuronal changes could be direct, as described above, or indirect, via the release of sensitizing mediators from trkA expressing inflammatory cells, e.g. mast cells and monocytes (7, 24, 56, 57, 59). Mediators released from mast cells, such as histamine and arachidonic acid metabolites have been suggested to affect neuronal function (60, 61).

Antibodies directed against NGF inhibited the *acute* bronchoconstriction after allergen challenge in guinea pigs (chapter 4). Sanico and colleagues, who reported

a short-term increase in nasal lavage fluid of NGF after allergen provocation, suggest that this increase in NGF could underlie exaggerated nasal reflexes (62-67). We demonstrated that NGF can induce neuronal changes, presumably via substance P. Other studies show that substance P and other tachykinins are involved in acute allergic responses in the airways. For instance, a role for substance P in the early allergic response was demonstrated in antigen-induced microvascular leakage (68). This suggests that NGF could underlie acute changes of sensory nerve endings by allergic responses (fig. 1). In conclusion, we cannot exclude that a change in neurokinin-1 receptor functioning underlies the short-term effects by NGF.

3.2. Substance P synthesis

We discussed effects of NGF in relationship to substance P, which do not involve increased synthesis of substance P. Besides these effects of NGF on the sensory nerve ending, NGF-mediated changes seem to involve upregulation of substance P in the ganglia as well (fig. 1). Hunter and colleagues showed that NGF induced an increase in substance P positive neurons in the nodose ganglia, 24 hrs after application of NGF in the tracheal wall (22).

Besides studies that estimated the effect of exogenously applied NGF, there are a number of studies revealing a role for NGF in inflammatory models. In models for allergic asthma there is an increase in substance P in the airways and in the number of substance P positive neurons (16, 69). We showed a prevention of this increase in substance P by inhibition of the tyrosine kinases of trkA in a guinea pig model for allergic asthma (chapter 5).

Similarities between allergic asthma and inflammatory pain are abundant. NGF has been suggested to play an important role in the induction of inflammatory pain (70-72). Furthermore, in models for pain, NGF was associated with an increase in substance P (56). In models of inflammatory pain trkA immunoreactivity is increased (73), pointing to a role for trkA as well.

Interestingly, the development of airway hyperresponsiveness in our allergic asthma model coincided with an increase in NGF levels and in substance P in bronchoalveolar lavage fluid 24 hrs after allergen challenge (chapter 5). We hypothesize that allergen provocation induces NGF release and that NGF in its turn activates trkA. Subsequently, substance P synthesis is upregulated, resulting in an increase in substance P in the airways leading to enhanced responsiveness to bronchoconstrictive agents (fig.1).

3.3. Cholinergic impact

Tachykinin containing nerves innervate cholinergic ganglia in the airways (74, 75). Baseline airway cholinergic nerve activity is necessarily dependent upon afferent nerve activity arising from the intrapulmonary airways and lungs, presumably via

sensory nerves (76, 77). Excessive activity of cholinergic nerves may be important in asthma (78, 79).
Vagal activation can induce bronchospasm, which can be reversed by tachykinin receptor antagonists (80). Furthermore, in the airways contraction is partly cholinergic when evoked by substance P (81-84) or neurokinin A (85). Additionally, tachykinins can augment cholinergic transmission (85, 86). Moreover, NGF could increase histamine-induced smooth muscle contraction and this could be blocked by a neurokinin-1 receptor antagonists (chapter 2 and 3). We can not exclude involvement of cholinergic neurotransmission in this respect. Parasympathetic ganglia are located in the trachea, thus in a tracheal preparation NGF-induced tracheal hyperreactivity could involve (augmented) release of acetylcholine (chapter 3; fig. 2). Further experiments using a cholinergic blocker are needed to reveal whether or not NGF affects cholinergic action in the airways.

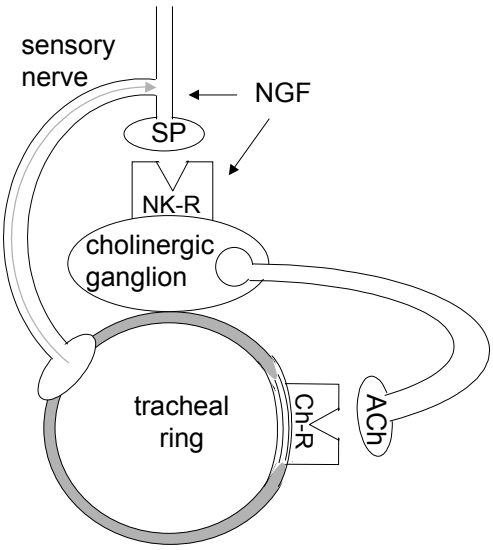


Figure 2. Hypothetical scheme of action of NGF via cholinergic ganglion, which is positioned in the trachea. NGF could change the substance P (SP) release from the sensory nerve ending, innervating a cholinergic ganglion, or could enhance neurokinin receptor (NK-R) function on a cholinergic ganglion. This could induce an enhanced acetylcholine release (ACh), which could induce an enhanced smooth muscle contraction, via cholinergic receptor (Ch-R) activation.

3.4. Vanilloid receptor

The receptor involved in reaction to the exogenous compound capsaicin, earlier known as the capsaicin receptor, has now been idetified as vanilloid receptor 1 (VR_1). Endogenous ligands of this receptor were unknown till recently

anandamide, an endogenous cannabinoid, was shown to act as a full agonist on VR_1 (87). Very recently, it was shown that products of lipoxygenases directly activate capsaicin receptors (88). These products are, in order of increasing potency: 12-hydroperoxyeicosatetraenoic acid (12-HPETE), 15-HPETE, 5-hydroxyeicosatetraenoic acid (5-HETE) and leukotriene B_4. This indicates that, in case of an inflammatory situation, in which different lipoxygenase products are upregulated, VR_1 receptors could be activated. As previously shown, mediators like leukotriene B_4 and other leukotrienes play a role in allergic asthma (89, 90) and pathological effects can be reversed by blocking leukotriene synthesis or leukotriene receptor activation in models for allergic asthma (91-93).

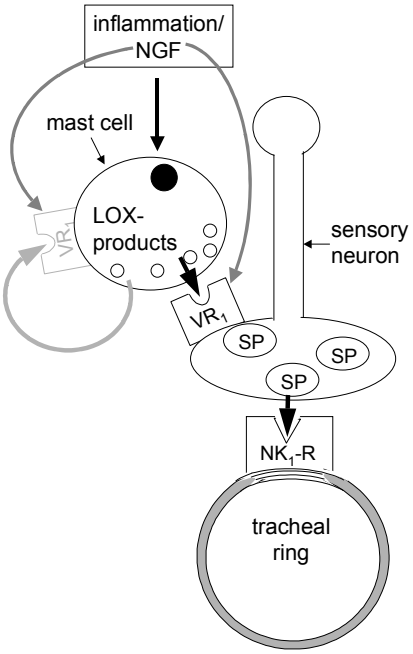


Figure 3. Schematic representation of possible involvement of the vanilloid receptor-1 (VR_1) in induction of tracheal hyperresponsiveness. Either inflammation or possibly NGF could induce changes in mast cells or sensory nerve endings. Lipoxygenase (LOX) products released from mast cells, or from other cells releasing lipoxygenase products, could activate the VR_1 on the sensory neuron, thereby inducing release of tachykinins, for instance substance P (SP), which could in turn activate the neurokinin-1 receptor (NK_1-R), and subsequently induce airway hyperresponsiveness. Alternatively, VR_1 receptors present on the mast cell could be activated as well, inducing an enhanced release of inflammatory mediators.

NGF induces a rapid enhancement of the response to capsaicin (94). This implies that NGF enhances the effects of the lipoxygenase products on the VR_1 as well.

This mechanism could account for an exacerbation of the pathological effects in allergic inflammation.
With respect to the data presented in the previous chapters, we postulate a novel mechanism. In the guinea pig model for allergic asthma (chapter 4 and 5), inflammatory mediators and presumably lipoxygenase products, were released (89-93). Such lipoxygenase products can activate VR_1 while NGF could have enhanced VR_1 activation (fig. 3). This cascade could have induced an increase in the release of substance P and thus enhanced neurogenic inflammation, leading to, for instance, airway hyperresponsiveness. It would be interesting to have a closer look at activation of the VR_1.
Whether or not this sequence of events is also operational in vivo in an inflammatory situation remains open to investigation. In favor of this view are two recent studies in which VR_1 knockout mice do not show inflammatory thermal hyperalgesia (95, 96). This implies that in a normal inflammatory situation VR_1 is activated and induces inflammatory pain. Caterina and colleagues speculated on anandamide, a cannabinoid, as an endogenous ligand for VR_1 (87, 95, 97). Interestingly, tissue injury increases the levels of endogenous cannabinoids (98). However, there are no other studies, to our knowledge, that have measured anandamide in inflammatory situations. Therefore, at this moment we can merely suggest that lipoxygenase products could have been involved in inflammatory pain.
We speculated on a role for VR_1 in inflammatory models. The direct effects of NGF, mediated by substance P on airway function (chapter 2 and 3), could have been mediated by VR_1 as well. Activation of VR_1 induces release of sensory peptides including substance P. In an allergic situation, increases in lipoxygenase products have been reported, though not in naïve animals. As NGF stimulates the release of mediators from a variety of inflammatory cells, maybe NGF induces release of lipoxygenase products (9, 11, 24, 30, 43, 49, 99, 100). Therefore, activation of the VR_1 could play a role in induction of hyperresponsiveness by NGF in naïve animals when lipoxygenase products are released after NGF application.
Could activation of VR_1 receptors be responsible for the increase of substance P in the airways in a model for allergic asthma? Normally, tachykinergic innervation in guinea pig airways is derived almost exclusively from small unmyelinated C-fibers. In allergic models, an increase in substance P producing large diameter, myelinated neurons is found in the nodose ganglia, being Aδ-fibers (16, 69). As mentioned before, this increase in substance P in nodose ganglia can be mimicked by application of NGF (22). Unlike C-fibers, Aδ-fibers are insensitive to bradykinin and capsaicin (16, 22). This indicates that these neurons do not express VR_1 and thus stimulation of VR_1 can not responsible for the increased content of substance P derived from Aδ-fibers in the airways. However VR_1 can be responsible for increases in tachykinins from C-fibers (chapter 5; 16, 69). Another

interesting experiment would be to have a closer look at the capsaicin receptors themselves: either at the number or the function of the receptors.
VR_1 receptors could be involved in other pathologic changes in airway inflammation, besides the changes in tachykinin release. Indeed, mast cells express VR_1 and respond directly to capsaicin (101). Using capsaicin provides further indirect evidence. Capsaicin has tachykinin-independent effects on smooth muscle in bronchi (102) and capsaicin induces release of inflammatory cytokines from bronchial epithelium (103). This all points to the possibility that VR_1 mediates effects which do not involve tachykinin release but could be involved in the induction of airway hyperresponsiveness.
Some previously published studies should be carefully reevaluated and assumptions made could be changed in view with the posibility of activation of the VR_1. For instance, 15-HPETE has been shown to induce airway hyperresponsiveness by affecting nerve endings (104). We could postulate that 15-HPETE activated VR_1, thereby inducing release of sensory neuropeptides. Furthermore, inhibition of a 5-lipoxygenase diminishes electrically evoked tachykinergic mediated contractions in airway tissue (105). Other inflammatory mediators, such as bradykinin, 5-hydroxytryptamine and prostaglandin E_2 do activate the capsaicin receptor, as the capsaicin antagonist capsazepine could completely block currents induced by these mediators (106). Caterina and colleagues suggest that this effect of bradykinin is mediated via the release of lipoxygenase products (95). Very many studies show effects of bradykinin via release of sensory neuropeptides (e.g. 107, 108). This implicates that bradykinin acts directly or indirectly on VR_1. Studies in our department provide evidence that the lipid mediator 13-hydroxyoctadecadienoic acid (13-HODE) induces airway hyperresponsiveness via sensory neuropeptides (109). As 13-HODE is another product of 15-lipoxygenase, it could be that 13-HODE acts via VR_1 as well.

4. NGF and hyperresponsiveness

4.1. NGF induces hyperresponsiveness

A single injection of NGF in guinea pigs induced airway hyperresponsiveness (chapter 2). This effect was transient as at 24 hrs after administration of NGF no airway hyperresponsiveness could be measured anymore (chapter 2). In patients suffering from allergic asthma, an airway hyperresponsiveness is a phenomenon which can be found at any moment (113). How can we correlate this to our data? We showed recently, that intravenous administration of NGF for three days every day could induce airway hyperresponsiveness 24 hrs after the last of the three injections in guinea pigs (fig. 4). Furthermore, as patients are normally exposed to allergens continuously and NGF is upregulated after allergen challenge (1, 13, 114) they are likely to suffer from sustained high levels of NGF. Therefore, our results

with repeated application of NGF provides more evidence for the importance of NGF in the development of airway hyperresponsiveness in allergic asthma.

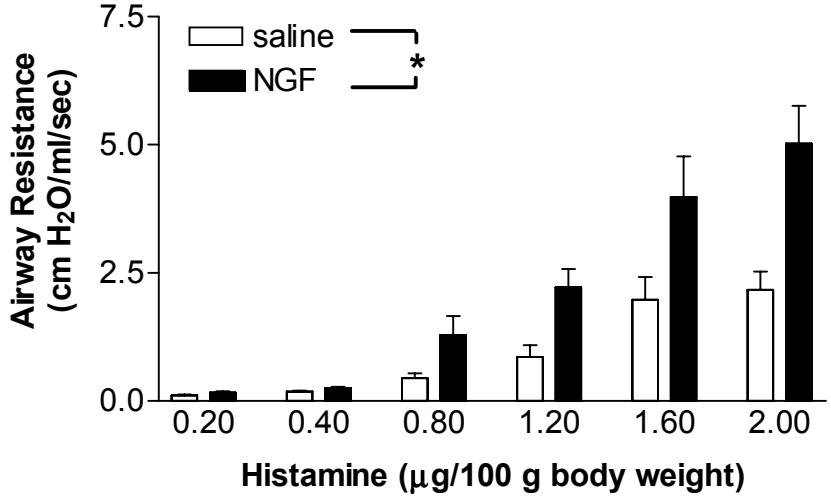


Figure 4. Intravenous application of NGF (80 ng/kg) to guinea pigs for 3 days every 24 hrs results in an airway hyperresponsiveness to histamine 24 hrs after the last intravenous injection of NGF (* P<0.0001 two-way ANOVA). See chapter 2 for materials and methods.

Our observation that anti-NGF does not attenuate the airway hyperresponsiveness in our guinea pig model is in contrast with the findings of Braun and colleagues, who did demonstrate a reduction in airway hyperresponsiveness by treatment with antibodies directed against NGF (3). The difference in effects could be due to a species difference, as our data are obtained from guinea pigs whereas Braun and colleagues used mice. Furthermore, it is hard to compare both studies since they involve different parameters: we measured changes in peptide expression in nerves in guinea pigs, whereas Braun and colleagues measured a shift from Th1 to Th2 type lymphocytes in mice (3). Recently, it has been shown that in models for pain as well that administration of NGF antibodies was insufficient to inhibit the induction of pain (110). Though in other studies neutralization of NGF was successfully inhibiting pain (111, 112).

4.2. Substance P

Blocking tyrosine kinases of the trkA completely prevented the induction of airway hyperresponsiveness and of substance P increases; this suggests a causal relationship between substance P and the induction of airway hyperresponsiveness (fig. 1). Moreover, a role for substance P in the induction of airway hyperresponsiveness has been reported in animal models (19-21) as well as for other tachykinins (109, 115; recently reviewed in 116). Also, tachykinin levels are

elevated in asthmatics (117) and protective effects of neurokinin receptor antagonists have been reported (118, 119). Here also, a similarity between the induction of inflammatory pain and allergic asthma seems to exist, as increases in substance P and NGF have been associated with pain (112, 120).

4.3. Neurotrophin 3

The partial inhibitory effect of anti-NGF on increases of substance P and the lack of effect of anti-NGF on ovalbumin-induced airway hyperresponsiveness could be explained by the action of neurotrophin 3 (NT-3). NGF is the neurotrophic factor with the highest binding affinity for trkA (121). NT-3 binds preferentially to its high-affinity receptor trkB, but can also bind and activate trkA, though with a lower affinity than NGF (122). As NT-3 is elevated in bronchoalveolar lavage fluid after allergen challenge in patients with allergic asthma (1) and NT-3 has been shown to be able to induce substance P release (123), we suggested that NT-3 could still activate trkA and thereby causing airway hyperresponsiveness (chapter 5).

4.4. TrkA and p75

Apart from NT-3 involvement, an alternative explanation for the lack of effect of treatment with antibodies directed against NGF in our guinea pig model for allergic asthma could imply an activation of p75. This receptor can also bind brain derived neurotrophic factor (BDNF), NT-3 and NT-4. The two receptors for NGF, p75 and trkA, have been shown to change each other's functioning negatively or positively. It could be that, in case of administration of antibodies against NGF, activation of p75 by BDNF or NT-3 could have a positive effect on trkA function, and therefore enhance NT-3 action on trkA. This could occur as NGF, NT-3 and BDNF are increased in bronchoalveolar lavage fluids 18 hr after allergen challenge (1). These neurotrophic factors bind with similar binding affinities, but p75 can distinguish between them and therefore exert different effects (121).

The p75 does not use tyrosine kinases for its signal transduction, therefore its functioning is not affected when using tyrosine kinase inhibitors. The p75 binds each of the neurotrophins with low nanomolar affinity (124). Receptor complexes and functional interactions between p75 and trkA have been reported and the balance between effects exerted by p75 and trkA is very complex (121). The final effect exerted is dependent upon the type of cells and the microenvironment (124).

So far, no studies addressed this issue in airway tissue. Further experiments are needed to reveal whether or not interactions between the two NGF receptors are important for the development of airway pathologies.

4.5. Immune cells as a source for substance P

So far we merely speculated about the induction and release of substance P from sensory nerves. However, substance P is not only derived from nerves, but also from various other immune cells: eosinophils, monocytes, macrophages, lymphocytes and dendritic cells (125-127). In patients with asthma, the concentrations of substance P was significantly related to the eosinophil cell count in induced sputum (128, 129). The substance P could be directly released by the eosinophils, or mediators released from the eosinophils induced substance P release from sensory neurons (130). Furthermore, substance P by itself affects immune cell function, for instance recruitment of eosinophils and neutrophils (131), mast cell activation and threshold lowering (132-134) and lymphocyte proliferation (127). In this way, immune cell-derived substance P could further worsen the pathologic effects in allergic asthma (79). These alternative pathways of substance P release and effects on immune cell functioning could be involved in the development of allergic asthma and airway hyperresponsiveness.

5. NGF in other inflammatory diseases

NGF is elevated and has a role in other inflammatory diseases besides allergic asthma. For instance, NGF and trkA are upregulated in the gut in patients suffering from inflammatory bowel disease (135). Furthermore, NGF levels are elevated in a number of autoimmune states (136, 137) and in allergic diseases such as rhinoconjunctivis, urticaria-angioedema, vernal keratoconjunctivitis and allergic rhinitis (2, 4, 13, 138, 139).

NGF plays a role in inflammatory pain, as mentioned previously (70, 112, 140). We showed a reducing effect of NGF blockade on airway pathology in a model for allergic asthma (chapter 4 and 5). Likewise, a tyrosine kinase inhibitor specific for trkA has beneficial effects in a model for experimental autoimmune encephalomyelitis, by its ability to suppress macrophage activation (141). Also, blocking NGF has a beneficial effect in inflammatory pain (111, 142). Taken together, this indicates that blocking of NGF is a potential target in inflammatory diseases.

On the other hand, a lack of NGF induces neural pathology (143, 144). In type 1 diabetes a downregulation of available NGF is measured. Diabetes is associated with severe neuro-pathologies (145, 146). Depletion in substance P (147), which is another phenomenon of diabetes, can be reversed by application of NGF in streptozotocin-induced diabetes in rats (148). Very interesting is a recent observation that shows that children with type-1 diabetes and their unaffected siblings have fewer symptoms of asthma (149). When co-existence does occur, the cases are generally mild, and effective treatment of one disease frequently exacerbates the other (150). Obviously, asthma has a typical Th2 type pathology,

and type 1 diabetes is a typical Th1-mediated disease. However, non-co-existence of asthma and diabetes could be caused by a lack or increase in NGF, especially since NGF has profound effects on T-cells as well (3, 9, 24, 36-39). This shows a critical balance for NGF, as too little is associated with neuropathologies and maybe a Th1 shift, whereas too much NGF seems to enhance inflammatory pathology and Th2 cell development.

References

1. Virchow J. C., Julius P., Lommatzsch M., Luttmann W., Renz H., Braun A. Neurotrophins are increased in bronchoalveolar lavage fluid after segmental allergen provocation. Am. J. Respir. Crit. Care Med. 1998; 158:2002-2005.
2. Bonini S., Lambiase A., Bonini S., Angelucci F., Magrini L., Manni L., Aloe L. Circulating nerve growth factor levels are increased in humans with allergic diseases and asthma. Proc. Natl. Acad. Sci. USA 1996; 93:10955-10960.
3. Braun A., Appel E., Baruch R., Herz U., Botchkarev V., Paus R., Brodie C., Renz H. Role of nerve growth factor in a mouse model of allergic airway inflammation and asthma. Eur. J. Immunol. 1998; 28:3240-3251.
4. Bonini S., Lambiase A., Levi-Schaffer F., Aloe L. Nerve growth factor: an important molecule in allergic inflammation and tissue remodelling. Int. Arch. Allergy Immunol. 1999; 118:159-162.
5. Braun A., Lommatzsch M., Renz H. The role of neurotrophins in allergic bronchial asthma. Clin. Exp. Allergy 2000; 30:178-186.
6. Leon A., Buriani A., Dal Toso R., Fabris M., Romanello S., Aloe L., Levi-Montalcini R. Mast cells synthesize, store, and release nerve growth factor. Proc. Natl. Acad. Sci. USA 1994; 91:3739-3743.
7. Nilsson G., Forsberg-Nilsson K., Xiang Z., Hallbook F., Nilsson K., Metcalfe D. D. Human mast cells express functional TrkA and are a source of nerve growth factor. Eur. J. Immunol. 1997; 27:2295-2301.
8. Hattori A., Iwasaki S., Murase K., Tsujimoto M., Sato M., Hayashi K., Kohno M. Tumor necrosis factor is markedly synergistic with interleukin 1 and interferon-gamma in stimulating the production of nerve growth factor in fibroblasts. FEBS Lett. 1994; 340:177-180.
9. Lambiase A., Bracci-Laudiero L., Bonini S., Bonini S., Starace G., D'Elios M. M., De Carli M., Aloe L. Human CD4+ T cell clones produce and release nerve growth factor and express high affinity nerve growth receptors. J. Allergy Clin. Immunol. 1997; 100:408-414.

10. Barouch R., Appel E., Kazimirsky G., Braun A., Renz H., Brodie C. Differential regulation of neurotrophin expression by mitogens and neurotransmitters in mouse lymphocytes. J. Neuroimmunol. 2000; 103:112-121.
11. Solomon A., Aloe L., Pe'er J., Frucht-Pery J., Bonini S., Levi-Schaffer F. Nerve growth factor is preformed in and activates human peripheral blood eosinophils. J. Allergy Clin. Immunol. 1998; 102:454-460.
12. Fox A. J., Barnes P. J., Belvisi M. G. Release of nerve growth factor from human airway epithelial cells (Abstract). Am. J. Respir. Crit. Care Med. 1997; 155:A157.
13. Sanico A. M., Stanisz A. M., Gleeson T. D., Bora S., Proud D., Bienenstock J., Koliatsos V. E., Togias A. Nerve growth factor expression and release in allergic inflammatory disease of the upper airways. Am. J. Respir. Crit. Care Med. 2000; 161:1631-1635.
14. Aloe L., Simone M. D., Properzi F. Nerve growth factor: a neurotrophin with activity on cells of the immune system. Microsc. Res. Tech. 1999; 45:285-291.
15. Riccio M. M., Myers A. C., Undem B. J. Immunomodulation of afferent neurons in guinea-pig isolated airway. J. Physiol. (Lond.) 1996; 491:499-509.
16. Undem B. J., Hunter D. D., Liu M., Haak-Frendscho M., Oakragly A., Fischer A. Allergen-induced sensory neuroplasticity in airways. Int. Arch. Allergy Immunol. 1999; 118:150-153.
17. Suzuki M., Furuno T., Teshima R., Sawada J., Nakanishi M. Soluble factors from rat basophilic leukemia (RBL-2H3) cells stimulated cooperatively the neurite outgrowth of PC12 cells. Biol. Pharm. Bull. 1998; 21:1267-1270.
18. Janiszewski J., Bienenstock J., Blennerhassett M. G. Activation of rat peritoneal mast cells in coculture with sympathetic neurons alters neuronal physiology. Brain Behav. Immun. 1990; 4:139-150.
19. Hunter D. D., Satterfield B. E., Huang J., Fedan J. S., Dey R. D. Toluene diisocyanate enhances substance P in sensory neurons innervating the nasal mucosa. Am. J. Respir. Crit. Care Med. 2000; 161:543-549.
20. Wu Z. X., Morton R. F., Lee L. Y. Role of tachykinins in ozone-induced airway hyperresponsiveness to cigarette smoke in guinea pigs. J. Appl. Physiol. 1997; 83:958-965.
21. Biyah K., Molimard M., Esmonds-Alt X., Advenier C. SR140333 prevents potentiation by citric acid of plasma exudation induced by histamine in airways. Eur. J. Pharmacol. 1996; 308:325-328.

22. Hunter D. D., Myers A. C., Undem B. J. Nerve growth factor-induced phenotypic switch in guinea pig airway sensory neurons. Am. J. Respir. Crit. Care Med. 2000; 161:1985-1990.
23. Sawada J., Itakura A., Tanaka A., Furusaka T., Matsuda H. Nerve growth factor functions as a chemoattractant for mast cells through both mitogen-activated protein kinase and phosphatidylinositol 3-kinase signaling pathways. Blood 2000; 95:2052-2058.
24. Matsuda H., Coughlin M. D., Bienenstock J., Denburg J. A. Nerve growth factor promotes human hemopoietic colony growth and differentiation. Proc. Natl. Acad. Sci. USA 1988; 85:6508-6512.
25. Matsuda H., Switzer J., Coughlin M. D., Bienenstock J., Denburg J. A. Human basophilic cell differentiation promoted by 2.5S nerve growth factor. Int. Arch. Allergy Appl. Immunol. 1988; 86:453-457.
26. Matsuda H., Kannan Y., Ushio H., Kiso Y., Kanemoto T., Suzuki H., Kitamura Y. Nerve growth factor induces development of connective tissue-type mast cells in vitro from murine bone marrow cells. J. Exp. Med. 1991; 174:7-14.
27. Kannan Y., Matsuda H., Ushio H., Kawamoto K., Shimada Y. Murine granulocyte-macrophage and mast cell colony formation promoted by nerve growth factor. Int. Arch. Allergy Immunol. 1993; 102:362-367.
28. Getchell M. L., Kulkarni-Narla A., Takami S., Albers K. M., Getchell T. V. Age-dependent phenotypic switching of mast cells in NGF-transgenic mice. Neuroreport 1995; 6:1261-1266.
29. Marshall J. S., Gomi K., Blennerhassett M. G., Bienenstock J. Nerve growth factor modifies the expression of inflammatory cytokines by mast cells via a prostanoid-dependent mechanism. J. Immunol. 1999; 162:4271-4276.
30. Tal M., Liberman R. Local injection of nerve growth factor (NGF) triggers degranulation of mast cells in rat paw. Neurosci. Lett. 1997; 22:129-132.
31. Kawamoto K., Okada T., Kannan Y., Ushio H., Matsumoto M., Matsuda H. Nerve growth factor prevents apoptosis of rat peritoneal mast cells through the trk proto-oncogene receptor. Blood 1995; 86:4638-4644.
32. Tam S. Y., Tsai M., Yamaguchi M., Yano K., Butterfield J. H., Galli S. J. Expression of functional TrkA receptor tyrosine kinase in the HMC-1 human mast cell line and in human mast cells. Blood 1997; 90:1807-1820.
33. Welker P., Grabbe J., Gibbs B., Zuberbier T., Henz B. M. Nerve growth factor-beta induces mast-cell marker expression during in vitro culture of human umbilical cord blood cells. Immunol. 2000; 99:418-426.
34. Pearlman D. S. Pathophysiology of the inflammatory response. J. Allergy Clin. Immunol. 1999; 104:S132-137.

35. Lipscomb M. F., Wilder J. A. Immune dysregulation as a cause for allergic asthma. Curr. Opin. Pulm. Med. 1999; 5:10-20.
36. Ehrhard P. B., Erb P., Graumann U., Otten U. Expression of nerve growth factor and nerve growth factor receptor tyrosine kinase Trk in activated CD4-positive T-cell clones. Proc. Natl. Acad. Sci. USA 1993; 90:10984-10988.
37. Santambrogio L., Benedetti M., Chao M. V., Muzaffar R., Kulig K., Gabellini N., Hochwald G. Nerve growth factor production by lymphocytes. J. Immunol. 1994; 153:4488-4495.
38. Brodie C., Goldreich N., Haiman T., Kazimirsky G. Functional IL-4 receptors on mouse astrocytes: IL-4 inhibits astrocyte activation and induces NGF secretion. J. Neuroimmunol. 1998; 81:20-30.
39. Brodie C. Differential effects of Th1 and Th2 derived cytokines on NGF synthesis by mouse astrocytes. FEBS Lett. 1996; 394:117-120.
40. Villoslada P., Hauser S. L., Bartke I., Unger J., Heald N., Rosenberg D., Cheung S. W., Mobley W. C., Fisher S., Genain C. P. Human nerve growth factor protects common marmosets against autoimmune encephalomyelitis by switching the balance of T helper cell type 1 and 2 cytokines within the central nervous system. J. Exp. Med. 2000; 191:1799-1806.
41. Melamed I., Kelleher C. A., Franklin R. A., Brodie C., Hempstead B., Kaplan D., Gelfand E. W. Nerve growth factor signal transduction in human B lymphocytes is mediated by gp140trk. Eur. J. Immunol. 1996; 26:1985-1992.
42. Schenone A., Gill J. S., Zacharias D. A., Windebank A. J. Expression of high- and low-affinity neurotrophin receptors on human transformed B lymphocytes. J. Neuroimmunol. 1996; 64:141-149.
43. Otten U., Ehrhard P., Peck R. Nerve growth factor induced growth and differentiation of human B lymphocytes. Proc. Natl. Acad. Sci. USA 1989; 86:10059-10063.
44. Djukanovic R., Wilson J. W., Britten K. M., Wilson S. J., Walls A. F., Roche W. R., Howarth P. H., Holgate S. T. Quantitation of mast cells and eosinophils in the bronchial mucosa of symptomatic atopic asthmatics and healthy control subjects using immunohistochemistry. Am. Rev. Respir. Dis. 1990; 142:863-871.
45. O'Byrne P. M., Gauvreau G. M., Wood L. J. Interaction between haemopoietic regulation and airway inflammation. Clin. Exp. Allergy 1999; 29 Suppl. 2:27-32.
46. Wu Z. X., Zhou D., Chen G., Lee L. Y. Airway hyperresponsiveness to cigarette smoke in ovalbumin-sensitized guinea pigs. Am. J. Respir. Crit. Care Med. 2000; 161:73-80.

47. Hamada A., Watanabe N., Ohtomo H., Matsuda H. Nerve growth factor enhances survival and cytotoxic activity of human eosinophils. Br. J. Haematol. 1996; 93:299-302.
48. Garaci E., Caroleo M. C., Aloe L., Aquaro S., Piacentini M., Costa N., Amendola A., Micera A., Calio R., Perno C. F., Levi-Montalcini R. Nerve growth factor is an autocrine factor essential for the survival of macrophages infected with HIV. Proc. Natl. Acad. Sci. USA 1999; 96:14013-14018.
49. Susaki Y., Shimizu S., Katakura K., Watanabe N., Kawamoto K., Matsumoto M., Tsudzuki M., Furusaka T., Kitamura Y., Matsuda H. Functional properties of murine macrophages promoted by nerve growth factor. Blood 1996; 88:4630-4637.
50. Susaki Y., Tanaka A., Honda E., Matsuda H. Nerve growth factor modulates Fc gamma receptor expression on murine macrophage J774A.1 cells. J. Vet. Med. Sci. 1998; 60:87-91.
51. Ehrhard P. B., Ganter U., Stalder A., Bauer J., Otten U. Expression of functional trk protooncogene in human monocytes. Proc. Natl. Acad. Sci. USA 1993; 90:5423-5427.
52. Lindholm D., Heumann R., Meyer M., Thoenen H. Interleukin-1 regulates synthesis of nerve growth factor in non-neuronal cells of rat sciatic nerve. Nature 1987; 330:658-659.
53. Zhou D., Zheng X., Bai T. R. Both nerve growth factor (NGF) and leukemia inhibitory factor (LIF) augment substance P release in airway explants (Abstract). Am. J. Respir. Crit. Care Med. 2000; 161:A839.
54. Knipper M., Beck A., Rylett J., Breer H. Neurotrophin induced second messenger responses in rat brain synaptosomes. Neuroreport 1993; 4:483-486.
55. Dupont J. L., Janoshazi A., Bellahcene M., Mykita S., de Barry J. Reversible protein kinase C activation in PC12 cells: effect of NGF treatment. Eur. J. Neurosci. 2000; 12:215-226.
56. Woolf C. J. Phenotypic modification of primary sensory neurons: the role of nerve growth factor in the production of persistant pain. Phil. Trans. R. Soc. Lond. B 1996; 351:441-448.
57. Woolf C. J., Ma Q. P., Allchorne A., Poole S. Peripheral cell types contributing to the hyperalgesic action of nerve growth factor in inflammation. J. Neurosci. 1996; 16:2716-2723.
58. Hohmann A. G., Herkenham M. Localization of central cannabinoid CB1 receptor messenger RNA in neuronal subpopulations of rat dorsal root ganglia: a double-label in situ hybridization study. Neurosci. 1999; 90:923-931.

59. Vedder H., Affolter H. U., Otten U. Nerve growth factor (NGF) regulates tachykinin gene expression and biosynthesis in rat sensory neurons during early postnatal development. Neuropeptides 1993; 24:351-357.
60. Silver R., Silverman A.-J., Vitkovic L., Lederhendler I. I. Mast cells in the brain: evidence and functional significance. Trends Neurosci. 1996; 19:25-31.
61. Purcell W. M., Atterwill C. K. Mast cells in neuroimmune function: neurotoxicological and neuropharmacological perspectives. Neurochem. Res. 1995; 20:521-532.
62. Sanico A. M., Koliatsos V. E., Stanisz A. M., Bienenstock J., Togias A. Neural hyperresponsiveness and nerve growth factor in allergic rhinitis. Int. Arch. Allergy Immunol. 1999; 118:154-158.
63. Sanico A. M., Philip G., Proud D., Naclerio R. M., Togias A. Comparison of nasal mucosal responsiveness to neuronal stimulation in non-allergic and allergic rhinitis: effects of capsaicin nasal challenge. Clin. Exp. Allergy 1998; 28:92-100.
64. Riccio M. M., Proud D. Evidence that enhanced nasal reactivity to bradykinin in patients with symptomatic allergy is mediated by neural reflexes. J. Allergy Clin. Immunol. 1996; 97:1252-1263.
65. Sanico A. M., Philip G., Lai G. K., Togias A. Hyperosmolar saline induces reflex nasal secretions, evincing neural hyperresponsiveness in allergic rhinitis. J. Appl. Physiol. 1999; 86:1202-1210.
66. Sanico A. M., Atsuta S., Proud D., Togias A. Plasma extravasation through neuronal stimulation in human nasal mucosa in the setting of allergic rhinitis. J. Appl. Physiol. 1998; 84:537-543.
67. Philip G., Jankowski R., Baroody F. M., Naclerio R. M., Togias A. G. Reflex activation of nasal secretion by unilateral inhalation of cold dry air. Am. Rev. Respir. Dis. 1993; 148:1616-1622.
68. Kudlacz E., Shatzer S., Logan D., Olsen K., Knippenberg R., Hsieh L., Esteve H., Maynard G. A role for histamine and substance P in immediate allergic responses in guinea pig airways: characterization of MDL 108,207DA, a dual H1/NK-1 receptor antagonist. Int. Arch. Allergy Immunol. 1998; 115:169-178.
69. Fischer A., McGregor G. P., Saria A., Philippin B., Kummer W. Induction of tachykinin gene and peptide expression in guinea pig nodose primary afferent neurons by allergic airway inflammation. J. Clin. Invest. 1996; 9:2284-2291.
70. McMahon S. B. NGF as a mediator of inflammatory pain. Phil. Trans. R. Soc. Lond. B 1996; 351:431-440.
71. Mendell L. M., Albers K. M., Davis B. M. Neurotrophins, nociceptors, and pain. Microsc. Res. Tech. 1999; 45:252-261.

72. Saragovi H. U., Gehring I. Development of pharmacological agents for targeting neurotrophins and their receptors. Trends Pharmacol. Sci. 2000; 21:93-98.
73. Pezet S., Ont niente B., Grannec G., Calvino B. Chronic pain is associated with increased TrkA immunoreactivity in spinoreticular neurons. J. Neurosci. 1999; 19:5482-5492.
74. Sonea I. M., Bowker R. M., Robinson N. E., Broadstone R. V. Substance P and calcitonin gene-related peptide-like immunoreactive nerve fibers in lungs from adult equids. Am. J. Vet. Res. 1994; 55:1066-1074.
75. Myers A., Undem B., Kummer W. Anatomical and electrophysiological comparison of the sensory innervation of bronchial and tracheal parasympathetic ganglion neurons. J. Auton. Nerv. Syst. 1996; 61:162-168.
76. Kesler B. S., Canning B. J. Regulation of baseline cholinergic tone in guinea-pig airway smooth muscle. J. Physiol. (Lond.) 1999; 518:843-855.
77. Belvisi M. G., Miura M., Stretton D., Barnes P. J. Endogenous vasoactive intestinal peptide and nitric oxide modulate cholinergic neurotransmission in guinea-pig trachea. Eur. J. Pharmacol. 1993; 231:97-102.
78. Shigyo M., Aizawa H., Inoue H., Matsumoto K., Takata S., Hara N. Pituitary adenylate cyclase activating peptide regulates neurally mediated airway responses. Eur. Respir. J. 1998; 12:64-70.
79. Joos G. F., Germonpre P. R., Pauwels R. A. Neural mechanisms in asthma. Clin. Exp. Allergy 2000; 30:60-65.
80. Boni P., Ballati L., Evangelista S. Tachykinin NK1 and NK2 receptors mediate the non-cholinergic bronchospastic response to capsaicin and vagal stimulation in guinea pigs. J. Auton. Pharmacol. 1995; 15:49-54.
81. Colasurdo G. N., Loader J. E., Graves J. P., Larsen G. L. SP-induced contraction of airway smooth muscle in normal and allergen-sensitized rabbits: mechanism of action. J. Appl. Physiol. 1995; 78:428-432.
82. Tanaka D. T., Grunstein M. M. Mechanisms of substance P-induced contraction of rabbit airway smooth muscle. J. Appl. Physiol. 1984; 57:1551-1557.
83. Nemmar A., Delaunois A., Beckers J. F., Sulon J., Bloden S., Gustin P. Modulatory effect of imetit, a histamine H3 receptor agonist, on C-fibers, cholinergic fibers and mast cells in rabbit lungs in vitro. Eur. J. Pharmacol. 1999; 371:23-30.
84. John C., Brunner S., Tanaka D. T. Neuromodulation mediated by neurokinin-1 subtype receptors in adult rabbit airways. Am. J. Physiol. 1993; 265:L228-233.

85. Shioya T., Kagaya M., Onodera A., Miura S., Miura M. Post-synaptic potentiation of vagal contractile responses by neurokinin A. Pulm. Pharmacol. 1993; 6:209-216.
86. Myers A. C., Kajekar R., Canning B. J. Effects of selective neurokinin-3 (NK3) receptor agonists and antagonists on modulation of cholinergic neurotransmission in the airway (abstract). Am. J. Resp. Crit. Care Med. 2000; 161:A433
87. Smart D., Gunthorpe M. J., Jerman J. C., Nasir S., Gray J., Muir A. I., Chambers J. K., Randall A. D., Davis J. B. The endogenous lipid anandamide is a full agonist at the human vanilloid receptor (hVR1). Br. J. Pharmacol. 2000; 129:227-230.
88. Hwang S. W., Cho H., Kwak J., Lee S. Y., Kang C. J., Jung J., Cho S., Min K. H., Suh Y. G., Kim D., Oh U. Direct activation of capsaicin receptors by products of lipoxygenases: endogenous capsaicin-like substances. Proc. Natl. Acad. Sci. USA 2000; 97:6155-6160.
89. Piacentini G. L., Kaliner M. A. The potential roles of leukotrienes in bronchial asthma. Am. Rev. Respir. Dis. 1991; 143:S96-S99.
90. Profita M., Sala A., Riccobono L., Paterno A., Mirabella A., Bonanno A., Guerrera D., Pace E., Bonsignore G., Bousquet J., Vignola A. M. 15-Lipoxygenase expression and 15(S)-hydroxyeicoisatetraenoic acid release and reincorporation in induced sputum of asthmatic subjects. J. Allergy Clin. Immunol. 2000; 105:711-716.
91. Nijkamp F. P., Ramakers A. G. Prevention of anaphylactic bronchoconstriction by a lipoxygenase inhibitor. Eur. J. Pharmacol. 1980; 62:121-122.
92. Van Oosterhout A. J., van Ark I., Hofman G., Van Der Linde H. J., Fattah D., Nijkamp F. P. Role of interleukin-5 and substance P in development of airway hyperreactivity to histamine in guinea-pigs. Eur. Respir. J. 1996; 9:493-499.
93. Gardiner P. J., Cuthbert N. J., Francis H. P., Fitzgerald M. F., Thompson A. M., Carpenter T. G., Patel U. P., Newton B. B., Mohrs K., Mueller-Peddinghaus R., Taylor W. A. Inhibition of antigen-induced contraction of guinea-pig aiways by a leukotriene synthesis inhibitor, BAY x1005. Eur. J. Pharmacol. 1994; 258:95-102.
94. Nicholas R. S., Winter J., Wren P., Bergmann R., Woolf C. J. Peripheral inflammation increases the capsaicin sensitivity of dorsal root ganglion neurons in a nerve growth factor-dependent manner. Neuroscience 1999; 91:1425-1433.
95. Caterina M. J., Leffler A., Malmberg A. B., Martin W. J., Trafton J., Petersen-Zeitz K. R., Koltzenburg M., Basbaum A. I., Julius D. Impaired

nociception and pain sensation in mice lacking the capsaicin receptor. Science 2000; 288:306-313.
96. Davis J. B., Gray J., Gunthorpe M. J., Hatcher J. P., Davey P. T., Overend P., Harries M. H., Latcham J., Clapham C., Atkinson K., Hughes S. A., Rance K., Grau E., Harper A. J., Pugh P. L., Rogers D. C., Bingham S., Randall A., Sheardown S. A. Vanilloid receptor-1 is essential for inflammatory thermal hyperalgesia. Nature 2000; 405:183-187.
97. Zygmunt P. M., Petersson J., Andersson D. A., Chuang H., Sorgard M., Di Marzo V., Julius D., Hogestatt E. D. Vanilloid receptors on sensory nerves mediate the vasodilator action of anandamide. Nature 1999; 400:452-457.
98. Schmid H. H., Schmid P. C., Natarajan V. N-acylated glycerophospholipids and their derivatives. Prog. Lipid Res. 1990; 29:1-43.
99. Burgi B., Otten U. H., Ochensberger B., Rihs S., Heese K., Ehrhard P. B., Ibanez C. F., Dahinden C. A. Basophil priming by neurotrophic factors. Activation through the trk receptor. J. Immunol. 1996; 15:5582-5588.
100. Levi-Montalcini R., Skaper S. D., Dal Tosco R., Petrelli L., Leon A. Nerve growth factor: from neurotrophin to neurokine. Trends Neurosci. 1996; 19:514-520.
101. Biro T., Maurer M., Modarres S., Lewin N. E., Brodie C., Acs G., Acs P., Paus R., Blumberg P. M. Characterization of functional vanilloid receptors expressed by mast cells. Blood 1998; 91:1332-1340.
102. Ellis J. L., Sham J. S. K., Undem B. J. Tachykinin-independent effects of capsaicin on smooth muscle in human isolated bronchi. Am. J. Respir. Crit. Care Med. 1997; 155:751-755.
103. Veronesi B., Carter J. D., Devlin R. B., Simon S. A., Oortgiesen M. Neuropeptides and capsaicin stimulate the release of inflammatory cytokines in a human bronchial epithelial cell line. Neuropeptides 1999; 33:447-456.
104. Riccio M. M., Matsumoto T., Adcock J. J., Douglas G. J., Spina D., Page C. P. The effect of 15-HPETE on airway responsiveness and pulmonary cell recruitment in rabbits. Br. J. Pharmacol. 1997; 122:249-256.
105. McAlexander M. A., Myers A. C., Undem B. J. Inhibition of 5-lipoxygenase diminishes neurally evoked tachykinergic contraction of guinea pig isolated airway. J. Pharmacol. Exp. Ther. 1998; 285:602-607.
106. Vyklicky L., Knotkova-Urbancova H., Vitaskova Z., Vlachova V., Kress M., Reeh P. W. Inflammatory mediators at acidic pH activate capsaicin receptors in cultured sensory neurons from newborn rats. J. Neurophysiol. 1998; 79:670-676.
107. Geppetti P. Sensory neuropeptide release by bradykinin: mechanisms and pathophysiology implications. Regul. Pept. 1993; 47:1-23.

108. Baumgarten C. R., O'Connor A., Dokic D., Schultz K. D., Kunkel G. Substance P is generated in vivo following nasal challenge of allergic individuals with bradykinin. Clin. Exp. Allergy 1997; 27:1322-1327.
109. Engels F., Van Houwelingen A. H., Buckley T. L., Van de Velde M. J., Henricks P. A. J., Nijkamp F. P. Airway hyperresponsiveness induced by 13-hydroxyoctadecadienoic acid (13-HODE) is mediated by sensory neuropeptides. Adv. Prostaglandin Thromboxane Leukot. Res. 1995; 23:361-363.
110. Safieh-Garabedian B., Dardenne M., Kanaan S. A., Atweh S. F., Jabbur S. J., Saade N. E. The role of cytokines and prostaglandin-E_2 in thymulin induced hyperalgesia. Neuropharmacol. 2000; 39:1653-1661.
111. Koltzenburg M., Bennett D. L., Shelton D. L., McMahon S. B. Neutralization of endogenous NGF prevents the sensitization of nociceptors supplying inflamed skin. Eur. J. Neurosci. 1999; 11:1698-1704.
112. Jaggar S. I., Scott H. C., Rice A. S. Inflammation of the rat urinary bladder is associated with a referred thermal hyperalgesia which is nerve growth factor dependent. Br. J. Anaesth. 1999; 83:442-448.
113. Pauwels R. The pharmacology of airway hyperresponsiveness and inflammation. Lung 1990; 168:221-229.
114. Braun A., Lommatzsch M., Lewin G. R., Virchow J. C., Renz H. Neurotrophins: a link between airway inflammation and airway smooth muscle contractility in asthma? Int. Arch. Allergy Immunol. 1999; 118:163-165.
115. Kraneveld A. D., Nijkamp F. P., Van Oosterhout A. J. M. Role for neurokinin-2 receptor in interleukin-5-induced airway hyperresponsiveness but not eosinophilia in guinea pigs. Am. J. Respir. Crit. Care Med. 1997; 156:367-374.
116. Joos G. F., Germonpre P. R., Pauwels R. A. Role of tachykinins in asthma. Allergy 2000; 55:321-337.
117. Lundberg J. M. Tachykinins, sensory nerves, and asthma- an overview. Can. J. Physiol. Pharmacol. 1995; 73:908-914.
118. Joos G. F., van Schoor J., Kips J. C., Pauwels R. A. The effect of inhaled FK224, a tachykinin NK-1 and NK-2 receptor antagonist, on neurokinin A-induced bronchoconstriction in asthmatics. Am. J. Respir. Crit. Care Med. 1996; 153:1781-1784.
119. Van Schoor J., Joos G. F., Chasson B. L., Brouard R. J., Pauwels R. A. The effect of the NK2 tachykinin receptor antagonist SR 48968 (saredutant) on neurokinin A-induced bronchoconstriction in asthmatics. Eur. Respir. J. 1998; 12:17-23.

120. Cao Y. Q., Mantyh P. W., Carlson E. J., Gillespie A. M., Epstein C. J., Basbaum A. I. Primary afferent tachykinins are required to experience moderate to intense pain. Nature 1998; 392:390-394.
121. Ibanez C. F. Emerging themes in structural biology of neurotrophic factors. Trends Neurosci. 1998; 21:438-444.
122. Clary D. O., Reichardt L. F. An alternatively spliced form of the nerve growth factor receptor TrkA confers an enhanced response to neurotrophin 3. Proc. Natl. Acad. Sci. USA 1994; 91:11133-11137.
123. Malcangio M., Ramer M. S., Boucher T. J., McMahon S. B. Intrathecally injected neurotrophins and the release of substance P from the rat isolated spinal cord. Eur. J. Neurosci. 2000; 12:139-144.
124. Barker P. A. p75NTR: A study in contrasts. Cell. Death. Differ. 1998; 5:346-356.
125. Maggi C. A. The effects of tachykinins on inflammatory and immune cells. Regul. Pept. 1997; 70:75-90.
126. Lai J. P., Douglas S. D., Rappaport E., Wu J. M., Ho W. Z. Identification of a delta isoform of preprotachykinin mRNA in human mononuclear phagocytes and lymphocytes. J. Neuroimmunol. 1998; 91:121-128.
127. Lambrecht B. N., Germonpre P. R., Everaert E. G., Carro-Muino I., De Veerman M., de Felipe C., Hunt S. P., Thielemans K., Joos G. F., Pauwels R. A. Endogenously produced substance P contributes to lymphocyte proliferation induced by dendritic cells and direct TCR ligation. Eur. J. Immunol. 1999; 29:3815-3825.
128. Tomaki M., Ichinose M., Miura M., Hirayama Y., Yamauchi H., Nakajima N., Shirato K. Elevated substance P content in induced sputum from patients with asthma and patients with chronic bronchitis. Am. J. Respir. Crit. Care Med. 1995; 151:613-617.
129. Kraneveld A. D., Folkerts G., van Oosterhout A. J. M., Nijkamp F. P. Airway hyperresponsiveness: First eosinophils and then neuropeptides. Int. J. Immunopharmacol. 1997; 19:517-527.
130. Garland A., Necheles J., White S. R., Neeley S. P., Leff A. R., Carson S. S., Alger L. E., McAllister K., Solway J. Activated eosinophils elicit substance P release from cultured dorsal root ganglion neurons. Am. J. Physiol. 1997; 273:L1096-1102.
131. Kudlacz E. M., Knippenberg R. W. In vitro and in vivo effects of tachykinins on immune cell function in guinea pig airways. J. Neuroimmunol. 1994; 50:119-125.
132. Karimi K., Redegeld F. A., Blom R., Nijkamp F. P. Stem cell factor and interleukin-4 increase responsiveness of mast cells to substance P. Exp. Hematol. 2000 28: 626-634.

133. Joos G. F., Pauwels R. A. The in vivo effects of tachykinins on airway mast cells of the rat. Am. Rev. Respir. Dis. 1993; 148:922-926.
134. Janiszewski J., Bienenstock J., Blennerhassett M. G. Picomolar doses of substance P trigger electrical responses in mast cells without degranulation. Am. J. Physiol. 1994; 276:C138-C145.
135. di Mola F. F., Friess H., Zhu Z. W., Koliopanos A., Bley T., Di Sebastiano P., Innocenti P., Zimmermann A., Buchler M. W. Nerve growth factor and trk high affinity receptor (TrkA) gene expression in inflammatory bowel disease. Gut 2000; 46:670-679.
136. Simone M. D., De Santis S., Vigneti E., Papa G., Amadori S., Aloe L. Nerve growth factor: a survey of activity on immune and hematopoietic cells. Hematol. Oncol. 1999; 17:1-10.
137. Aloe L., Skaper S. D., Leon A., Levi-Montalcini R. Nerve growth factor and autoimmune diseases. Autoimmunity 1994; 19:141-150.
138. Lambiase A., Bonini S., Micera A., Tirassa P., Magrini L., Aloe L. Increased plasma levels of substance P in vernal keratoconjunctivitis. Invest. Ophthalmol. Vis. Sci. 1997; 38:2161-2164.
139. Aloe L., Bracci-Laudiero L., Bonini S., Manni L. The expanding role of nerve growth factor: from neurotrophic activity to immunologic diseases. Allergy 1997; 52:883-894.
140. Lewin G. R., Rueff A., Mendell L. M. Peripheral and central mechanisms of NGF-induced hyperalgesia. Eur. J. Neurosci. 1994; 6:1903-1912.
141. Lazarovici P., Rasouly D., Friedman L., Tabekman R., Ovadia H., Matsuda Y. K252a and staurosporine microbial alkaloid toxins as prototype of neurotrophic drugs. Adv. Exp. Med. Biol. 1996; 391:367-377.
142. Ro L. S., Chen S. T., Tang L. M., Jacobs J. M. Effect of NGF and anti-NGF on neuropathic pain in rats following chronic constriction injury of the sciatic nerve. Pain 1999; 79:265-274.
143. Anand P., Terenghi G., Warner G., Kopelman P., Williams-Chessnut R. E., Sinicropi D. V. The role of endogenous nerve growth factor in human diabatic neuropathy. Nature Med. 1996; 2, 6:703-707.
144. Anand P. Neurotropihins and peripheral neuropathy. Phil. Trans. R. Soc. Lond. B 1996; 351:449-454.
145. Hellweg R., Hartung H. D. Endogenous levels of nerve growth factor (NGF) are altered in experimental diabetes mellitus: a possible role for NGF in the pathogenesis of diabetic neuropathy. J. Neurosci. Res. 1990; 26:258-267.
146. el-Sakka A. I., Lin C. S., Chui R. M., Dahiya R., Lue T. F. Effects of diabetes on nitric oxide synthase and growth factor genes and protein expression in an animal model. Int. J. Impot. Res. 1999; 11:123-132.

147. Rittenhouse P. A., Marchand J. E., Chen J., Kream R. M., Leeman S. E. Streptozotocin-induced diabetes is associated with altered expression of peptide-encoding mRNAs in rat sensory neurons. Peptides 1996; 17:1017-1022.
148. Diemel L. T., Brewster W. J., Fernyhough P., Tomlinson D. R. Expression of neuropeptides in experimental diabetes; effects of treatment with nerve growth factor or brain-derived neurotrophic factor. Brain Res. Mol. Brain Res. 1994; 21:171-175.
149. Douek I. F., Leech N. J., Gillmor H. A., Bingley P. J., Gale E. A. Children with type-1 diabetes and their unaffected siblings have fewer symptoms of asthma. Lancet 1999; 353:1850.
150. Lasser E. Asthma and diabetes mellitus: a biochemical basis for antithetical features. Med. Hypotheses 1987; 23:95-106.

Nederlandse samenvatting

Nerve growth factor (NGF) is al jarenlang bekend als groeifactor voor zenuwen. Het zorgt bijvoorbeeld voor de uitgroei van zenuwen tijdens de ontwikkeling van een embryo. Recentelijk is gebleken dat dit soort groeifactoren ook een rol speelt in ontsteking. Tijdens een ontsteking is de hoeveelheid NGF ter plekke verhoogd. Allergisch astma wordt o.a. door ontsteking in de luchtwegen gekenmerkt. In dit proefschrift is onderzocht in hoeverre NGF een rol speelt in allergisch astma.
Gezonde mensen reageren niet of nauwelijks op koude lucht of andere prikkels, maar patiënten met astma reageren hierop met een luchtwegvernauwing. Deze overgevoeligheid van de luchtwegen kan in de cavia worden nagebootst door simpelweg een kleine hoeveelheid NGF toe te dienen. Dit is beschreven in hoofdstuk 2.
Er zijn zenuwen die weliswaar signalen afgeven aan de luchtwegen, maar bovenal signalen doorgeven van de luchtwegen naar de hersenen. Dit zijn de zogenaamde sensorische zenuwen. Een bepaalde soort sensorische zenuwen brengt een aantal zeer specifieke peptiden tot expressie. Dit zijn de tachykininen, waaronder substance P en neurokinine A. De tachykininen kunnen luchtwegovergevoeligheid veroorzaken. NGF lijkt zijn effecten te bewerkstelligen door deze zenuwen overgevoelig te maken. Dit betekent dat de zenuwen op een prikkel sneller reageren of meer substance P afgeven. Indien vlak vóór de toediening van NGF de receptor voor substance P wordt geblokkeerd, dan kan de overgevoeligheid van de luchtwegen voorkomen worden (hoofdstuk 2). Hoofdstuk 3 laat zien dat NGF overgevoeligheid van de luchtwegen veroorzaakt via de zenuwuiteinden, maar zonder dat er meer substance P wordt geproduceerd.
Hoofdstuk 3 bevat nog een aanwijzing dat zenuwuiteinden betrokken zijn bij de inductie van luchtwegovergevoeligheid door NGF. Een cannabinoid blijkt in staat de effecten van NGF in zowel het trachea-preparaat als in het hele dier te blokkeren. De gebruikte cannabinoid stimuleert specifiek de cannabinoid receptor op sensorische zenuwen, en zorgt ervoor dat de zenuwen minder signalen doorgeven.
Ook is in een diermodel voor allergisch astma gekeken naar de rol van NGF. Een cavia kan overgevoelig worden gemaakt voor ovalbumine, een eiwit uit het kippen-ei. Op het moment dat de cavia ovalbumine inademt, reageert het dier met een allergische aanval in de luchtwegen. Als vlak voor de ovalbumine antistoffen tegen NGF worden toegediend, die er dus voor zorgen dat de hoeveelheid NGF sterk verlaagd wordt, is de acute allergische luchtwegvernauwing sterk verminderd (hoofdstuk 4).
Verder treedt er in dit diermodel een dag na de inademing van ovalbumine een overgevoeligheid van de luchtwegen op. Dit gaat gepaard met een verhoging van

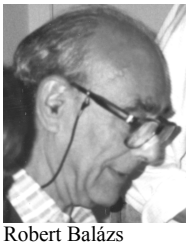
substance P in de zenuwen. In hoofdstuk 5 is aangetoond dat tegelijkertijd de hoeveelheid NGF in de longen toeneemt. De dieren zijn behandeld met tyrosine kinase remmers specifiek voor de receptor met een hoge affiniteit voor NGF: de tyrosine kinase receptor A (trkA). Deze remmers zorgen ervoor dat NGF zijn signalen via de trkA receptor niet meer kan doorgeven. Als de dieren met deze tyrosine kinase remmers behandeld worden, is de overgevoeligheid van de luchtwegen verdwenen en is ook de hoeveelheid substance P niet meer verhoogd in het diermodel voor allergisch astma (hoofdstuk 5).
Opvallend is dat antistoffen tegen NGF de overgevoeligheid van de luchtwegen niet kunnen voorkomen, terwijl blokkade van trkA signalering wel effectief is in dit diermodel voor astma. De acute-astma aanval daarentegen, zoals beschreven in hoofstuk 4, kan wel door antistoffen tegen NGF en niet door remming van de tyrosine kinases van de trkA receptor worden verminderd. Onze hypothese is dat er nog een andere neurotrofe factor betrokken is bij allergisch astma die ook via de trkA receptor werkt.
NGF kan uit verschillende cellen worden vrijgezet. Eén van die cellen is de mestcel. Al vaker is in de literatuur directe communicatie tussen mestcellen en sensorische zenuwen gesuggereerd. Deze twee typen cellen zijn samen gekweekt en er is onderzocht in hoeverre de cellen elkaars functie beïnvloeden (hoofdstuk 6). In onze experimenten hechten de cellen specifiek aan elkaar. Verder beïnvloeden deze cellen elkaar wel, maar slechts in geringe mate. Meer studies zijn nodig om deze interacties nader te bestuderen. Op dit moment kunnen we nog geen uitspraak doen of de mestcellen betrokken zijn bij de vrijzetting of effecten van NGF in de luchtwegen.
Concluderend kan gesteld worden dat de effecten van NGF in de luchtwegen bewerkstelligd worden door veranderingen in de sensorische zenuwen. In dit proefschrift is aangetoond dat NGF een rol in allergisch astma speelt. Dit kan de aanleiding vormen tot de ontwikkeling van een nieuwe klasse geneesmiddelen tegen astma.

Dankbeeld

I would like to thank many people for their contribution to the completion of this thesis. Besides functioning as the names of the categories suggest, some people helped in a scientific way, others by listening to my complaints and, most importantly, by having fun/dinner/drinks together. I hope the pictures will help to memorize these special people. Besides the persons shown here, I would like to thank all who spend time on great scientific discussions on meetings or elsewhere.

Annick

the initiator



Robert Balázs

family

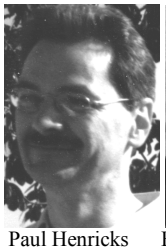
Hans de Vries

Jeichien de Vries

Nynke de Vries en Michel Peeters

supervisors

Ferdi Engels



Paul Henricks

Frans Nijkamp

collaborators

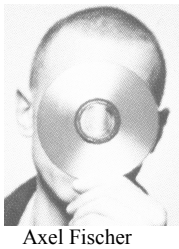
Mieke Struik

Paul Dijkhuizen

Angela d'Aprile

Roy Goldie

Silke Wiegand



Axel Fischer

Petra Hartmann

students


Mark Dessing


Carolien van Rijnsoever


Paula Mommersteeg

ex-colleagues


Cornelie v Petersen

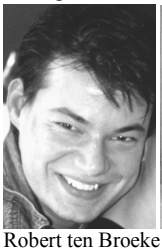

Peet v/d Loo


Joris de Bie


John Hagreis


Nebal Shamki

colleagues

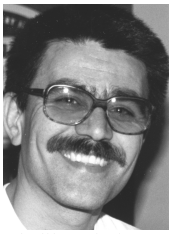

Robert ten Broeke


Richard Muijsers


Thea Leusink-Muis


Joris Kloek


Janine Schuurman


Khalil Karimi


Andrys Weitenberg


Anneke v Houwelingen


Mirjam Kool


Hanneke v/d Kleij


Judith Scheerens


Nanne Bloksma


Ingird v Ark


Jeroen v Bergenhenegouwen


Gert Folkerts

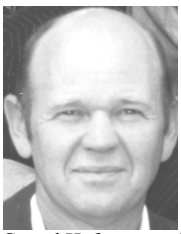

Frank Redegeld

Dankbeeld


Andries Koster


René Houtman


Peter Groot


Gerard Hofman


Diny v/d Pal-Heij


Richard Blom


Anne Lamers


Betty Lobato- v.Esch


Marcel Schijf


Esmael Mortaz


Daphne Deurloo


Joost Smit


Ilse Dijkgraaf


Saskia de Jager


Antoon v Oosterhout


Inge Valstar


Aletta Kraneveld


Sjef de Kimpe

Beyond Biology Lectures


Harald van Mil


Mieke Struik


Ellen ter Gast


Jechiam Gural


Catelijne Stortelers

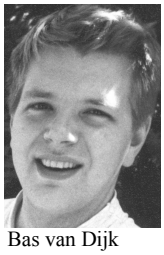
friends....


Liesbeth Oostergetel


Marianne Rots


Mariane ter Veen


Janneke Visser

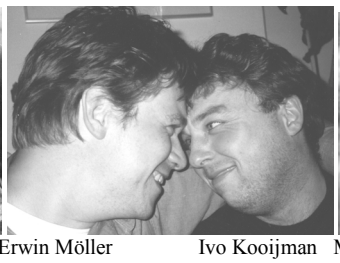

Bas van Dijk


Kasper Spaan


Rob Mutsaers


Melissa Verburg


Cynthia Kneppers


Erwin Möller

Ivo Kooijman


Madeleine Brouns


Renske Brune


Jennifer Kahle


Nick Callamaras


Irene Krämer


Wilko Steinhoff

paranimfen


Aloys Thielen


Sandra Mol

Beyond Biology Lectures


Rob Ruitenbeek


David Claessen


Frans Jacobs


Jennifer Baker

Curriculum Vitae Annick de Vries

May 1996 -Sept 2000	**Neurogenic aspects of the airways** Ph.D. student; dep. Pharmacology and Pathophysiology, Utrecht Institute of Pharmaceutical Sciences. Supervisors: Prof. Dr. F.P. Nijkamp and Dr. G.M.H. Engels, Dr. P.A.J. Henricks. Nov 1999 - Dec 1999 **The effect of Nerve Growth Factor on excitatory Non-adrenergic-Non-cholinergic induced contraction**. Temporary stay in the lab of Dr. Darryl Knight and Prof. Dr. Roy Goldie at the Faculty of Medicine & Dentistry, Department of Pharmacology, University of Western Australia, Australia
Sept 1995- April 1996	teaching-assistant at the Faculty of Biology, the University of Amsterdam.
Sept 1989- Aug 1996	M.Sc. degree in **Medical Biology**, at the University of Amsterdam March 1995- Aug 1995 **Desensitization of the metabotropic glutamate receptor 5 in astrocyte cultures.** Internship; Institute for Brain Aging and Dementia, University of California Irvine, supervisors: Dr. Robert Balázs and Prof. Carl W. Cotman. Feb. 1994- Feb 1995 **Kindling epileptogenisis induces no changes in [^{3}H]AMPA and [^{3}H]Nimodipine binding in rat hippocampus**. Internship; Institute for Neurobiology, Faculty of Biology, University of Amsterdam; supervisors: Dr. Willem Kamphuis and Prof. Dr. Fernando H. Lopes da Silva. Dec1993- Jan 1994 **The role of prostaglandins in the central nervous system.** Literature Survey, Dep. Pharmacology, Faculty of Medicine, Free University of Amsterdam; supervisor: Prof. Dr. Fred J. Tilders. Nov 1992- Nov 1993 **The effect of ORG2766, an ACTH(4-9) analog, on memory deficits induced by frontal cortex lesions in the rat**. Internship; Rudolf Magnus Institute, Faculty of Medicine, University Utrecht; supervisor: Dr. Gerrit Wolterink and Prof. dr. Jan M. van Ree. Feb 1992- Oct 1992 **The resistance against chloroquine of Plasmodium Falciparum in a rural area in Zambia**. Internship; Dep. Parasitology/Microbiology, Faculty of Medicine, University of Amsterdam; supervisor dr. Jan Vetter and Prof. dr. A.C. Muller. This involved three months work in Zambia, Africa.

List of Publications

Aletta D. Kraneveld, Deborah E. James, Annick de Vries and Frans P. Nijkamp 2000, Excitatory Non-Adrenergic-Non-Cholinergic neuropeptides: key players in asthma, Eur. J. Pharmacol. in press

Gert Folkerts, Janneke Westra-de Vlieger, Annick de Vries, Stephan Faas, Henk van der Linde, Ferdi Engels, Jan C. de Jong, Fons A. K. C. P. Verheyen, Dicky van Heuven-Nolsen, and Frans P. Nijkamp, 2000, Virus- and Bradykinin-induced airway hyperresponsiveness in guinea pigs, Am. J. Resp. Crit. Care Med. 161: 1666-1671

Annick de Vries, Mark C. Dessing, Ferdi Engels, Paul A.J. Henricks, and Frans P. Nijkamp, 1999, Nerve growth factor induces a neurokinin-1 receptor mediated airway hyperresponsiveness in guinea pigs, Am. J. Resp.Crit. Care Med. 159. 1541-1544

Axel Fischer, Annick de Vries, Frans P. Nijkamp, Bradley J. Undem, Pierangelo Geppetti, and Wolfgang Kummer, 2000, Plasticity of tachykinins in allergic airway inflammation, Eur. Respir. Reviews 10: 269-271

Annick de Vries, Mark C. Dessing, Ferdi Engels, Paul A.J. Henricks, and Frans P. Nijkamp, 2000, Nerve Growth Factor induces airway hyperresponsiveness in guinea pigs, Eur. Respir. Reviews 10: 324-325

R. Balázs, S. Miller, C. Romano, A. de Vries. Y. Chun, and C.W. Cotman, 1997. Metabotropic Glutamate Receptor mGluR5 in Astrocytes: Pharmacological Properties and Agonist Regulation, J. Neurochem. 69: 151-163