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Brain-derived neurotrophic factor (BDNF) contributes to neuronal dysfunction in a model of allergic airway inflammation

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1 Brain-derived neurotrophic factor (BDNF) is a candidate molecule for mediating functional neuronal changes in allergic bronchial asthma. Recently, enhanced production of BDNF during allergic airway inflammation caused by infiltrating T-cells and macrophages as well as by resident airway epithelial cells has been described. It was the aim of this study to investigate the effect of enhanced BDNF levels on lung function and airway inflammation in a mouse model of allergic inflammation.

2 Ovalbumin-sensitised BALB/*c* mice were challenged in two consecutive allergen challenges. Prior to the challenge, the mice were treated with either anti-BDNF antibodies or isotype-matched control antibodies. Airway responsiveness to methacholine, capsaicin and electric field stimulation, as well as airway inflammation and chronic airway obstruction 1 week after the last allergen challenge were assessed.

3 Anti-BDNF blocked enhanced reactivity in response to capsaicin, but not airway smooth muscle hyper-reactivity *in vivo*. Furthermore, persistent airway obstruction, as observed 1 week after the last allergen challenge, was to a large extent prevented by anti-BDNF treatment. *In vitro*, BDNF and anti-BDNF treatment had a profound effect on local neuronal hyper-reactivity, as shown by electric field stimulation experiments. In contrast, neither BDNF nor anti-BDNF treatment affected airway inflammation.

4 Our data indicate that development of allergen-induced neuronal hyper-reactivity in mice is partially mediated by BDNF.
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Keywords: Bronchial asthma; neurotrophins; brain-derived neurotrophic factor; airway inflammation; airway hyper-responsiveness

Abbreviations: BAL(F), bronchoalveolar lavage (fluid); BDNF, brain-derived neurotrophic factor; BSA, bovine serum albumin; EFS, electrical field stimulation; EF_{50}, mid-expiratory airflow; HBP, head-out body plethysmography; NGF, nerve growth factor; OVA, ovalbumin; TB, time of braking

Introduction

Allergic bronchial asthma is a chronic inflammatory disease characterised by a variable degree of airway remodelling, airway hyper-responsiveness and recurrent attacks of wheezing and airway obstruction (for review, see Kay, 1996). Development of airway hyper-responsiveness to unspecific pharmacological stimuli is a pathogenic hallmark of asthma (Wills-Karp, 1999). Though the immunological network in asthma has been well characterised, the relationship between airway inflammation and changes in lung function is not completely understood (Wilder *et al*., 1999; Wills-Karp, 1999). Airway tone is predominantly regulated by vagal cholinergic and sensory nerves (Barnes, 1996; Kesler & Canning, 1999). Over the last decade, it has been demonstrated that allergic asthma is associated with altered neuronal control of the airways, including sensory hyper-reactivity (Ellis & Undem, 1992; Nieber *et al*., 1992; Fischer *et al*., 1996; Kaltreider *et al*., 1997; Undem *et al*., 1999). However, concepts describing the link between allergic airway inflammation and neuronal dysfunction are still lacking. Several studies demonstrated that neurotrophins such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) can mediate neuronal plasticity in inflammatory conditions (Lindsay & Harmar, 1989; Donnerer *et al*., 1992; Dmitrieva *et al*., 1997). There is recent evidence for a specific role of NGF in allergic asthma (Braun *et al*., 1998; Päth *et al*., 2002). However, experiments on the functional role of elevated BDNF levels in asthma have not been described yet.

To evaluate the influence of BDNF on lung function *in vivo*, body plethysmography was performed as described before (Päth *et al*., 2002; Kerzel *et al*., 2003). This system analyses the

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breathing pattern of mice by continuously registering changes in airflow during ins- and expiration. By using specific stimuli, it can distinguish between bronchoconstriction and sensory irritation. For determination of the sensory function, we used the reflex prolongation of stage I of expiration ('time of braking (TB)') that is caused by stimulating sensory airway nerves. Electrical or chemical stimulations of laryngeal, vagus or carotid sinus nerves were shown to prolong the period of depolarisation in postinspiratory neurons without changing the duration of expiratory or inspiratory stages, indicating a fairly selective prolongation of the first stage of expiration (Remmers *et al*., 1986). This characteristic modification of the normal breathing pattern has been demonstrated as a sensitive and specific parameter for sensory nerve function in the lung. This system was validated and the results documented in a series of papers (Vijayaraghavan *et al*., 1993; 1994; Alarie, 1998). It was adapted for asthma research recently (Braun *et al*., 1999; Neuhaus-Steinmetz *et al*., 2000; Glaab *et al*., 2001). In this study, capsaicin was used as a stimulus for sensory neurons, since it is known to act specifically on sensory neurons *via* a vanilloid receptor (VR-1) (Michael & Priestley, 1999; Szallasi & Blumberg, 1999). In contrast, methacholine acts *via* direct stimulation of airways smooth muscle cells, resulting in a characteristic dose-dependent decrease in midexpiratory flow rate (EF_{50}). EF_{50} represents an established parameter of airway obstruction (Neuhaus-Steinmetz *et al*., 2000; Glaab *et al*., 2001).

Experimental data from animal models suggest that the effect of neurotrophins may be responsible for neuronal hyperreactivity following allergic inflammation of the airways (Braun *et al*., 1998; Hoyle *et al*., 1998; de Vries *et al*., 1999; Undem *et al*., 1999; Päth *et al*., 2002; Kerzel *et al*., 2003). Indeed, allergic asthmatic patients respond with increased levels of the neurotrophins NGF and BDNF in the bronchoalveolar lavage fluid (BALF) following allergen challenge (Virchow *et al*., 1998). Utilising a well-characterised mouse model of asthma, we have identified T cells and macrophages as additional sources of BDNF in allergic airway inflammation (Braun *et al*., 1999). Based on these observations, we have developed the hypothesis that BDNF may provide a link between airway inflammation and neuronal dysfunction in allergic asthma. It was the aim of this study to test this hypothesis in our mouse model of allergic airway inflammation.

Methods

Animals

Female BALB/*c* mice, 6–8 weeks old, were obtained from Harlan Winkelmann, Borchen, Germany and maintained under controlled conditions. Figure 1 and Table 1 show all experiments including the treatment scheme and total animal number.

Protocol of allergic sensitisation

Mice were sensitised to ovalbumin (OVA; 10 μg per injection) (Sigma, Deisenhofen, Germany) adsorbed to 1.5 mg $Al(OH)_3$ (Pierce, Rockford, U.S.A.) or vehicle alone by intraperitoneal injections, as detailed in Figure 1. Aerosol challenges were performed with 1% OVA (w v^{-1}) diluted in PBS or PBS alone delivered by a Pari-Jet nebuliser aerosolisation (median mass diameter MMD = 3 μm). All animals were analysed 24 h after the last challenge.

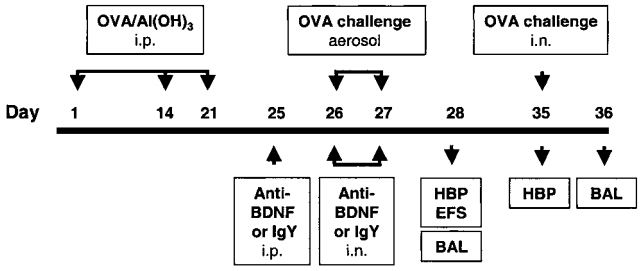


Figure 1 Animal treatment protocol. Mice were sensitised to OVA adsorbed to $Al(OH)_3$ or vehicle alone by intraperitoneal injections on days 1, 14 and 21. Prior to analysis, animals received two consecutive local aerosol challenges of 1% OVA (w v^{-1}) diluted in PBS or PBS alone and delivered by 20-min aerosolisation on days 26 and 27. Intranasal application of polyclonal chicken IgY (isotype antibody) or anti-mouse BDNF was performed 3 h before each airway allergen challenge. In addition, animals received the antibodies i.p. on day 25. The response to acute allergen exposure was measured on day 35 in the body plethysmograph. All animals were analysed 24 h after the last challenge. Abbreviations: intraperitoneally (i.p.), intranasally (i.n.), aerosol challenge (aerosol).

Intranasal BDNF or anti-BDNF treatment

Intranasal application of antibodies was performed as described before (Braun *et al*., 1998), and lung distribution of nasally administered substances was controlled by evans blue instillation (data not shown). Polyclonal chicken IgY anti-mouse BDNF ($2 \times 25\,\mu$l) (500 μg ml^{-1}) or isotype antibody (500 μg ml^{-1}) (Promega, Madison, U.S.A.) solved in sterile PBS was instilled intranasally 3 h before each airway allergen challenge. In addition, $1 \times 100\,\mu$l polyclonal chicken IgY anti-mouse BDNF (500 μg ml^{-1}) or isotype antibody (500 μg ml^{-1}) solved in sterile PBS was given intraperitoneally (i.p.) 24 h before first allergen challenge (Figure 1). Recombinant BDNF (5 μg ml^{-1}) (Biosource) or BSA (0.1%) was administered intranasally. Before application, mice were anaesthetised by subcutaneous injection of 2.6 mg ketamin hydrochloride (Ketanest©, Parke Davis, Berlin, Germany) and 0.18 mg xylazin hydrochloride (Rompun©, Bayer, Leverkusen, Germany) for approx. 10 min.

Bronchoalveolar lavage (BAL)

Animals were killed by cervical dislocation and their tracheae cannulated. Airways were lavaged twice with 0.8 ml ice-cold PBS and cell numbers, and cytokine contents were determined as described previously (Braun *et al*., 1999). BAL recovery was 1.4 ± 0.2 ml in all groups. Cell-free BALF was frozen at −20°C until analysis.

Assessment of airway responsiveness by electrical field stimulation (EFS)

Airway smooth muscle responsiveness was assessed by EFS as described before (Braun *et al*., 1998; Herz *et al*., 1998). Mice were killed by cervical dislocation 24 h after the last allergen

Table 1 Overview of experiments and animal numbers

	Allergen		*Treatment*		*Analysis*	
Figure	*i.p.*	*Aerosol*	*i.p.*	*i.n.*	*Method*	*Animals*
—	—	—	—	—	EFS	8
—	—	—	—	—	EFS	8
—	OVA	OVA	—	—	EFS	10
3 A	—	—	—	BDNF	EFS	12
3 A	—	—	—	BSA	EFS	12
3 B	OVA	OVA	Anti-BDNF	Anti-BDNF	EFS	12
3 B	OVA	OVA	IgY	IgY	EFS	12
5	—	—	—	—	HBP	8
5	OVA	OVA	Anti-BDNF	Anti-BDNF	HBP	10
5	OVA	OVA	IgY	IgY	HBP	10
6+7	—	OVA	—	—	HBP/BAL	11
6+7	OVA	OVA	Anti-BDNF	Anti-BDNF	HBP/BAL	11
6+7	OVA	OVA	IgY	IgY	HBP/BAL	11
7	—	—	—	BDNF	BAL	12
7	—	—	—	BSA	BAL	12

Figures showing the results of these experiments are detailed in the first column. Sensitisation (intraperitoneally, i.p.) to allergen (ovalbumin, OVA) and allergen challenge (aerosol) are detailed in the following two columns. Intraperitoneal (i.p.) or intranasal (i.n.) treatment with anti-BDNF, isotype-matched control antibodies (IgY), BDNF or bovine serum albumin (BSA) is specified in the two columns 'treatment'. The last two columns 'analysis' detail the methods (electric field stimulation, EFS; head-out body plethysmography, HBP; and bronchoalveolar lavage, BAL) and the number of animals used for analysis in each experiment. Ganglia for neuropeptide experiments were taken from animals of the last two experiments.

challenge. Tracheal smooth muscle segments ($\sim$0.5 cm) were removed and hung between stainless steel wire triangular supports. There was a standard pretreatment and treatment regime in all EFS experiments. During the 90 min equilibration in the bath, tracheal segments were stimulated with KCl as a viability control. KCl stimulation was performed at three time points, with a final concentration in the organ bath of 120 mM KCl. Afterwards, tissues were rinsed with fresh buffer and allowed to relax to their initial tension. Then, the maximum contractile response was assessed using the following parameters: 16 V, 200 mA, 2 ms pulse duration, 1 ms delay, 30 Hz. The degree of maximal contraction in response to EFS and to KCl stimulation was comparable for each animal. Finally, the stimulus–response curves were generated by stimulating the tracheal segments with 0.5–30 Hz (12 V, 200 mA), with 2 min recovery time between each stimulation step (Braun *et al*., 1998; Herz *et al*., 1998). In each stimulation step, tracheal segments were stimulated until reaching the peak of the contractile response (for approximately 20–25 s). The contraction in response to EFS stimulus was measured *via* an isometric force transducer (Grass Instruments, Quincy, U.S.A.). The frequency that caused 50% of the maximal contraction was calculated from logarithmic plots of the contractile response *versus* the frequency of EFS, and expressed as the ES_{50}.

Assessment of lung function by head-out bodyplethysmography

Meanwhile, airway function was assessed by head-out body plethysmography (HBP), as previously described (Vijayaraghavan *et al*., 1993; 1994; Braun *et al*., 1999). Four mice were placed in four body plethysmographs attached to an exposure chamber (Crown Glass, Somerville, N.J., U.S.A.). For acclimatisation, animals were put into the exposure chamber 15 min prior to exposure. The slight decrease in respiratory flow during the baseline period is due to acclimatisation of mice in the body plethysmograph (Figure 6). There was no further decrease in respiratory flow after 10 min of acclimatisation (data not shown). Airflow was measured with a PTM 378/1.2 pneumotachograph (Hugo Sachs Electronics, March-Hugstetten, FRG) and a 8-T2 differential pressure transducer (Gaeltec, Dunvegan, U.K.). For determination of bronchoconstriction, mid-expiratory airflow (EF_{50}, i.e. the expiratory airflow when 50% of the tidal volume is exhaled) was measured in response to various concentrations of methacholine (4–75 mg ml^{-1}, 1 min) delivered by a jet nebuliser (Pari-Boy®; Pari-Werke, Starnberg, Germany) (Neuhaus-Steinmetz *et al*., 2000; Glaab *et al*., 2001). For determination of sensory irritation, 'TB' was determined according to Vijayaraghavan *et al*. (1993; 1994), in response to various concentrations of capsaicin (10, 50, 100, 500, 1000 μg ml^{-1}, 1 min) delivered by a jet nebuliser (Pari-Boy®; Pari-Werke, Starnberg, Germany). OVA (20 μl, 1%) was given intranasally to mice placed in the body plethysmographs, to measure allergen-induced airway obstruction.

ELISA, RIA and immunohistochemistry

Cytokine levels were measured in cell-free BAL supernatants using ELISA, as previously described (Braun *et al*., 1999). Neuropeptide concentrations and distribution were analysed by RIA and immunohistochemistry, respectively. The methods are described in detail in an earlier study (Fischer *et al*., 1996). In brief, tissue concentrations of substance P/neurokinin A and CGRP were measured by a combination of HPLC and RIA. Immunohistochemistry was performed using Zamboni's fixed vagal sensory ganglia and antigen preabsorption (10 ng peptide ml^{-1} diluted primary antiserum) for the control of specificity. Figure 2 shows substance P/neurokinin A staining of vagal sensory ganglia (a) as well as a control section incubated with antigen-preabsorbed antiserum (b).

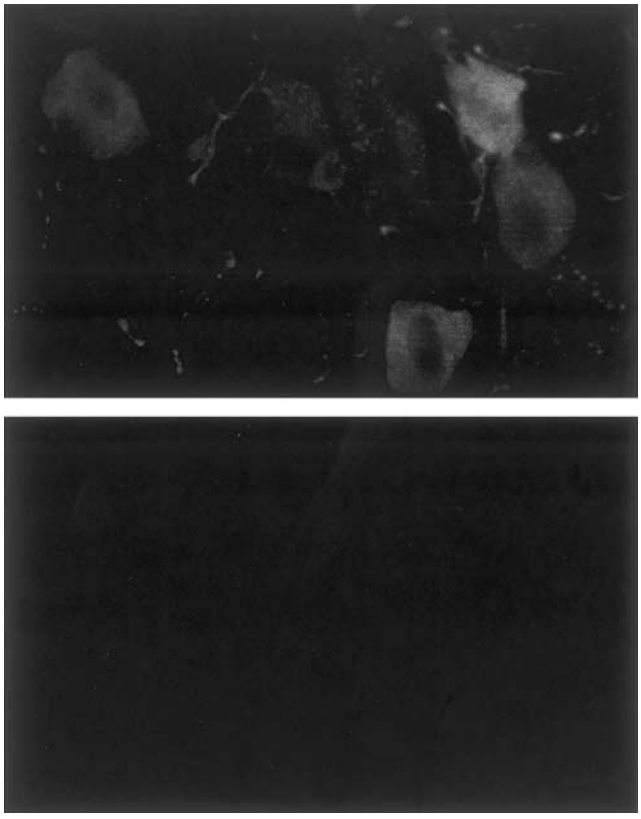


Figure 2 Immunohistochemical detection of tachykinins. Substance P/neurokinin A immunohistochemistry was performed using Zamboni's fixed vagal sensory ganglia and antigen preabsorption for the control of specificity. The upper picture (a) shows substance P/neurokinin A staining of vagal sensory ganglia, the lower picture a control section incubated with antigen-preabsorbed antiserum (b). Bars represent 50 μm.

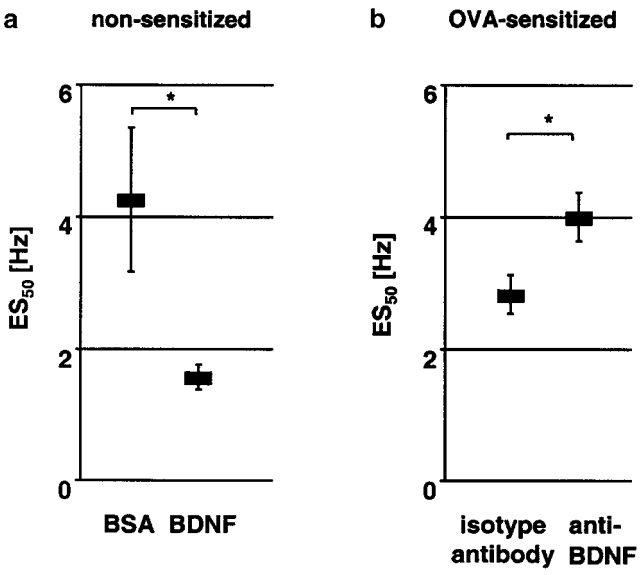


Figure 3 Effect of BDNF on airway hyper-responsiveness in response to EFS. Airway hyper-responsiveness was measured in response to EFS. The frequency that caused 50% of maximal airway smooth muscle constriction was defined as ES_{50}. Airway contractility was expressed as mean $\pm$ s.d. Statistics were performed using *t*-test with Welch's correction. $P<0.05$ was regarded as a statistically significant difference. (a) Nonsensitised BALB/*c* mice were treated intranasally either with recombinant human BDNF (5 μg ml^{-1}, 50 μl) or BSA (0.1%, 50 μl) as a control on two consecutive days. AHR was measured 24 h after the last treatment. In all, 12 animals were examined in each group in two separate experiments. (b) OVA-sensitised Balb/*c* mice were treated intranasally either with anti-BDNF (500 μg ml^{-1}, 50 μl) or isotype IgY (500 μg ml^{-1}, 50 μl) as a control on two consecutive days, 3 h before OVA aerosol challenge. In addition, the animals received 100 μl of the antibody solution i.p. 24 h before the first OVA challenge (day 25). AHR was measured 24 h after the last treatment. A total of 12 animals were examined in each group in two separate experiments.

Ganglia for neuropeptide experiments were taken from animals of the experiments for Figure 7.

Statistical analysis

Results are presented as mean values $\pm$ s.d., unless otherwise stated. Student's *t*-test was used to determine the level of difference between animal groups, unless otherwise stated. $P<0.05$ was regarded as a statistically significant difference.

Results

Effect of BDNF on airway hyper-responsiveness

Electrical field stimulation To investigate the specific contribution of BDNF to the development of airway hyper-responsiveness in our animal model of allergic asthma, airway hyper-responsiveness was assessed with various methods and stimuli. First, we assessed airway hyper-responsiveness *in vitro* by EFS of tracheal segments. The frequency that causes 50% of maximal airway smooth muscle constriction (ES_{50}) was at 4.1 Hz in nonsensitised animals after nasal BSA control treatment and at 4.2 Hz in completely untreated mice. In contrast, OVA-sensitised and challenged mice developed airway hyper-responsiveness, as indicated by a significant decrease in ES_{50} values to 2.4 Hz (data not shown). In a further experiment, allergen-sensitised and challenged animals were treated with anti-BDNF antibodies or isotype-matched control antibodies by intranasal application of an anti-BDNF polyclonal antibody delivered on days 26 and 27. There was one additional i.p. application of anti-BDNF polyclonal antibodies or isotype-matched control antibodies on day 25 (Figure 1). Mice treated with isotype-matched control antibodies showed a regular hyper-responsiveness following allergen challenge (Figure 3b). Anti-BDNF treatment almost completely eliminated the decrease in ES_{50} values (Figure 3b). We then investigated whether BDNF treatment by itself could be sufficient to alter airway responsiveness. Nonsensitised naive mice were treated nasally with BDNF or BSA on two consecutive days, and airway responsiveness was assessed 24 h later. BDNF-treated mice showed marked airway hyper-responsiveness in response to EFS as compared to BSA-treated control mice (Figure 3a). This airway hyper-responsiveness seemed to be even stronger than that observed in sham-treated allergen-challenged mice, though a direct comparison of these data is not possible due to the different experimental settings (Figure 3a, b).

Head-out body plethysmography To further analyse the underlying mechanisms, a mouse head-out body plethysmographic system was used. This system analyses the breathing pattern of each animal by continuously registering changes in airflow during ins- and expiration. By using specific stimuli, it can distinguish between bronchoconstriction due to direct stimulation of airway smooth muscle and changes in the breathing pattern due to sensory nerve activation. Capsaicin is a potent and selective stimulant of sensory nerves and induces a characteristic modification of the normal breathing pattern, which is quantitatively and qualitatively assessed by measuring the duration of braking ('TB') (see Introduction for further explanation). As shown in Figures 4 and 5d, capsaicin induced a marked dose-dependent increase in 'TB' in normal mice. In contrast, methacholine acts via direct stimulation of airways smooth muscle cells. As shown in Figure 5c, methacholine has no effect on 'TB', but results in a characteristic dose-dependent decrease in mid-expiratory flow rate (EF_{50}) (Figure 5a).

Methacholine and capsaicin were used to further analyse the specific contribution of BDNF to the development of airway hyper-responsiveness (Figure 5). As shown in Figure 5a, allergen-sensitised and -challenged animals develop increased airway responsiveness to methacholine, characterised by a significant ($P<0.05$) leftward shift of the dose–response curves to aerosolised methacholine. This response was not altered by anti-BDNF treatment. In contrast, hyper-reactivity to capsaicin was almost completely blocked by anti-BDNF treatment.

Effect of BDNF on allergen-induced airway obstruction

As recently demonstrated (Neuhaus-Steinmetz *et al*., 2000), a re-challenge of OVA-sensitised and allergic mice at weekly intervals causes chronic airway obstruction, as indicated by a decrease in baseline EF_{50} values (Figure 6). In this model, additional acute intranasal OVA-challenge further decreased EF_{50} values 10–30 min post challenge (Figure 6). This model was employed to assess the contribution of BDNF to allergen-induced airway obstruction. Anti-BDNF treatment partially prevented chronic airway obstruction, as indicated by a significant ($P<0.05$) increase in baseline EF_{50} value when compared to sham-treated animals. After an acute allergen challenge, these mice showed a rapid decrease in EF_{50} values within 10 min post challenge, which persisted for at least 30 min. The magnitude of this response was similar in both groups, indicating that anti-BDNF treatment did not affect the strength of the acute response following allergen challenge. On the contrary, the differences in the mid-expiratory flow following allergen challenge seem to reflect pre-existing differences in airway calibre between the groups.

Effect of BDNF on airway inflammation

Murine allergic airway inflammation is characterised by a characteristic change of the cytokines interleukin-4 (IL-4), interleukin-5 (IL-5) and interferon-γ (IFN-γ), as well as increased eosinophil, monocyte and lymphocyte counts in the bronchoalveolar lavage (Braun *et al*., 1999). BDNF might decrease airflow by affecting the inflammatory response in the airways. To investigate this possibility, we assessed IL-4, IL-5 and IFN-γ levels as well as differential cell counts in BALFs obtained from anti-BDNF or sham-treated mice. Blocking of BDNF neither quantitatively nor qualitatively affected the pattern of allergic inflammation (Figures 7b, d). In addition, anti-BDNF treatment had no effect on the cellular influx of eosinophils and lymphocytes in acutely OVA-challenged mice on day 36 (Figure 7e). Furthermore, nasal administration of recombinant BDNF to nonsensitised naive mice did not induce a local inflammatory response (Figures 7a, c).

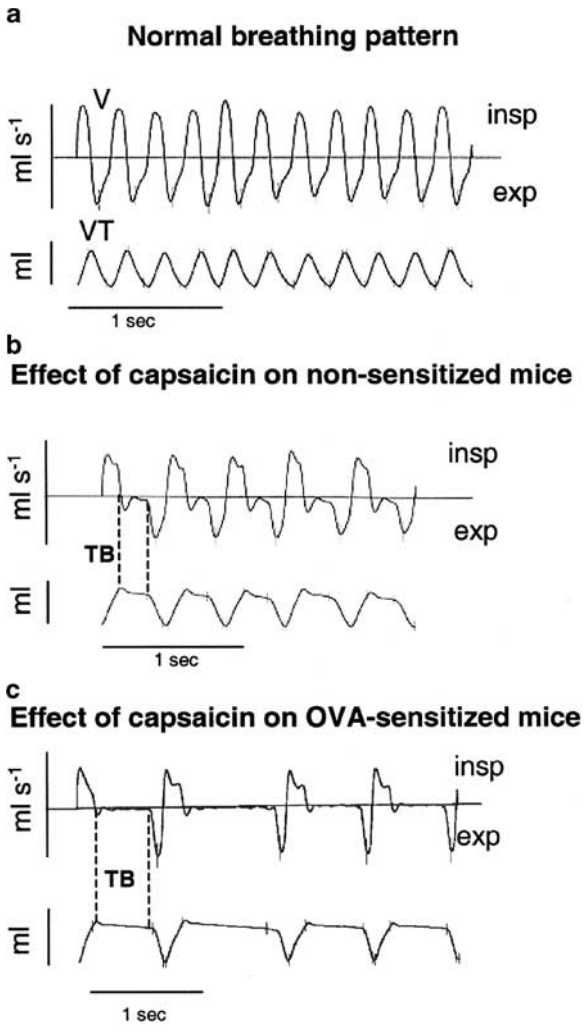
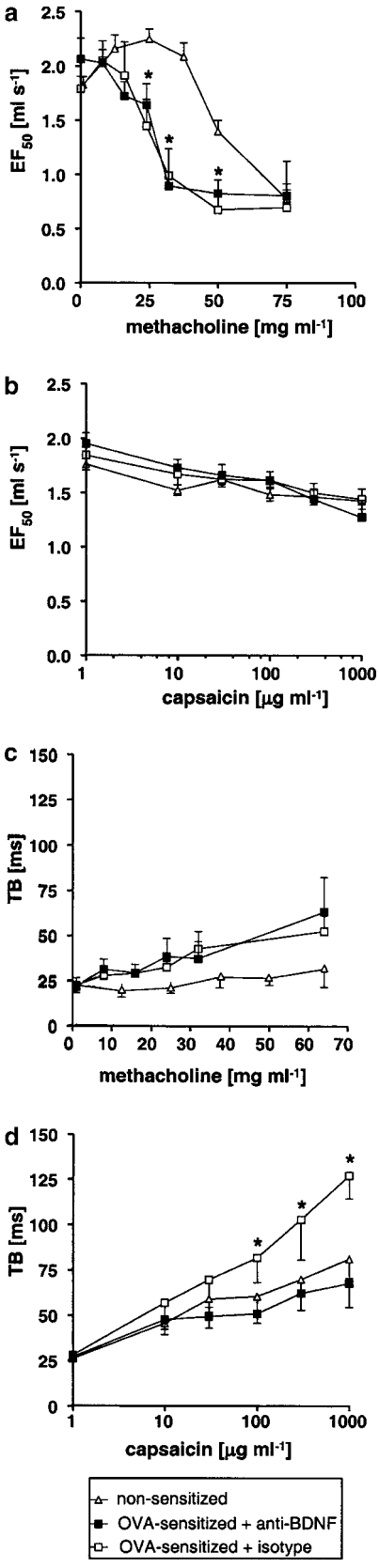


Figure 4 Characteristic modifications to the normal breathing pattern in response to capsaicin measured by HBP. (a) Normal breathing pattern (airflow) of nonsensitised BALB/*c* mice while breathing room air. Normal breathing patterns were virtually identical in sensitised and nonsensitised mice. (b) Characteristic pattern of sensory irritation (lengthening of TB) of nonsensitised BALB/*c* mice in response to capsaicin (500 μg ml^{-1}). The breathing pattern before capsaicin challenge is identical to breathing pattern shown in (a). (c) Characteristic pattern of sensory irritation (lengthening of TB) of OVA-sensitised BALB/*c* mice in response to capsaicin (500 μg ml^{-1}).

Effect of BDNF on neuronal neuropeptide content

Neurotrophins may modulate afferent nerve functions *via* several mechanisms, including enhanced neuropeptide production and releasability. In order to assess whether the functional changes in sensory neurons are associated with altered neuropeptide levels, tachykinin and CGRP content in sensory ganglia was measured 24 h after the last allergen challenge. The

data presented in Table 2 compare the results obtained with anti-BDNF and sham-treated allergic mice. There was no detectable difference between the groups in (a) number of tachykinin or CGRP-containing neurons or (b) tachykinin or CGRP content in various ganglia.



Discussion

The aim of this study was to analyse the role of BDNF with respect to airway function in allergic asthma, using a well-established murine model of allergic airway inflammation. In order to characterise the specific contribution of BDNF to altered lung function, we measured allergen-induced airway obstruction as well as airway hyper-responsiveness to various nonsensitising stimuli in anti-BDNF treated and in sham-treated control mice. We were able to distinguish between airway dysfunctions mediated by smooth muscle changes and those mediated by neuronal alterations. This differentiation is especially important for the evaluation of airway hyper-responsiveness. Airway hyper-responsiveness following allergic inflammation is mediated by multiple independent and additive pathways working in concert (Herz *et al*., 1998; Wilder *et al*., 1999; Wills-Karp, 1999). Dependent on the method chosen for the measurement of airway hyper-responsiveness, different pathways can be distinguished. They include: (a) altered neuronal regulation of airway tone, (b) increased smooth muscle content or function, and (c) increased epithelial mucus production and airway edema (Wills-Karp, 1999). Recent studies with asthmatic patients revealed an individual contribution of neuronal and smooth muscle hyper-reactivity to airway hyper-responsiveness (Millqvist *et al*., 1998).

Neutralisation of BDNF by local and systemic treatment with specific antibodies reduced allergen-induced chronic airway obstruction *in vivo*, as shown by HBP. Allergen-induced airway obstruction has recently been demonstrated in mice using body plethysmography (Cieslewicz *et al*., 1999; Neuhaus-Steinmetz *et al*., 2000). The time course of BDNF in BALF showed an elevation over 2–3 weeks and a late peak on day 7 after the last allergen challenge (Lommatzsch *et al*., 2003). Therefore, we examined the effects of BDNF neutralisation on airway obstruction on day 7 after the last allergen challenge. Interestingly, EF_{50} values were still decreased in allergen-challenged mice *versus* control mice at this late time point. Neutralisation of BDNF significantly increased these EF_{50} values. The decrease of EF_{50} in response to allergen re-challenge was concomitantly altered. However, the latter effect seems to reflect only an altered basal airway obstruction, since the differences in EF_{50} between anti-BDNF-treated and control-antibody-treated groups are nearly the same before

Figure 5 Effect of anti-BDNF treatment on airway hyper-responsiveness in response to methacholine and capsaicin by HBP. (a) Dose–response curve of expiratory flow (EF_{50}) in response to inhaled methacholine. (b) Dose–response curve of expiratory flow (EF_{50}) in response to inhaled capsaicin. (c) Dose–response curve of sensory irritation (TB) in response to inhaled methacholine. (d) Dose–response curve of sensory irritation (TB) in response to inhaled capsaicin. The error bars represent standard error of the mean (s.e.m.), $n=8$–10 animals in each group in three separate experiments. Student's *t*-test: $^*P<0.05$. (a) Nonsensitised against OVA-sensitised + isotype, (d) OVA-sensitised + isotype against OVA-sensitised + anti-BDNF.

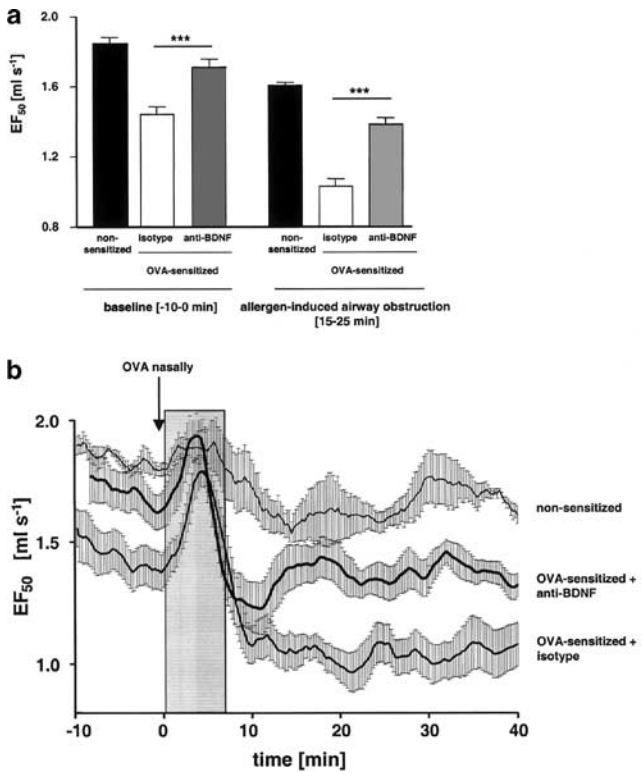


Figure 6 Effect of anti-BDNF treatment on allergen-induced airway obstruction by HBP. Measurement of expiratory flow in response to OVA (intranasal application during continuous measurement of airflow in the HBP). (a) Integration of EF_{50} values between 15 and 25 min after OVA application. (b) Time course of $n = 11$ animals in each group. Data from three separate experiments are shown. Grey bar: possible artefacts from OVA application. The error bars represent standard error of the mean (s.e.m.). Student's *t*-test: $***P < 0.001$.

and after allergen re-challenge. We conclude that neutralisation of BDNF has profound effects on the basal allergen-induced airway obstruction, even 1 week after the initial allergen challenge. One explanation for this phenomenon could be an enhanced basal parasympathetic tone of the airways. An alternative explanation could be an inhibition of sensory nerve-induced plasma leakage, mucus production and oedema, which could affect airway calibre as well. Significant obstructions due to altered inflammatory conditions are unlikely, since anti-BDNF did not affect inflammatory parameters.

Using EFS *in vitro*, we have further shown that anti-BDNF treatment was able to prevent local neuronal hyper-reactivity in the airways. It has been previously demonstrated that *in vitro* EFS of tracheal segments specifically reflects neuronal airway obstruction. Administration of both atropine (disruption of cholinergic pathways) and capsaicin (depletion of sensory neurons) completely blocks responsiveness of tracheal segments to electric field stimulation (Andersson & Grundstrom, 1983; Ellis & Undem, 1992). We used, therefore, *in vitro* EFS to examine the effects of BDNF on neuronal dysfunction. Locally administered BDNF was sufficient to induce airway hyper-responsiveness in response to EFS in the absence of inflammation. In addition, neutralisation of BDNF in allergen-sensitised and -challenged mice prevented airway hyper-responsiveness to EFS, which normally follows allergic airway inflammation. Thus, BDNF seems to have direct effects on neuronal control of the airway diameter. These effects seem to be independent of the local inflammatory situation, since neither BDNF nor anti-BDNF affected any of the inflammatory parameters measured.

In vivo experiments strengthened the idea that anti-BDNF treatment primarily affects neuronal parameters. Anti-BDNF treatment did affect the altered reactivity of sensory neurons (to capsaicin), but not the altered reactivity of airway smooth muscle (to methacholine). It needs to be emphasised that increased sensory irritation in response to capsaicin, as shown by a lengthening of the first phase of expiration, does not necessarily imply airway obstruction. In fact, it appears that capsaicin and neuropeptides such as substance *P* are bronchodilators in rodents (Manzini, 1992; Szarek *et al*., 1998). In addition, altered capsaicin reactivity does not necessarily imply altered neuropeptide levels in sensory neurons, as shown by our immunohistochemical studies. The capsaicin experiments in our present study were solely carried out to answer the general question whether anti-BDNF treatment can affect the reactivity of sensory neurons. The mechanisms of enhanced reactivity in response to capsaicin and the effects on airway diameter are species-specific and cannot be extrapolated from our results.

Our data support the idea that BDNF could primarily act on the reactivity of innervating neurons. Since BDNF neither affected inflammatory conditions in allergen-challenged mice, nor induced any inflammation by itself, it seems unlikely that it affects airway functions indirectly *via* inflammatory alterations. In addition, direct effects on airway smooth muscle cells seem to be unlikely, since anti-BDNF treatment did not affect airway hyper-responsiveness in response to methacholine. In contrast, the profound effects of BDNF and anti-BDNF on airway hyper-responsiveness measured by *in vitro* EFS, as well as the effects of BDNF on the capsaicin response *in vivo*, demonstrate that BDNF could directly affect neuronal control. We hypothesise, therefore, that enhanced BDNF production by immune cells in the lung (e.g., T-cells or macrophages) (Virchow *et al*., 1998; Braun *et al*., 1999) could substantially contribute to neuronal dysfunction.

It has been well established that a variety of sensory and motor neurons innervating the adult viscera express the high-affinity BDNF receptor trkB (Wetmore & Olson, 1995; Kashiba *et al*., 1997; Michael *et al*., 1997; Zhou *et al*., 1998). On the other hand, BDNF receptors are not detectable on resident non-neuronal cells in the adult lung (Lommatzsch *et al*., 1999). The abundance of BDNF in the adult viscera, especially in the lung, and the retrograde transport of BDNF in sensory and motor neurons with their axons in the vagus nerve indicate that target-derived BDNF could indeed influence innervating neurons (Helke *et al*., 1998; Lommatzsch *et al*., 1999). Effects on both sensory and motor neurons have to be considered. For example, altered reactivity of sensory neurons to capsaicin could be mediated by BDNF-induced VR-1 receptor changes in vagal sensory neurons (Winter, 1998). Effects of BDNF on motor neurons may be especially important regarding airway obstruction. BDNF is retrogradely transported in vagal motor neurons and can affect a variety of motor neuron functions, including excitability, ion channel expression, synaptic composition and neurotransmitter production (Gonzalez & Collins, 1997; Fernandes *et al*.,

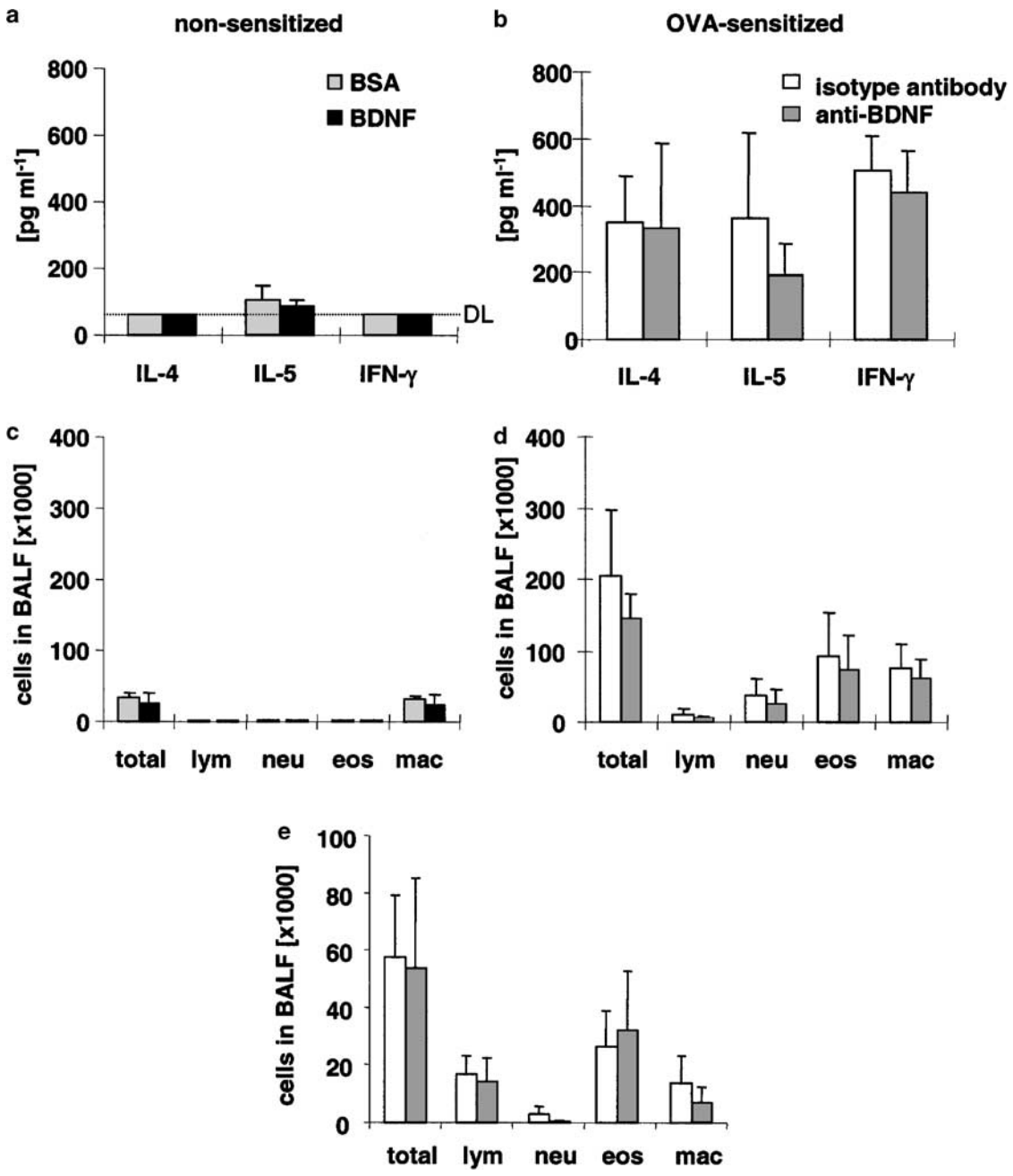


Figure 7 Effect of BDNF and anti-BDNF treatment on inflammation. (a, b) Cytokine levels in BALF. IL-4, IL-5 and IFN-γ were measured in BALF from nonsensitised or OVA-sensitised mice 24 h after aerosol challenge by ELISA. The BAL recovery was 1.4 ± 0.2 ml in all groups. (c, d) Cell differentiation in BALF after OVA aerosol challenge on day 28. Lymphocytes, macrophages, eosinophils and neutrophils were differentiated according to morphological criteria in BALF from nonsensitised or OVA-sensitised mice 24 h after the last aerosol challenge. (e) Cell differentiation in BALF 24 h after nasal OVA challenge on day 36. Student's *t*-test did not reveal any statistically significant difference ($P<0.05$) between groups. In all, 12 mice were analysed in each group, in two separate experiments. The error bars represent standard deviation (s.d.).

Table 2 Tachykinin containing sensory neurons from ovalbumin-sensitised mice

	Ganglion V *SP/NKA* (pg mg^{-1} wet weight)	*Ganglion IX/X* *SP/NKA* (pg mg^{-1} wet weight)	*Ganglion IX/X* *SP/NKA positive neurons* (%)
IgY	15.8 ± 1.7	7.9 ± 2.4	6.5 ± 0.8
Anti-BDNF	13.0 ± 1.8	8.0 ± 0.8	6.0 ± 1.1

Immunohistochemistry for SP/NKA revealed immunoreactive perikarya that were counted for each ganglion and are expressed as % of total neurons. The neuropeptide content of each ganglion is expressed as pg mg^{-1} wet weight. The ganglia were prepared 24 h after the last allergen provocation. Each value represents the mean of four animals $\pm$ s.e.m. Student's *t*-test did not reveal any statistically significant difference ($P<0.05$) between groups.

1998; Helke *et al*., 1998; Baldelli *et al*., 1999; Novikov *et al*., 2000). Effects of the closely related neurotrophin NGF on enhanced reactivity in response to capsaicin have already been shown (Hoyle *et al*., 1998; Winston *et al*., 2001). Interestingly, NGF seems to have similar effects as BDNF on airway sensory nerves (Päth *et al*., 2002). This could be due to the fact that NGF affects airway sensory neurons by activating the pan-neurotrophin (p75) receptor (Kerzel *et al*., 2003). Notably, NGF does have profound effects on allergic inflammation (Päth *et al*., 2002), whereas BDNF does not. This could be due to specific effects of NGF on neuronal tachykinin production, as well as direct trkA-mediated effects on immune cells (Päth *et al*., 2002).

In conclusion, our experiments demonstrate that BDNF could serve as a link between allergic airway inflammation and neuronal dysfunction in allergic asthma. We have found evidence that BDNF is directly acting on neuronal tissues in the lung, inducing neuronal hyper-reactivity and chronically enhancing the basal airway tone.

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References

ALARIE, Y. (1998). Computer-based bioassay for evaluation of sensory irritation of airborne chemicals and its limit of detection. *Arch. Toxicol.,* **72,** 277–282.

ANDERSSON, R.G. & GRUNDSTROM, N. (1983). The excitatory non-cholinergic, non-adrenergic nervous system of the guinea-pig airways. *Eur. J. Respir. Dis. Suppl.,* **131,** 141–157.

BALDELLI, P., MAGNELLI, V. & CARBONE, E. (1999). Selective upregulation of P- and R-type Ca^{2+} channels in rat embryo motoneurons by BDNF. *Eur. J. Neurosci.,* **11,** 1127–1133.

BARNES, P.J. (1996). Neuroeffector mechanisms: the interface between inflammation and neuronal responses. *J. Allergy Clin. Immunol.,* **98,** S73–S81 discussion S81–S83.

BRAUN, A., APPEL, E., BARUCH, R., HERZ, U., BOTCHKAREV, V., PAUS, R., BRODIE, C. & RENZ, H. (1998). Role of nerve growth factor in a mouse model of allergic airway inflammation and asthma. *Eur. J. Immunol.,* **28,** 3240–3251.

BRAUN, A., LOMMATZSCH, M., MANNSFELDT, A., NEUHAUS-STEINMETZ, U., FISCHER, A., SCHNOY, N., LEWIN, G.R. & RENZ, H. (1999). Cellular sources of enhanced brain-derived neurotrophic factor production in a mouse model of allergic inflammation. *Am. J. Respir. Cell Mol. Biol.,* **21,** 537–546.

CIESLEWICZ, G., TOMKINSON, A., ADLER, A., DUEZ, C., SCHWARZE, J., TAKEDA, K., LARSON, K.A., LEE, J.J., IRVIN, C.G. & GELFAND, E.W. (1999). The late, but not early, asthmatic response is dependent on IL-5 and correlates with eosinophil infiltration. *J. Clin. Invest.,* **104,** 301–308.

DE VRIES, A., DESSING, M.C., ENGELS, F., HENRICKS, P.A. & NIJKAMP, F.P. (1999). Nerve growth factor induces a neurokinin-1 receptor-mediated airway hyperresponsiveness in guinea pigs. *Am. J. Respir. Crit. Care Med.,* **159,** 1541–1544.

DMITRIEVA, N., SHELTON, D., RICE, A.S. & MCMAHON, S.B. (1997). The role of nerve growth factor in a model of visceral inflammation. *Neuroscience,* **78,** 449–459.

DONNERER, J., SCHULIGOI, R. & STEIN, C. (1992). 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,* **49,** 693–698.

ELLIS, J.L. & UNDEM, B.J. (1992). Antigen-induced enhancement of noncholinergic contractile responses to vagus nerve and electrical field stimulation in guinea-pig isolated trachea. *J. Pharmacol. Exp. Ther.,* **262,** 646–653.

FERNANDES, K.J., KOBAYASHI, N.R., JASMIN, B.J. & TETZLAFF, W. (1998). Acetylcholinesterase gene expression in axotomized rat facial motoneurons is differentially regulated by neurotrophins: correlation with trkB and trkC mRNA levels and isoforms. *J. Neurosci.,* **18,** 9936–9947.

FISCHER, A., MCGREGOR, G.P., SARIA, A., PHILIPPIN, B. & KUMMER, W. (1996). Induction of tachykinin gene and peptide expression in guinea pig no dose primary afferent neurons by allergic airway inflammation. *J. Clin. Invest.,* **98,** 2284–2291.

GLAAB, T., DASER, A., BRAUN, A., NEUHAUS-STEINMETZ, U., FABEL, H., ALARIE, Y. & RENZ, H. (2001). Tidal midexpiratory flow as a measure of airway hyperresponsiveness in allergic mice. *Am. J. Physiol. Lung Cell Mol. Physiol.,* **280,** L565–L573.

GONZALEZ, M & COLLINS III, W.F. (1997). Modulation of motoneuron excitability by brain-derived neurotrophic factor. *J. Neurophysiol.,* **77,** 502–506.

HELKE, C.J., ADRYAN, K.M., FEDOROWICZ, J., ZHUO, H., PARK, J.S., CURTIS, R., RADLEY, H.E. & DISTEFANO, P.S. (1998). Axonal transport of neurotrophins by visceral afferent and efferent neurons of the vagus nerve of the rat. *J. Comp. Neurol.,* **393,** 102–117.

HERZ, U., BRAUN, A., RUCKERT, R. & RENZ, H. (1998). Various immunological phenotypes are associated with increased airway responsiveness. *Clin. Exp. Allergy,* **28,** 625–634.

HOYLE, G.W., GRAHAM, R.M., FINKELSTEIN, J.B., NGUYEN, K.P., GOZAL, D. & FRIEDMAN, M. (1998). Hyperinnervation of the airways in transgenic mice overexpressing nerve growth factor. *Am. J. Respir. Cell Mol. Biol.,* **18,** 149–157.

KALTREIDER, H.B., ICHIKAWA, S., BYRD, P.K., INGRAM, D.A., KISHIYAMA, J.L., SREEDHARAN, S.P., WARNOCK, M.L., BECK, J.M. & GOETZL, E.J. (1997). Upregulation of neuropeptides and neuropeptide receptors in a murine model of immune inflammation in lung parenchyma. *Am. J. Respir. Cell Mol. Biol.,* **16,** 133–144.

KASHIBA, H., UEDA, Y., UEYAMA, T., NEMOTO, K. & SENBA, E. (1997). Relationship between BDNF- and trk-expressing neurones in rat dorsal root ganglion: an analysis by *in situ* hybridization. *Neuroreport,* **8,** 1229–1234.

KAY, A.B. (1996). Pathology of mild, severe, and fatal asthma. *Am. J. Resp. Crit. Care Med.,* **154,** S66–S69.

KERZEL, S., PATH, G., NOCKHER, W.A., QUARCOO, D., RAAP, U., GRONEBERG, D.A., DINH, Q.T., FISCHER, A., BRAUN, A. & RENZ, H. (2003). Pan-neurotrophin receptor p75 contributes to neuronal hyperreactivity and airway inflammation in a murine model of experimental asthma. *Am. J. Respir. Cell Mol. Biol.,* **28,** 170–178.

KESLER, B.S. & CANNING, B.J. (1999). Regulation of baseline cholinergic tone in guinea-pig airway smooth muscle. *J. Physiol.,* **518,** 843–855.

LINDSAY, R.M. & HARMAR, A.J. (1989). Nerve growth factor regulates expression of neuropeptide genes in adult sensory neurons. *Nature,* **337,** 362–364.

LOMMATZSCH, M., BRAUN, A., MANNSFELDT, A., BOTCHKAREV, V.A., BOTCHKAREVA, N.V., PAUS, R., FISCHER, A., LEWIN, G.R. & RENZ, H. (1999). Abundant production of brain-derived neurotrophic factor by adult visceral epithelia. Implications for paracrine and target-derived neurotrophic functions. *Am. J. Pathol.,* **155,** 1183–1193.

LOMMATZSCH, M., BRAUN, A. & RENZ, H. (2003). Neurotrophins in allergic airway dysfunction: what the mouse model is teaching us. *Ann. N.Y. Acad. Sci.,* **992,** 241–249.

MANZINI, S. (1992). Bronchodilatation by tachykinins and capsaicin in the mouse main bronchi. *Br. J. Pharmacol.,* **105,** 968–972.

MICHAEL, G.J., AVERILL, S., NITKUNAN, A., RATTRAY, M., BENNETT, D.L., YAN, Q. & PRIESTLEY, J.V. (1997). Nerve growth factor treatment increases brain-derived neurotrophic factor selectively in trkA-expressing dorsal root ganglion cells and in their central terminations within the spinal cord. *J. Neurosci.,* **17,** 8476–8490.

MICHAEL, G.J. & PRIESTLEY, J.V. (1999). Differential expression of the mRNA for the vanilloid receptor subtype 1 in cells of the adult rat dorsal root and nodose ganglia and its downregulation by axotomy. *J. Neurosci.,* **19,** 1844–1854.

MILLQVIST, E., BENDE, M. & LOWHAGEN, O. (1998). Sensory hyperreactivity – a possible mechanism underlying cough and asthma-like symptoms. *Allergy,* **53,** 1208–1212.

NEUHAUS-STEINMETZ, U., GLAAB, T., DASER, A., BRAUN, A., LOMMATZSCH, M., HERZ, U., KIPS, J., ALARIE, Y. & RENZ, H. (2000). Sequential development of airway hyperresponsiveness and acute airway obstruction in a mouse model of allergic inflammation. *Int. Arch. Allergy Immunol.,* **121,** 57–67.

NIEBER, K., BAUMGARTEN, C.R., RATHSACK, R., FURKERT, J., OEHME, P. & KUNKEL, G. (1992). Substance *P* and beta-endorphin-like immunoreactivity in lavage fluids of subjects with and without allergic asthma. *J. Allergy Clin. Immunol.,* **90,** 646–652.

NOVIKOV, L.N., NOVIKOVA, L.N., HOLMBERG, P. & KELLERTH, J. (2000). Exogenous brain-derived neurotrophic factor regulates the synaptic composition of axonally lesioned and normal adult rat motoneurons. *Neuroscience,* **100,** 171–181.

PÄTH, G., BRAUN, A., MEENTS, N., KERZEL, S., QUARCOO, D., RAAP, U., HOYLE, G.W., NOCKHER, W.A. & RENZ, H. (2002). Augmentation of allergic early-phase reaction by nerve growth factor. *Am. J. Respir. Crit. Care Med.,* **166,** 818–826.

REMMERS, J.E., RICHTER, D.W., BALLANTYNE, D., BAINTON, C.R. & KLEIN, J.P. (1986). Reflex prolongation of stage I of expiration. *Pflugers Arch.,* **407,** 190–198.

SZALLASI, A. & BLUMBERG, P.M. (1999). Vanilloid (Capsaicin) receptors and mechanisms. *Pharmacol. Rev.,* **51,** 159–212.

SZAREK, J., SPURLOCK, B., GRUETTER, C.A. & LEMKE, S. (1998). Substance P and capsaicin release prostaglandin E2 from rat intrapulmonary bronchi. *Am. J. Physiol.,* **275,** L1006–L1012.

UNDEM, B.J., HUNTER, D.D., LIU, M., HAAK-FRENDSCHO, M., OAKRAGLY, A. & FISCHER, A. (1999). Allergen-induced sensory neuroplasticity in airways. *Int. Arch. Allergy Immunol.,* **118,** 150–153.

VIJAYARAGHAVAN, R., SCHAPER, M., THOMPSON, R., STOCK, M.F. & ALARIE, Y. (1993). Characteristic modifications of the breathing pattern of mice to evaluate the effects of airborne chemicals on the respiratory tract. *Arch. Toxicol.,* **67,** 478–490.

VIJAYARAGHAVAN, R., SCHAPER, M., THOMPSON, R., STOCK, M.F., BOYLSTEIN, L.A., LUO, J.E. & ALARIE, Y. (1994). Computer assisted recognition and quantitation of the effects of airborne chemicals acting at different areas of the respiratory tract in mice. *Arch. Toxicol.,* **68,** 490–499.

VIRCHOW, J.C., JULIUS, P., LOMMATZSCH, M., LUTTMANN, W., RENZ, H. & BRAUN, A. (1998). Neurotrophins are increased in bronchoalveolar lavage fluid after segmental allergen provocation. *Am. J. Respir. Crit. Care Med.,* **158,** 2002–2005.

WETMORE, C. & OLSON, L. (1995). Neuronal and nonneuronal expression of neurotrophins and their receptors in sensory and sympathetic ganglia suggest new intercellular trophic interactions. *J. Comp. Neurol.,* **353,** 143–159.

WILDER, J.A., COLLIE, D.D., WILSON, B.S., BICE, D.E., LYONS, C.R. & LIPSCOMB, M.F. (1999). Dissociation of airway hyperresponsiveness from immunoglobulin E and airway eosinophilia in a murine model of allergic asthma. *Am. J. Respir. Cell Mol. Biol.,* **20,** 1326–1334.

WILLS-KARP, M. (1999). Immunologic basis of antigen-induced airway hyperresponsiveness. *Annu. Rev. Immunol.,* **17,** 255–281.

WINSTON, J., TOMA, H., SHENOY, M. & PASRICHA, P.J. (2001). Nerve growth factor regulates VR-1 mRNA levels in cultures of adult dorsal root ganglion neurons. *Pain,* **89,** 181–186.

WINTER, J. (1998). Brain-derived neurotrophic factor, but not nerve growth factor, regulates capsaicin sensitivity of rat vagal ganglion neurones. *Neurosci. Lett.,* **241,** 21–24.

ZHOU, X.F., CHIE, E.T. & RUSH, R.A. (1998). Distribution of brain-derived neurotrophic factor in cranial and spinal ganglia. *Exp. Neurol.,* **149,** 237–242.

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