



# Neuroendocrine cells of nasal mucosa are a cellular source of brain-derived neurotrophic factor

L. Jornot\*, J.S. Lacroix# and T. Rochat\*

**ABSTRACT:** There is growing evidence for extensive interaction between sensory neurons, immune and mucosal epithelial cells during airway inflammation and hyperreactivity. This neuro-immune cross-talk (neurogenic inflammation) involves different groups of mediators, which include the neurotrophin family (nerve growth factor, brain-derived neurotrophic factor (BDNF) and neurotrophin-3 and -4). Neurotrophins modulate airway inflammation by enhancing sensory nerve excitability and production of neuropeptides, and by interaction with different immune cell types.

In the present study, it was questioned whether airway epithelial cells express BDNF, and if proinflammatory cytokines (tumour necrosis factor- $\alpha$ , interleukin-1 $\beta$  and interferon- $\gamma$ ) and a glucocorticoid (budesonide) affect this expression. Primary cultures of nasal epithelial cells were used.

It was found that BDNF was stored in chromogranin A-containing secretory granules of specialised epithelial cells, *i.e.* neuroendocrine cells, and was secreted in a polarised manner. Apical secretion appears to be constitutive, whereas basolateral secretion is markedly enhanced upon stimulation with cytokines. This enhanced basolateral secretion was not due to enhanced synthesis and was not affected by inhibitors of the processing enzymes, such as furin and the metalloproteinases involved in the maturation of BDNF, but was considerably diminished by budesonide.

Therefore, airway mucosa might contribute to neurogenic inflammation through increased secretion of brain-derived neurotrophic factor by neuroendocrine cells under inflammatory conditions.

**KEYWORDS:** Budesonide, cytokines, furin, metalloproteinases, neurogenic inflammation

The neurotrophins (NTs) are a family of structurally related peptides whose actions are essential for the survival, death and differentiation of neurons [1]. The most prominent members are nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and NT-3 and NT-4/5. NTs are synthesised from large precursor proteins of ~30–35 kDa that contain a signal sequence for secretion. These precursors are then proteolytically cleaved in trans-Golgi or secretory granules by pro-protein convertases, among which furin is the most abundant and efficient, or extracellularly by plasmin and metalloproteinases, in a highly conserved dibasic amino acid cleavage to yield the mature forms of ~12–14 kDa [2]. The whole mature proteins, containing six cysteine residues at identically spaced positions, are very well conserved in all mammalian species and ~50% of the amino acids are common to all NTs, indicating functional homology. The biologically active NTs are secreted as protein dimers associated by

noncovalent bonds. Central nervous system neurons release mature NGF and NT-3 *via* the constitutive secretory pathway, whereas mature BDNF is packaged in vesicles and primarily sorted into the regulated pathway [3].

The cellular sources of NTs under physiological conditions are primarily neurons and nerve-associated cells, such as glia cells, Schwann cells and fibroblasts. During inflammation, NTs are also produced by haematopoietic cells, and a variety of nonneuronal and nonimmune cell types, such as endothelial and epithelial cells [4]. In the normal human lung, constitutive expression of BDNF and NGF is found in the bronchial epithelial cells and gland cells, as well as in pulmonary lymphocytes and macrophages [5]. NT concentration is normally low in bronchoalveolar lavage fluid and in nasal fluids, but increases dramatically during inflammation in allergic patients [6–8]. Recently, an increase in BDNF expression has been demonstrated in nasal

## AFFILIATIONS

\*Division of Pulmonary Medicine, Dept of Internal Medicine, and #Rhinology, Olfactology Unit, Dept of Otorhinolaryngology/Head and Neck Surgery, Geneva University Hospitals and University of Geneva, Geneva, Switzerland.

## CORRESPONDENCE

L. Jornot  
Division of Pulmonary Medicine  
Dept of Internal Medicine  
Geneva University Hospitals  
Geneva  
Switzerland  
Fax: 41 223729909  
E-mail: Lan.H.Jornot@hcuge.ch

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## STATEMENT OF INTEREST

None declared.

mucosa of patients with allergic rhinitis after allergen provocation, which correlates with an increase of total nasal symptom score [9]. NTs exert a dual role in asthma pathogenesis [10]. In the nervous system, NTs enhance the number of tachykinin-producing nerve fibres surrounding the airways, sensitise C fibres to irritants and increase the synthesis and release of neuropeptides, such as the tachykinins, substance P and neurokinin A/B. These neuropeptides are involved in several key features of asthma, including airway smooth muscle constriction, vascular dilatation, increased vascular permeability and mucus hypersecretion [11]. In the immune system, NTs induce differentiation of B-lymphocytes, induce cytokine synthesis by T-cells, promote the release of various mediators by mast cells, and increase survival and activation of eosinophils.

The airway epithelium was considered for many years simply as a physical barrier involved in airway defence. Recent studies suggest that airway epithelial cells can interact with immune and neuronal cells by producing cytokines and chemokines [12] and NTs [4, 13]. However, to date, most studies concerning NTs have been performed in the human pulmonary epithelial cell line A549 and focused essentially on NGF [14, 15]. In previous work, the current authors have shown that primary human nasal epithelial cells expressed and released BDNF constitutively [16]. Therein, these cells were identified as neuroendocrine cells (NECs) and it was shown that tumour necrosis factor (TNF)- $\alpha$ , interleukin (IL)-1 $\beta$  and interferon (IFN)- $\gamma$ , cytokines believed to play an important role in airway hyperresponsiveness, enhanced the secretion of mature BDNF into the basolateral compartment, which was prevented by budesonide, a glucocorticoid that is often used to treat asthma and bronchial hyperreactivity.

## MATERIALS AND METHODS

### Reagents

TNF- $\alpha$ , IL-1 $\beta$ , IFN- $\gamma$  and budesonide were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Collagen (PureCol) was purchased from Nutacon (Leimuiden, the Netherlands). Rabbit anti-BDNF for Western blot (sc-8856) and rabbit anti-chromogranin A (ChrA) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse anti-BDNF for immunofluorescence (MAB648) was from RD Systems Europe (Abingdon, UK).

### Cell culture

Human airway epithelial cells were obtained from different patients after partial middle turbinectomies. Patients gave informed consent and the protocol has been approved by the institution's ethical committee (Geneva University Hospitals, Geneva, Switzerland). Cells were isolated by pronase (Roche, Mannheim, Germany) digestion and seeded onto collagen-coated Millicell polycarbonate filters (Millipore, Molsheim, France) as previously described [16]. The mucosal media was removed 24 h after plating and the cells are allowed to grow at the air-liquid interface. This allowed the cells to develop a morphological and functional phenotype that closely resembles the *in vivo* airway epithelium. The culture media consisted of a 1:1 mix of Dulbecco's modified Eagle's medium:F-12 (Invitrogen Life Technologies, Basel, Switzerland), 2% Ultrosor

G (Pall-Biosepra, Cergy-St-Christophe, France), 100 U·mL<sup>-1</sup> penicillin and 100  $\mu$ g·mL<sup>-1</sup> streptomycin.

### Experimental conditions

Cytokine treatment: after 3 weeks of culture, polarised cells were treated apically and basolaterally with 50 ng·mL<sup>-1</sup> of "cytomix" (a mixture of TNF- $\alpha$ , IL-1 $\beta$ , and IFN- $\gamma$ ) for 2 days. Budesonide treatment: cells were pretreated apically and basolaterally with 500 nM budesonide for 24 h prior to exposure to cytokines; budesonide was maintained throughout the 48-h treatment with cytokines.

### Western blot analysis

Secreted proteins in the apical and basolateral media were separated by SDS-PAGE (15% polyacrylamide gel), transferred to nitrocellulose filters (Hybond<sup>TM</sup> ECL<sup>TM</sup>; Amersham Biosciences Europe, Freiburg, Germany) and immunoblotted with rabbit anti-BDNF (1  $\mu$ g·mL<sup>-1</sup>). The signal was developed with the SuperSignal West Pico Substrate from Pierce (Rockford, IL, USA).

Prior to Western blot analysis, the apical and basolateral media were concentrated using the Amicon Centrifuge and Microcon centrifugal filter devices (Millipore, Billerica, MA, USA) with a molecular weight cut-off of 3,000 Da, according to the protocol of the manufacturer.

### Immunostaining

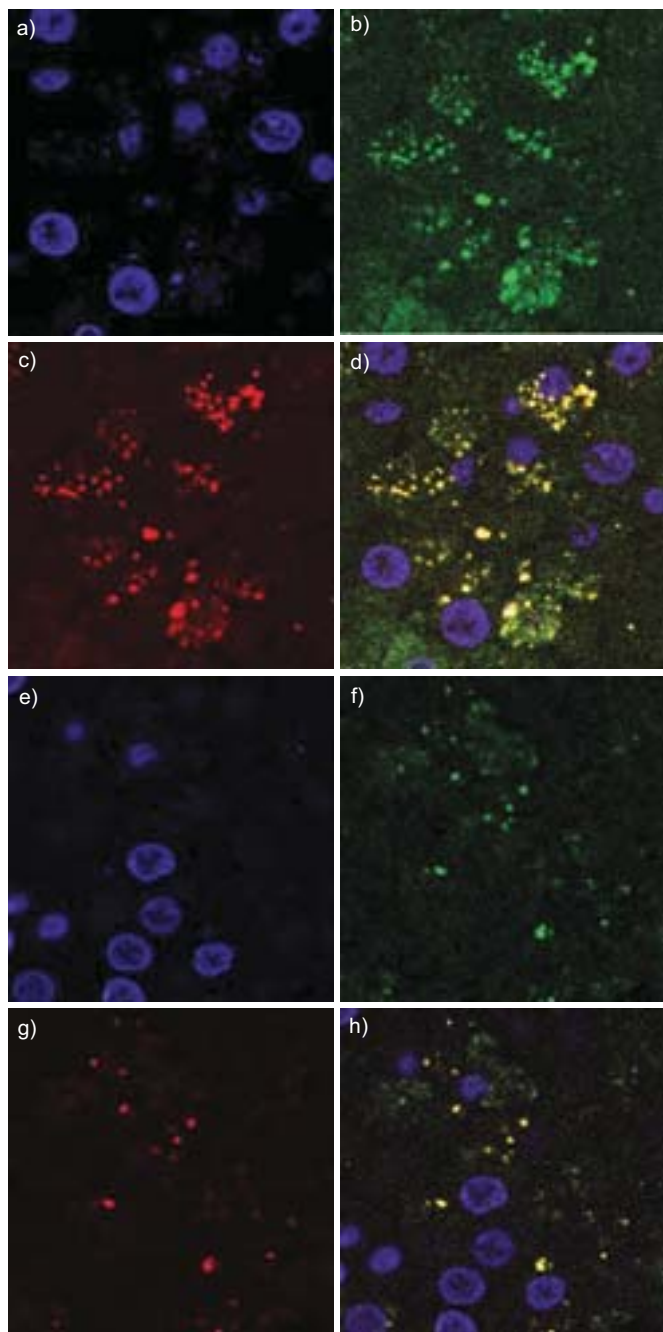
Epithelial cells on filters were fixed in 4% paraformaldehyde, permeabilised in 0.2% Triton-X100, and incubated for 3 h with mouse anti-BDNF and rabbit anti-ChrA antibodies diluted in PBS containing 1% bovine serum albumin and 0.2% Triton-X100. After several washes with PBS, the tissues were incubated again for 1 h with an appropriate secondary antibody. Filters were cut off from the culture inserts, mounted in Vectorshield-DAPI (4,6-diamidino-2-phenylindole; Vector Laboratories, Burlingame, CA, USA) between glass coverslips. Images were acquired on a Zeiss LSM-510 Meta confocal microscope utilising a Zeiss Axiovert 100 microscope equipped with an X63 oil immersion objective (Carl Zeiss, Jena, Germany). An argon laser excited fluorescein isothiocyanate (FITC) at 488 nm and rhodamine at 561 nm, and bandpass filters of 530 and 590 nm collected the signals from FITC and rhodamine, respectively. For each set of experiments, the gain on the confocal system was kept constant for all recordings in order to allow quantitative comparison. A total of 15–20 optical sections of the preparation were obtained using a z-axis step size of 0.35  $\mu$ m.

### Three-dimensional reconstruction of the confocal images by IMARIS

The three-dimensional reconstruction of the secretory vesicles was obtained using confocal laser scanning images using IMARIS software (Bitplane AG, Saint Paul, MN, USA).

### Statistical analysis

The significance of the difference between groups ( $p < 0.05$ ) was determined with the nonparametric Kruskal–Wallis test and the Wilcoxon signed rank test for paired samples. Results are given as box plots with the quartile, maximum and minimum values.



**FIGURE 1.** Immunocytochemical localisation of brain-derived neurotrophic factor (BDNF) and chromogranin A (ChrA) in untreated (a–d) and cytomix-treated (e–h) nasal epithelial cells. a) and e) Nuclei stained with 4,6-diamidino-2-phenylindole; b) and f) BDNF stained with fluorescein isothiocyanate; c) and g) ChrA stained with rhodamine; d) and h) merged images showing colocalisation of BDNF with ChrA. The images shown are from one representative of six consistent experiments.

## RESULTS

### **BDNF colocalises with ChrA in secretory granules**

In order to investigate the intracellular localisation of BDNF, cells were labelled with specific antibodies against BDNF and ChrA respectively, and analysed by indirect immunofluorescence microscopy. About one-half (56%) of the cultures

showed a significant amount (10–30%) of positively labelled cells. Figure 1 shows punctate staining of BDNF and ChrA in both untreated (fig. 1a–d) and cytomix treated cells (fig. 1e–h). In cells treated with cytomix for 48 h, a significant decrease in the punctate staining was observed. The identity of the subcellular storage compartment for BDNF is determined by colocalisation of BDNF with ChrA, an acidic sulphated glycoprotein present in dense core granules of the regulated secretory pathway of NECs. Figure 2 shows the three-dimensional reconstruction of the original confocal images using the IMARIS software (Bitplane AG). Analysis of BDNF/ChrA double-labelled cells revealed that almost all ChrA-immunopositive granules were also positive for BDNF. These observations indicate that in nasal mucosa BDNF is expressed in NECs and stored in dense core secretory granules.

### **Cytomix enhances basolateral secretion of BDNF**

The decreased BDNF staining in cytomix-treated cells is indicative of enhanced release of BDNF in the extracellular space. As shown in figure 3, in untreated cells, appreciable amount of the mature 12-kDa BDNF was secreted into the apical compartment, whereas a very low level of basal secretion was released in the basolateral compartment. A 48-h treatment with cytomix did not affect the apical secretion but significantly enhanced the release of BDNF into the basolateral compartment (48-fold increase). This enhanced basolateral secretion was not associated with increased mRNA level for BDNF, and treatment with inhibitors of furin and matrix metalloproteinases did not alter the increased release of BDNF (data not shown).

### **Budesonide attenuates cytokine-enhanced release of BDNF**

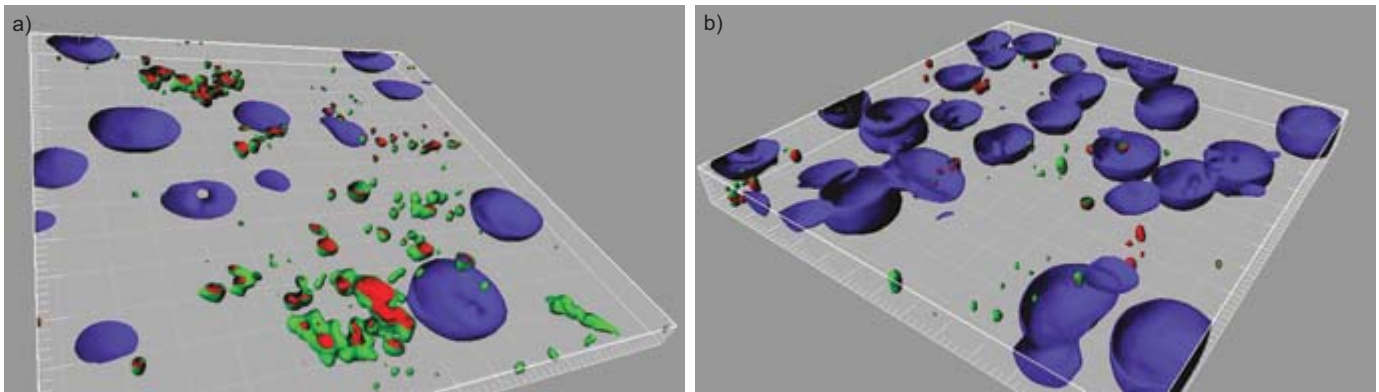
As corticosteroids are commonly used to decrease inflammation in the airways, it was investigated whether budesonide was capable of inhibiting BDNF secretion by nasal epithelial cells. As shown in figure 4, budesonide reduced cytomix-stimulated basolateral secretion of BDNF by ~50%, but the difference did not reach statistical significance ( $p=0.06$ ).

## DISCUSSION

In the present study, the presence of NECs has been indentified for the first time in primary cultures of epithelial cells derived from human nasal mucosa. Most importantly, it has been shown that these cells in culture: 1) constitutively expressed BDNF that was stored in secretory granules with typical endocrine-like characteristics, the so-called dense-cored vesicles; and 2) secreted BDNF in a polarised manner.

NECs are specialised epithelial cells distributed as single cells or in clusters called neuroepithelial bodies. They are very sparsely located in the nasal respiratory epithelium, laryngeal mucosa [17, 18] and throughout the entire respiratory tract, from the trachea to the terminal airways [19, 20]. So far, they are known to synthesise, store and secrete various neuropeptides such as vasoactive intestinal peptide, neurotensin, substance P and calcitonin gene-related peptide, and general neuroendocrine markers such as ChrA. These bioactive peptides, secreted into the stroma, play a crucial role in the regulation of immunological reactions in inflammatory conditions of the respiratory tract. The current authors report that NECs within the nasal mucosa store the NT BDNF in secretory granules, and release BDNF in a polarised manner. Indeed, BDNF release in the apical compartment appears to be constitutive, whereas the release in

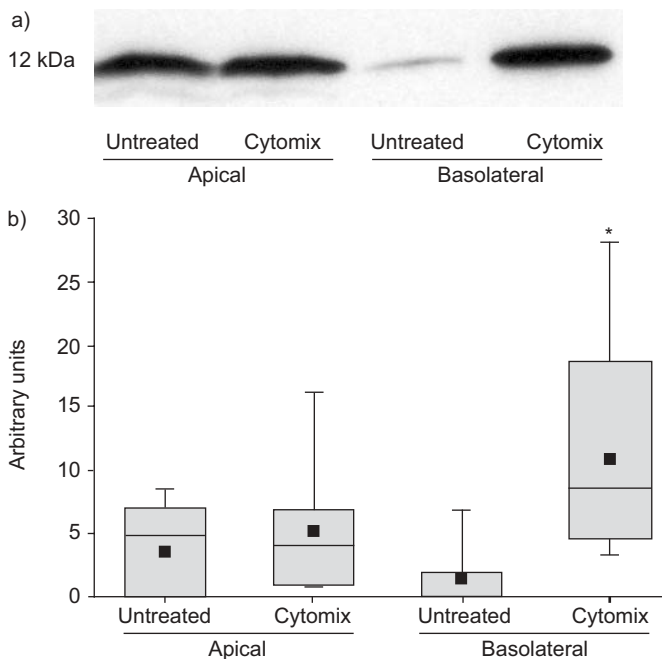




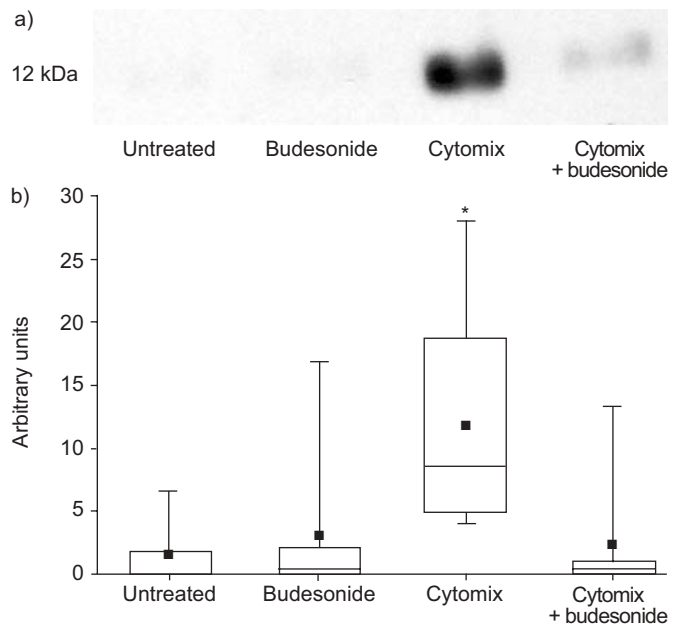
**FIGURE 2.** Three-dimensional reconstruction of the confocal images shown in figure 1 using the IMARIS software (Bitplane AG, Saint Paul, MN, USA). a) Untreated cells; b) cytomix-treated cells. Nuclei are stained in blue (4,6-diamidino-2-phenylindole), brain-derived neurotrophic factor is stained in green (fluorescein isothiocyanate) and chromogranin A is stained in red (rhodamine).

the basolateral compartment is regulated, since only the latter is significantly increased in response to proinflammatory cytokines and is, in great part, prevented by the glucocorticoid budesonide. To date, data concerning the polarity of NT secretion are not related to airway cells. Thus far, the vectorial secretion of NTs has been demonstrated only in the kidney epithelial cell line MDCK transfected with cDNA for NGF, BDNF and NT-3, and data are contradictory: in one study, all three NTs were predominantly (~75%) secreted basolaterally [21], and in the other study >90% of BDNF was secreted apically [22]. Since these aforementioned studies utilised transfected cell lines, it is possible that this

divergence might reflect differences in the expression levels of the transfected BDNF gene that may overload the transport machinery differently. Untreated NECs of the human nasal mucosa secreted BDNF mostly in the apical compartment. However, this must be taken with caution, since it cannot be completely discounted that this released amount derived from apoptotic and necrotic cells. Cytokine treatment did not affect the apical release but significantly increased the basolateral secretion of BDNF. This finding is of particular interest because only NT secretion towards the basement membrane is of biological significance, since in the airways the basal pole of neuroepithelial



**FIGURE 3.** Effects of cytokines on the secretion of brain-derived neurotrophic factor into the apical and basolateral compartments. a) Representative Western blot. b) Densitometric analysis of six immunoblots. Data are presented as box plots with the median, interquartile range, the mean (■), maximum and minimum values. \*:  $p < 0.05$ .



**FIGURE 4.** Effect of the glucocorticoid budesonide on the basolateral secretion of brain-derived neurotrophic factor in untreated and cytomix-treated cells. a) Representative Western blot. b) Densitometric analysis of six immunoblots. Data are presented as box plots with the median, interquartile range, the mean (■), maximum and minimum values. \*:  $p < 0.05$ .

bodies is contacted by several nerve fibre populations that are both sensory and motor [23]. It is established that NTs stimulate growth of sensory and sympathetic nerves, and induce expression and release of neuropeptides by these sensory nerves. These neuropeptides are known to elicit a multitude of effects on airway function which include mucus secretion, contraction of airway smooth muscle, vasodilation and plasma extravasation, stimulation of mast cells, macrophages, lymphocytes, eosinophils and neutrophils chemotaxis and vascular adhesion of neutrophils [11, 24]. Conversely, NTs are survival and activation factors for eosinophils after migration in the lung [13, 25].

The increase in basolateral BDNF release triggered by cytokines may result from enhanced synthesis and/or processing of pro-BDNF and/or release of BDNF. No increase in BDNF mRNA level was detected by real-time RT-PCR after cytokine treatment (data not shown). Additionally, cytokines did not affect the maturation step since inhibitors of furin and matrix metalloproteinases, enzymes known to process pro-BDNF to mature BDNF, did not alter the increase in basolateral secretion of BDNF induced by cytokines (data not shown). This clearly indicates that cytokines might have a direct effect on the secretory process. The mechanism responsible for this effect is unknown at the present time, and further studies are necessary to clarify this phenomenon.

The present study used differentiated nasal epithelial cells grown at an air-liquid interface with bioelectric and physiological function closely resembling native airway epithelium [26]. Although widely accepted, this cell culture model does not take into account interactions with other cells such as fibroblasts, immune cells and nerve fibres. It should be noted that the density of ChrA/BDNF immunoreactive NECs is not identical for all cell cultures examined: NECs were present in significant amounts (~10–30%) in more than one half (56%) of the cultures, and absent in the rest. This may relate to the airway pathological conditions of the patients, but no evidence was found for a correlation of the number of detected NECs and clinical scores for inflammation of nasal mucosa before surgery. Alternatively, the cell culture conditions used may lead to NEC enrichment in some cultures and absence in others, despite the strictly standardised procedure. The finding that secretion of BDNF by NECs is enhanced upon stimulation with proinflammatory cytokines is of particular interest because NTs have effects that are relevant to clinical airway inflammation. NEC proliferation and hyperplasia have been observed in many airway diseases associated with cigarette smoking [27]. Since these conditions are also associated with enhanced production and secretion of proinflammatory cytokines by locally recruited immune cells, cytokine-induced release of NTs represents a further amplification pathway of neuroimmune interaction.

In conclusion, neuroendocrine cells within the nasal mucosa are an important source of brain-derived neurotrophic factor, and thus might contribute to airway neurogenic inflammation. The present data extend previous knowledge on the effects of proinflammatory cytokines and corticosteroids on neurotrophins in other structural airway cell types, such as fibroblasts and smooth muscle cells [28–30]. Therefore, the concomitant use of corticosteroids and specific antagonists to neurotrophins might be helpful in the treatment of inflammation in airway diseases.

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