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Neurotrophin-4 dependency of intraepithelial vagal sensory nerve terminals that selectively contact pulmonary NEBs in mice

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Summary. Important physiological functions of neurotrophins (NTs) in airways and lungs are the early development, differentiation and maintenance of peripheral sensory neurons. The main pulmonary sensory innervation is of vagal origin, with several nerve fibre populations that selectively contact complex morphologically well-characterized receptor end-organs, called neuroepithelial bodies (NEBs). NEBs in mouse lungs are innervated by at least two separate myelinated vagal sensory nerve fibre populations, of which the neurochemical coding is suggestive of a mechanosensory function. Since neurotrophin-4 (NT-4) has been especially described to be important for the maintenance of mechanosensory nerve terminals, the present study aimed at investigating the NT-4 dependency of the two myelinated vagal sensory nerve fibre populations innervating mouse pulmonary NEBs.

Multiple immunostaining in 21-day-old and adult mouse lungs revealed the expression of the NT-4 receptor TrkB on the two different myelinated vagal sensory nerve fibre populations, i.e., the vesicular glutamate transporter/calbindin-positive and the $P2X_{2/3}$-positive fibres, which selectively contact pulmonary NEBs.

Examination of the effect of the lack of NT-4 on these NEB-related nerve fibre populations, by comparing adult NT-$4^{-/-}$ and wild-type mice, revealed that in NT-$4^{-/-}$ mice the percentage of NEBs contacted by $P2X_{2/3}$+ is reduced by 75%, while the VGLUT+/CB+ population seemed to be unaffected.

This study demonstrated that although mouse pulmonary NEBs are contacted by two distinct TrkB expressing populations of vagal myelinated afferents, only one is distinctly reduced in NT-4 deficient mice, suggesting the involvement of NTs. In view of the growing evidence for the involvement of NTs in neuronal plasticity associated with airway diseases, pulmonary NEBs innervated by NT-sensitive vagal afferents may play a significant role.

Key words: Neurotrophin-4, TrkB, Vagal sensory innervation, Pulmonary neuroepithelial bodies, Mouse

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Introduction

Neurotrophins (NTs) are a family of closely related proteins that present similarities in structure, receptor utility and physiological activities. Today, four members of this family have been characterised: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4(/5) (NT-4). The effects of neurotrophins depend upon their levels of availability, their affinity of binding to transmembrane receptors and the downstream signalling cascades that are stimulated after receptor activation [for review see (Chao et al., 2006)]. All members of the neurotrophin family use a common family of specific cell surface receptors, the 'high-affinity' tropomyosin-related tyrosine kinase (Trk) receptors (cognate binding preferences: TrkA/NGF; TrkB/BNDF and NT-4; TrkC/NT-3) (Bothwell, 1995), and can bind to the 'low-affinity' neurotrophin receptor $p75^{NTR}$ [for review see (Barbacid, 1994)].

In airways and lungs, evidence exists for major roles of neurotrophins on non-neuronal cells (Hikawa et al., 2002), especially in inflammatory lung diseases

(Nockher and Renz, 2003; Frossard et al., 2004). Nevertheless, important physiological functions of neurotrophins in the lung are the early development, differentiation and maintenance of peripheral sensory neurons (Nockher and Renz, 2003). The main sensory innervation in airways and lungs is vagal in origin, with cell bodies located in jugular-nodose ganglia (Dinh et al., 2004). Recent data suggest that mice possess a similar set of vagal airway sensors and pulmonary reflexes, as typically found in larger mammals (Zhang et al., 2006). Data on the possible neurotrophin dependence of vagal afferent nerve terminals in mouse lungs are, however, not available.

Pulmonary neuroepithelial bodies (NEBs) (Lauweryns et al., 1972) are complex sensory airway receptors (Widdicombe, 2001; Adriaensen et al., 2006), located in the epithelium of the intrapulmonary airways in air-breathing vertebrates. Our recent data obtained in mouse lungs (Brouns et al., 2009) pointed out that these organoid groups of neuroendocrine cells are contacted by several types of vagal sensory nerve terminals. Besides varicose calcitonin gene-related peptide (CGRP) and substance P (SP)-containing vagal sensory nerve terminals in close proximity to the basal pole of mouse NEBs, they are contacted by two different populations of myelinated vagal afferents. Both nerve fibre populations give rise to extensive arborisations of branching nerve terminals between the NEB cells. One is immunoreactive (ir) for vesicular glutamate transporters (VGLUTs) and calbindin D-28k (CB), while the other expresses $P2X_2$ and $P2X_3$ ATP receptors. The observation that NEBs are connected with myelinated vagal afferents expressing VGLUTs, calcium-binding proteins and $P2X_{2/3}$ receptors, suggests that vagal sensory terminals in mouse NEBs may account for the morphological counterparts of at least certain subpopulations of electrophysiologically identified vagal mechanosensors.

Literature data indicate that vagal mechanoreceptors in other organs (e.g. in the gastro-intestinal tract) are under the control of neurotrophins (Fox et al., 2001; Raab et al., 2003). In both NT-$3^{+/-}$ (Raab et al., 2003) and NT-$4^{-/-}$ (Fox et al., 2001) mice the number of intraganglionic laminar endings (IGLEs) was significantly reduced compared to wild-type mice. Quantification of neuronal cell bodies in vagal nodose ganglia of $NT3^{+/-}$ (Raab et al., 2003) and NT-$4^{-/-}$ (Conover et al., 1995; Liu et al., 1995; Fox et al., 2001) mice showed a reduction in the number of nodose neurons by 48% and 55%, respectively.

Homozygous NT-4 knock-out mice are attractive models to study sensory nerve fibre-related loss-of-function, since these mice are viable as adults. NT-4 appears to have major effects on developing (Conover et al., 1995; Liu et al., 1995) and mature (Davies et al., 1993; Wiklund and Ekström, 2000; Fox et al., 2001) vagal sensory neurons, both *in vivo* (Conover et al., 1995; Liu et al., 1995; Fox et al., 2001) and *in vitro* (Wiklund and Ekström, 2000).

Since NT-4 primarily acts via the TrkB receptor, it is not surprising that vagal sensory neuronal cell bodies in mice have been found to express TrkB (Wiklund and Ekström 2000). TrkB seems to be important for the survival and differentiation of peripheral mechano-receptors, since at least some populations of cutaneous rapidly and slowly adapting sensory receptors were reported to be dependent of TrkB (Perez-Pinera et al., 2008).

The present study aimed at investigating the presence of the NT-4 receptor TrkB on vagal sensory nerve terminals innervating mouse pulmonary NEBs, using multiple immunofluorescence staining. Antibodies against VGLUT1 and $P2X_3$ were used as selective markers for the two different myelinated vagal sensory nerve fibre populations that give rise to intraepithelial nerve terminals in NEBs, i.e., the VGLUT+/CB+ and the $P2X_{2/3}$+ population (Brouns et al., 2009). To study NT-4 dependency of the two myelinated vagal sensory nerve fibre populations, the percentage of NEBs contacted by a population of vagal sensory nerve terminals was compared in adult NT-$4^{-/-}$ and wild-type mice.

Materials and methods

Animals

Studies were performed on 21-day-old C57-Bl6 mice (Janvier, Bio Services, Uden, The Netherland), and in adult NT-4 knockout (NT-$4^{-/-}$; n=4) and congenic wild type mice (n=4) generated previously. Original breeding pairs of mice heterozygous for a null mutation in the NT-4 locus (Liu et al., 1995) were purchased from The Jackson Laboratory (Bar Habor, USA; stock number: 002497; 129/Sv-*Ntf5*^{tm1Jae}). Originally, the strain background of NT-4 mice was 129/SV. To create a pure C57-Bl6 background, homozygous males of the NT-4 mutant strain (NT-$4^{-/-}$) were primarily bred to C57-Bl6 females (The Jackson Laboratory; stock number: 000664) to get heterozygous NT-4 mutants (NT-$4^{+/-}$). From the fifth or older backcross generation, male and female NT-$4^{+/-}$ mice were bred to create NT-$4^{-/-}$ mutants on C57-Bl6 background. In the present study adult littermates (>3 month), consisting of wild type control mice (NT-$4^{+/+}$) and homozygous NT-4 mutants (NT-$4^{-/-}$), respectively, were used.

Genotype identification was performed on mouse tails. Tail genomic DNA was isolated by incubating mouse tail in lysis buffer (100mM EDTA; pH 8.0), 50mM Tris (pH 8.0), 1% SDS, 100mM NaCl and 16mg/ml proteinase K (Roth, Karlsruhe, Germany) for 2h at 55°C followed by isopropanol precipitation. Genotyping was carried out by polymerase chain reactions using a thermal cycler (Primus 96 thermal cycler, MWG-Biotech, Ebersberg, Germany). PCR amplified DNA was analysed on a 1.0% agarose gel stained with ethidium bromide and documented with Gel Doc 2000 (BioRad, München, Germany). Primers used for NT-4 genotyping (Cycling conditions: 94°C 30sec,

50°C 30sec, 72°C 1 min for 40 cycles) were oIMR0013 (5´-CTTGGGTGGAGAGGCTATTC-3´) and oIMR0014 (5´-AGGTGAGATGACAGGAGATC-3´), two neo generic primers amplifying a 280bp DNA fragment and oIMR0602 (5´-GTCAGTACTTCTTCGAGACGCG-3´) and oIMR0603 (5´-AGCTGTGTCGATCCGAATCC-3`), two wild type primers amplifying a 197bp DNA fragment (Fig. 1).

Animals were kept with their mothers (PD21) or in separate acrylic cages (adults max. 6/cage) with wood shavings, in an acclimatized room (12 h / 12 h light/dark cycle; 22± 3°C) and had free access to water and food pellets. Experiments were approved by the ethics committees of the Universities of Antwerp and Erlangen, and national and European principles of laboratory animal care were followed.

Tissue processing

Cryostat sections of whole mouse lungs

All mice were killed by an overdose of sodium pentobarbital (Nembutal 200 mg/kg; CEVA Santé Animale, Brussels, Belgium) containing heparin (500 U/kg; Rhône Poulenc Rorer 256S68F12; Brussels, Belgium). The pulmonary circulation was transcardially perfused with physiological solution and subsequently fixed for 15 min with 3% paraformaldehyde (in 0.1 M phosphate buffer, pH 7.4). Simultaneously, lungs were intratracheally instilled with 3% paraformaldehyde. The lungs were then dissected, degassed in a vacuum chamber, and immersion fixed for an additional 20 min. After rinsing in phosphate-buffered saline (PBS; 0.01 M, pH 7.4), lungs were stored overnight in 15% sucrose (in PBS; 4°C) and were subsequently mounted in Tissue Tek (Sakura Finetek Europe, Zoeterwoude, The Netherlands) on a cryostat chuck by freezing in a CO_2 chamber. Serial 25-μm-thick cryostat sections of the complete lung were thaw-mounted on poly-L-lysine-coated microscope slides, dried at 37°C (2h), and either processed for immunolabelling or stored at -80°C in a closed container until further use.

Immunohistochemistry

All incubations were carried out at room temperature in a humid incubation chamber. Characteristics and sources of antibodies and streptavidin-complexes used for immunohistochemistry are listed in Tables 1 and 2. The combinations of primary antisera and visualisation methods used for multiple immunohistochemical labelling are listed in Table 3. Single immunostaining was carried out as outlined in the first step of the multiple staining procedures. Unless indicated otherwise, all antisera were diluted in PBS containing 10% non-immune serum of the host species of the secondary antibodies, 0.1% bovine serum albumin, 0.05% thimerosal, and 0.01% NaN3 (PBS*). Prior to incubation with the primary antisera, cryostat sections were incubated for 30 min with PBS* containing 1% Triton X-100. After a final wash, all sections were mounted in citifluor (Ted Pella 19470, Redding, CA,

Table 1. List of primary antisera used for immunohistochemistry.

Primary antisera, Antigen	Host	Mc/Pc	Source
Calcitonin gene-related peptide (CGRP)	Rabbit	Pc	Affiniti CA1134, Exeter, UK
CGRP	Rabbit	Pc	Sigma C8198, Bornem, Belgium
$P2X_3$ receptor ($P2X_3$)	Rabbit	Pc	Gift from Roche Bioscience, Palo Alto, CA
Protein gene product 9.5 (PGP9.5)	Rabbit	Pc	Chemicon AB1761
PGP9.5	Rabbit	Pc	Biogenesis 7863-0504
Tropomyosin-related tyrosine kinase B (TrkB)	Rabbit	Pc	Santa Cruz Biotechnology inc 794 sc-12, Santa Cruz, CA
Vesicular Glutamate Transporter 1 (VGLUT1)	Guinea Pig	Pc	Chemicon AB5905

Table 2. List of secondary antisera and streptavidin complexes used for immunohistochemistry.

Secondary antisera, streptavidin complexes	Source	Dilution
Biotinylated donkey anti-guinea pig immunoglobulins (IgG) (DAGP-BIOT)	Jackson ImmunoResearch 706-065-148, West Grove, PA, USA	1:500
Biotinylated Fab fragments of goat anti-rabbit IgG (GAR-Fab-BIOT)	Rockland 811-1602, Gilbertsville, PA, USA	1:500
Cy™3- conjugated Fab Fragments of goat anti-rabbit IgG (GAR-Fab-Cy3)	Jackson ImmunoResearch 111-167-003	1:2000
Cy™3- conjugated Streptavidin (STR-Cy3)	Jackson ImmunoResearch 016-160-084	1:6000
Cy™5-conjugated Fab Fragments of goat anti-rabbit IgG (GAR-Fab-Cy5)	Jackson ImmunoResearch 111-177-003	1:500
ExtrAvidin™ horseradish peroxidase	Sigma E2886	1:1000
Fluorescein (FITC)-conjugated Steptavidin (STR-FITC)	Jackson ImmunoResearch 016-090-084	1:1000
FITC-conjugated Fab Fragments of goat anti-rabbit IgG (GAR-Fab-FITC)	Jackson ImmunoResearch 111-097-003	1:100
Unconjugated Fab fragments of goat anti-rabbit IgG (GAR-Fab)	Jackson ImmunoResearch 111-007-003	1:50

USA).

Conventional immunohistochemical double labeling

For double labeling of TrkB and PGP9.5, two primary antisera raised in rabbit, the binding sites of the antibody raised against TrkB were blocked with GAR-Fab-Cy3 (6h incubation) and additionally with unlabelled GAR-Fab (2h incubation) (Fab blocking; Negoescu et al., 1994; Brouns et al., 2002). The second primary antibody raised against PGP9.5, was detected in a conventional way using GAR-Fab-FITC.

Multiple immunohistochemical staining using tyramide signal amplification (TSA)

To enhance sensitivity and to allow the straightforward combination of two antisera raised in rabbits, biotin-conjugated tyramide signal amplification (TSA kit NEL700; PerkinElmer LAS, Zaventem, Belgium) was applied. Between subsequent steps, sections were rinsed in PBS containing 0.05% Tween 20. Prior to the immunohistochemical procedures, endogenous peroxidase activity in cryostat sections of mouse lungs was blocked by H_2O_2 (3% in 50% methanol in PBS; 15 min), followed by preincubation with PBS* and overnight incubation with the first primary antibody (see Tables 1, 3). Sections were then consecutively incubated with the appropriate biotinylated secondary antibodies (see Table 2), ExtrAvidin-horseradish peroxidase (in PBS; 1 h), biotin-conjugated tyramide (diluted 1:100 in 'amplification solution'; 12 min) and fluorochrome-conjugated streptavidin (12 min). In double immunohistochemical stainings, the second primary antiserum was detected using a conventional secondary antibody. For the simultaneous detection of three antigens, the first primary antiserum was detected using TSA, while the second and third primary antisera were detected by Fab blocking and conventional immunohistochemical labelling. TSA-enhanced immunostaining for the ATP receptor $P2X_3$ was performed as previously described (Brouns et al., 2000).

Control experiments for the immunohistochemical procedures

Negative staining controls for all immunohistochemical procedures were performed by substitution of primary or secondary antisera for non-immune sera. Controls for the amplification-based multiple staining were performed by omission of the primary antiserum of the second and third incubations. To check for possible cross-reactivity after consecutive multiple staining when using two or three rabbit primary antisera, the results of single immunostaining for the different antisera were evaluated and compared with those from multiple labelling experiments.

Microscopy and data analysis

An epifluorescence microscope (Zeiss Axiophot) equipped with filters for the visualisation of FITC (Zeiss 17; BP 485-20/ FT 510/ BP 515-565) and Cy3 (Zeiss 14; LP 510-KP 560/ FT 580/ LP 590) was used to quickly screen the results and to perform quantitative analysis.

All detailed images included in this work were obtained using a microlens-enhanced dual spinning disk confocal microscope (Ultra*VIEW* ERS, PerkinElmer) equipped with an argon-krypton laser source with three excitation lines (488, 568 and 647 nm) for excitation of FITC, Cy3 and Cy5, respectively. All presented images

Table 3. Multiple stainings

Double labelling using Fab blocking and conventional staining

Primary antisera antigen 1	Dilution	Visualisation	Primary antisera antigen 2 (Fab-blocking)	Dilution	Visualisation
TrkB	1:100	GAR-Fab-Cy3	PGP9.5	1:500	GAR-Fab-FITC

Double staining with one tyramide signal amplification (TSA)

Primary antisera antigen 1 (TSA)	Dilution	Visualisation	Primary antisera antigen 2 (conv.)	Dilution	Visualisation
$P2X_3$	1:1000	STR-Cy3	CGRP	1:2000	GAR-Fab-FITC
VGLUT1	1:10000	STR-FITC	CGRP	1:1000	GAR-Fab-Cy3

Triple labelling using TSA, Fab blocking and conventional staining

Primary antisera antigen 1 (TSA)	Dilution	Visualisation	Primary antisera antigen 2 (Fab blocking)	Dilution	Visualisation	Primary antisera antigen 3 (Conv.)	Dilution	Visualisation
$P2X_3$	1:1000	STR-FITC	TrkB	1:100	GAR-Fab-Cy3	PGP9.5	1:500	GAR-Fab-Cy5
VGLUT1	1:10000	STR-FITC	TrkB	1:100	GAR-Fab-Cy3	PGP9.5	1:500	GAR-Fab-Cy5

(Figs. 2-7) are maximum intensity projections of confocal optical sections. Images were processed using the reconstruction facilities of Volocity 3.5 software (Improvision, Coventry, United Kingdom) and Adobe Photoshop 7.0.

For comparative quantitative analysis, NEBs that are selectively contacted by VGLUT1 or $P2X_3$-ir vagal sensory nerve terminals were counted in adult NT-$4^{-/-}$ (n=4; n=2 for each subpopulation) and control mice (n=4; n=2 for each subpopulation). One out of five of all serial sections of each lung was double immunostained for either VGLUT1/CGRP or $P2X_3$/CGRP. Whole sections were screened, and the percentage of NEBs contacted by the selected nerve fibre population was calculated using CGRP immunoreactivity (IR) as the marker for all counted NEBs (Brouns et al., 2009). For either VGLUT1/CGRP or $P2X_3$/CGRP stainings, the average percentage of NEBs contacted by the selected nerve fibre population was compared between wild-type and NT-$4^{-/-}$ mice. In each group at least 450 NEBs were counted. The percentages were analyzed by an unpaired Student's t test and a probability of $p<0.05$ was set as the level of significance.

Results

TrkB immunostaining labels intraepithelial nerve terminals in neuroepithelial bodies

In both 21-day-old and adult wild-type mice, TrkB was detected in nerve endings that formed terminal arborisations at distinct locations in the epithelium of intrapulmonary airways (Fig. 2a). TrkB-ir nerve fibres could be observed to approach the airway epithelium, branch, and protrude between the epithelial cells, giving rise to extensive intraepithelial nerve endings. The staining intensity of the approaching nerve fibres and of intraepithelial terminals varied from fibre to fibre.

Subsequent labelling of lung sections that were processed for TrkB localisation, with antibodies against protein gene-product 9.5 (PGP9.5; a general neuronal and neuroendocrine marker), revealed that the intraepithelial TrkB-ir nerve endings always coincided with the presence of a NEB (Figs. 2b, 3). No TrkB IR was observed in NEB cells.

TrkB is expressed in both of the myelinated vagal sensory nerve fibre populations that contact pulmonary neuroepithelial bodies

When VGLUT1 was used as a marker for the myelinated VGLUT+/CB+ vagal sensory nerve fibre population that forms intraepithelial laminar terminals between mouse NEB-cells (Brouns et al., 2009), triple immunohistochemical staining showed that the intraepithelial VGLUT1-ir nerve terminals in PGP9.5-ir NEBs also express TrkB in 21-day-old (Fig. 4a-c) and adult (Fig. 5a-c) mice. All intraepithelial VGLUT1/TrkB-ir nerve terminals appeared to correspond with the presence of NEBs (Figs. 4c, 5c).

Multiple immunohistochemical staining for $P2X_3$ receptor and TrkB (Figs. 6a-c; 7a-c) showed that the myelinated vagal sensory $P2X_{2/3}$-ir nerve fibre population with terminals between pulmonary NEB cells (Brouns et al., 2009) also expresses TrkB receptors in 21-day-old (Fig. 6a-c) and adult (Fig. 7a-c) mice. In some NEBs, both $P2X_3$+/TrkB+ and $P2X_3$-/TrkB+ terminals could be observed in separate parts of the cluster of NEB cells (Fig. 6a-c).

Comparative quantitative analysis of both populations of myelinated vagal sensory nerve terminals that contact NEBs in NT-4 knock-out and wild-type mice

The effect of the lack of NT-4 on the VGLUT+/CB+ and $P2X_{2/3}$+ nerve fibre populations in contact with NEBs was studied by comparing wild-type and NT-$4^{-/-}$ mice.

Double immunohistochemical staining for VGLUT1 and CGRP revealed no significant difference (p=0.222) in the percentage of NEBs selectively innervated by VGLUT1-ir intraepithelial nerve terminals between wild-type (about 11% of the counted NEBs) and $NT4^{-/-}$ mice (about 9% of the counted NEBs) (Fig. 8). Lung sections double stained for $P2X_3$ receptor and CGRP, on the other hand, showed that the percentage of NEBs that received a $P2X_3$-ir innervation in adult wild-type mouse lungs (about 12% of the counted NEBs) was significantly reduced (p=0.015) by about 75% in $NT4^{-/-}$ mice (about 3% of the counted NEBs) (Fig. 9).

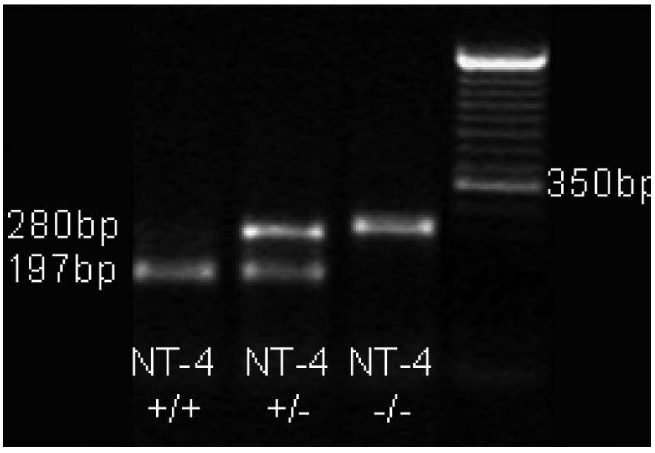
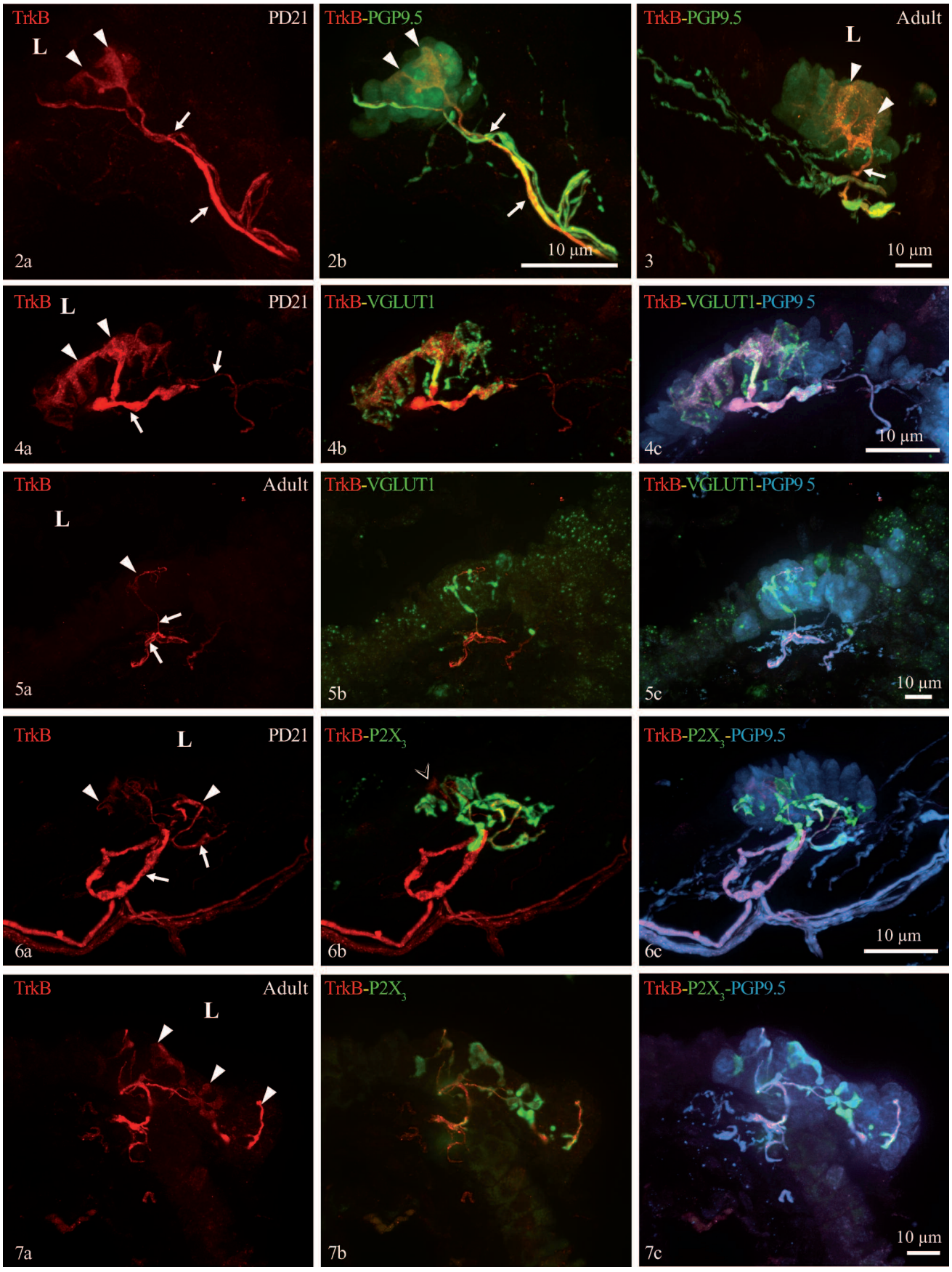


Fig. 1. Polymerase chain reaction (PCR) of NT-4 wild-type (NT-$4^{+/+}$), heterozygous (NT-$4^{+/-}$) and homozygous (NT-$4^{-/-}$) knock-out mice. In wild-type mice, only the 197-bp product from the wild type allel was generated (lane 1). NT-4 knock-out mice yielded the 280-bp product from the targeted allel (lane 3), while heterozygous mice express products from both the wild-type and targeted allel (lane 2). The two brighter bands of the 50-bp DNA ladder consist of an 800-bp fragment (at the top) and a 350-bp fragment (in between the ladder bands; lane 4).


TrkB
PD21
L
2a
TrkB-PGP9.5
10 µm
2b
TrkB-PGP9.5
Adult
L
10 µm
3
TrkB
L
PD21
4a
TrkB-VGLUT1
4b
TrkB-VGLUT1-PGP9 5
10 µm
4c
TrkB
Adult
L
5a
TrkB-VGLUT1
5b
TrkB-VGLUT1-PGP9 5
10 µm
5c
TrkB
L
PD21
6a
TrkB-P2X3
6b
TrkB-P2X3-PGP9.5
10 µm
6c
TrkB
Adult
L
7a
TrkB-P2X3
7b
TrkB-P2X3-PGP9.5
10 µm
7c

Control experiments

Omission of the primary or secondary antibodies did result in negative staining in all of the immunohistochemical procedures performed. The staining pattern in single labelled sections showed no obvious differences with that observed after multiple labelling for the

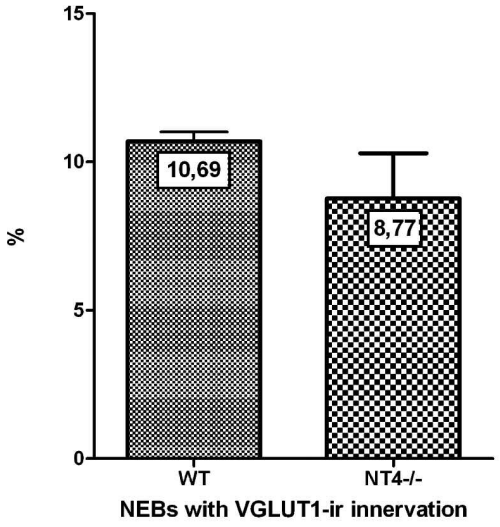


Fig. 8. Comparison of the VGLUT1-ir vagal sensory innervation of NEBs in adult NT-4 knock-out (n=2) and control (n=2) mouse lungs after double immunostaining for VGLUT1 and CGRP. The total number of NEBs in the lung sections was quantified using CGRP as a marker for NEBs. The given percentages indicate that also in control lungs only a subpopulation of the CGRP-ir NEBs is innervated by VGLUT1-ir terminals. Our data suggest that the lack of NT-4 does not significantly alter the percentage of NEBs contacted by VGLUT1-ir nerve terminals.

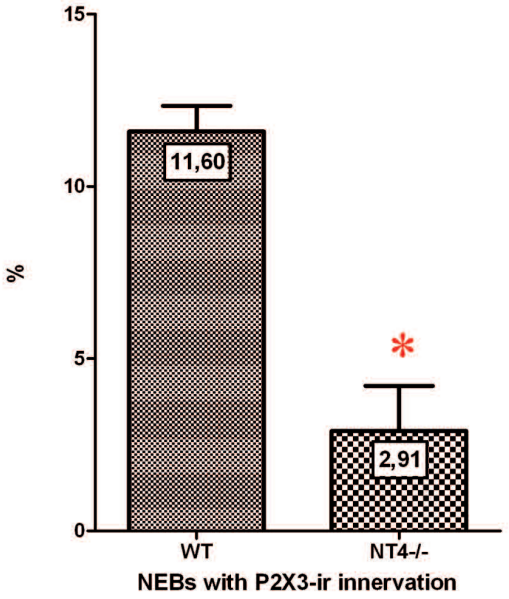


Fig. 9. Quantification of the percentage of NEBs selectively contacted by $P2X_3$-ir vagal sensory nerve terminals in adult control (n=2) and NT-4 knock-out (n=2) mice, in lung sections double stained for $P2X_3$ and CGRP. Percentages represent the fraction of NEBs innervated by $P2X_3$-ir nerve terminals. In NT-4 KO animals the percentage of NEBs receiving the $P2X_3$ terminals appears to be significantly decreased (asterisk)

Fig. 2. Double immunohistochemical staining of a mouse (PD21) bronchiole for the NT-4 receptor TrkB (red Cy3 fluorescence) and for PGP9.5 (green FITC fluorescence). **a.** TrkB-ir nerve fibres (arrows) approach the epithelium, branch and form laminar terminals (arrowheads) that seem to completely surround a small group of epithelial cells. **b.** Combination of the red and green channels shows that the TrkB-ir nerve terminals innervate a PGP9.5-ir NEB. L: lumen of the airway.

Fig. 3. Bronchiole of an adult mouse double immunostained for TrkB (red Cy3 fluorescence) and PGP9.5 (green FITC fluorescence). A TrkB-ir nerve fibre approaches the PGP9.5-ir NEB and forms laminar intraepithelial nerve terminals (arrowheads). L: lumen of the airway.

Fig. 4. Immunohistochemical triple staining for TrkB (red Cy3 fluorescence), VGLUT1 (green FITC fluorescence) and PGP9.5 (artificial blue colour of Cy5 fluorescence) in 21-day-old mouse lungs. **a.** Intraepithelial TrkB-ir nerve terminals (arrowheads) originating from TrkB-ir nerve fibres (arrows) with different fluorescence intensities. **b.** Combination of the red and green channels shows that VGLUT1-ir nerve terminals express TrkB. **c.** Combination of the red, green and blue channels reveals that the intraepithelial terminals contact a PGP9.5-ir NEB. L: lumen of the airway.

Fig. 5. Adult mouse lung immunostained for TrkB (red Cy3 fluorescence), VGLUT1 (green FITC fluorescence) and PGP9.5 (artificial blue colour of Cy5 fluorescence). Intraepithelial nerve terminals immunoreactive for both TrkB (**a**; arrowhead) and VGLUT1 (**b**) innervate PGP9.5-ir NEB cells (**c**). In (**a**) it is clear that the TrkB-ir nerve fibre (**a**; arrows) gives rise to the TrkB-ir intraepithelial nerve terminals. L: lumen of the airway

Fig. 6. Mouse PD21. Immunohistochemical triple staining of a bronchiole for TrkB (red Cy3 fluorescence), $P2X_3$ receptor (green FITC fluorescence) and PGP9.5 (artificial blue colour of Cy5 fluorescence). **a.** TrkB-ir nerve fibres (arrows) give rise to an intraepithelial receptor-like complex (arrowheads). The intensity of TrkB immunostaining differs from fibre to fibre **b.** Combination of the red and green channels shows that the intraepithelial $P2X_3$ receptor-stained nerve terminals express TrkB receptors. Note that not all TrkB-ir nerve terminals (open arrowhead) express $P2X_3$ receptors. **c.** Combination of the red, green and blue channel showing an extensively innervated PGP9.5-ir NEB. L: lumen of the airway.

Fig. 7. Adult mouse lung triple stained for TrkB (red Cy3 fluorescence), $P2X_3$ receptor (green FITC fluorescence) and PGP9.5 (artificial blue colour of Cy5 fluorescence). A PGP9.5-ir NEB **(c)** is contacted by an extensive complex of TrkB-ir intraepithelial nerve terminals (arrowheads in **a**), which also show $P2X_3$ receptor IR (**b**). L: lumen of the airway.

antigens studied. In multiple immunolabellings using TSA-enhanced and subsequent conventional staining, omission of the primary antiserum of the second or third incubation abolished all staining in the second or third step.

Discussion

For localisation of the NT-4 receptor TrkB, we applied the same antibody as has been used to demonstrate TrkB receptors in lungs of BALB/c mice (Nassenstein et al., 2006). While Nassenstein and co-workers (2006) focussed on the overall distribution of Trk receptors, and showed TrkB expression in large bundles of nerve fibres and on terminal 'subepithelial varicosities', our study visualised additional TrkB IR in intraepithelial nerve endings in mouse airways. Subepithelial TrkB-ir nerve fibres approached the epithelium, branched, and formed extensive local intraepithelial TrkB-ir nerve endings that appeared to invariably coincide with the presence of pulmonary NEBs. Given that all known intraepithelial nerve terminals in mouse NEBs have been reported to originate from myelinated vagal sensory nerve fibres (Brouns et al., 2009), TrkB was likely expressed by the latter nerve fibres.

Myelinated vagal afferents innervating mouse pulmonary NEBs can be divided in two different nerve fibre populations that give rise to intraepithelial terminals: (1) expressing VGLUTs and CB, and (2) expressing the ATP receptors $P2X_2$ and $P2X_3$ (Brouns et al., 2009). The possibility that one or both of these populations express TrkB was, therefore, taken into account. Multiple immunohistochemical staining revealed that both the VGLUT+/CB+ and $P2X_{2/3}$+ nerve terminals in NEBs express TrkB, and thus, theoretically, both might be NT-4 dependent.

NT-$4^{-/-}$ mice show a remarkable loss of vagal sensory neuronal cell bodies (Conover et al., 1995; Liu et al., 1995; Fox et al., 2001), and vagal receptor terminals in the gastro-intestinal tract have been reported to disappear in NT-$4^{-/-}$ mice (Fox et al., 2001). In the present study, quantitative analysis showed a loss of vagal sensory nerve terminals in contact with NEBs in NT-$4^{-/-}$ mice compared to wild-types.

Counting NEBs in lung cryostat sections double immunostained for $P2X_3$ and CGRP, revealed that the percentage of pulmonary NEBs receiving $P2X_{2/3}$+ fibres is significantly decreased by 75% in NT-$4^{-/-}$ mice, suggesting that this vagal sensory nerve fibre population is strongly dependent on the presence of NT-4. The percentage of NEBs innervated by VGLUT1-ir terminals, on the other hand, did not drastically alter between NT-$4^{-/-}$ and wild-type mice, indicating that the VGLUT+/CB+ nerve fibre population in contact with NEBs is largely independent of NT-4. In this way, the two different myelinated vagal sensory nerve terminals reported in mouse pulmonary NEBs (Brouns et al., 2009) seem to be differentially supported by NT-4 for their survival. This observation is similar to findings in the gastrointestinal tract, where in NT-$4^{-/-}$ mice similar vagal receptor terminals (i.e., IGLEs) disappeared in small intestine, but remained in the stomach (Fox et al., 2001). It has been proposed that gastric and intestinal IGLEs may represent different receptor phenotypes with specific properties that influence their survival in the absence of NT-4 (Fox et al., 2001).

In mice, the TrkB receptor is expressed by the majority of vagal nodose neurons, as shown by immunohistochemistry (Wiklund and Ekström, 2000). Null mutations in genes encoding TrkB result in the disappearance of large numbers of nodose neurons (Silos-Santiago et al., 1997). Since TrkB is the cognate receptor for NT-4, it is not surprising that NT-4 was found to support cultured developing nodose ganglion neurons (Davies et al., 1993), and induce neurite outgrowth from cultured nodose ganglia (Wiklund and Ekström, 2000). On the other hand, TrkB is also the cognate receptor for BDNF, and it has been shown that NT-3 also may be able to activate TrkB (Rydén and Ibáñez, 1996), although less efficiently (Kirstein and Fariñas, 2002). Nodose neurons seem to be heterogeneous with respect to their responses to TrkB ligands, and BDNF and NT-4 act in a complementary nonredundant fashion on discrete populations of nodose neurons (Liu et al., 1995). The VGLUT+/CB+ vagal sensory nerve fibre population in contact with NEBs may, therefore, be dependent on additional neurotrophins during development.

In NT-$4^{-/-}$ mice, Liu and co-workers (1995) tried to correlate the loss of nodose neurons to particular phenotypic subpopulations. Immunostaining with antibodies against CGRP, NPY, SP, TH, SOM or TrkB did, however, not reveal obvious differences between the mutant and control mice (Liu et al., 1995). Our observation that the $P2X_{2/3}$+ myelinated vagal sensory nerve terminals innervating NEBs are highly reduced in NT-$4^{-/-}$ mice, suggests that at least a subpopulation of the disappeared neurons belongs to the $P2X_{2/3}$ receptor-ir nodose population (Zhong et al., 2001).

Striking morphological similarities exists between taste buds and pulmonary NEBs, both being highly innervated organoid groups of neurosecretory epithelial cells. In taste buds, TrkB receptors have been identified in about 80% of taste bud cells, among others the Type III receptor cells that make afferent synaptic contacts with nerve terminals (Takeda et al., 2005). This results in a reduced number of fungiform papillae at birth in NT-4 KO mice (Liebl et al., 1999). Since no TrkB immunostaining was detected in any of the pulmonary neuroendocrine cells in NEB corpuscles in the present study, it may be suggested that, in contrast to taste buds, the lack of NT-4 possibly will not have a direct effect on the survival of NEBs.

It is generally agreed that NEBs are not dependent on their vagal innervation for survival (Sonstegard et al., 1979). In mice, both our former unilateral infranodosal vagal denervation studies (Brouns et al., 2009) and the

lack of the vagal sensory $P2X_{2/3}$ population in adult $NT4^{-/-}$ mice in the present study, suggest that removal of the vagal innervation is not critical for lung function in the healthy animal. It can, however, not be excluded that the vagal sensory $P2X_{2/3}$-ir NEB innervation may be important in changed environmental conditions or during disease.

In conclusion, the present study demonstrated that NEBs in mouse lungs are innervated by at least two separate TrkB-expressing myelinated vagal sensory nerve fibre populations, one that is drastically reduced in NT-4 deficient mice, and one that seems to be unaffected by the lack of NT-4. Growing evidence for the involvement of NTs in the neuronal plasticity associated with inflammatory airway diseases (Nassenstein et al., 2006), suggests that pulmonary NEBs innervated by NT-sensitive vagal afferents may play a significant role.

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