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Inge Brouns
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Novel Insights in the Neurochemistry and Function of Pulmonary Sensory Receptors

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With 24 figures

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Dedicated to emeritus
Prof. Dr. Dr. h.c. Dietrich W. Scheuermann, former Chair of Histology and Microscopic Anatomy, and Dean of the Faculty of Medicine at the University of Antwerp. He was one of the pioneers of pulmonary neuroepithelial body research and a mentor of many young scientists in the field.

Abstract

Afferent nerves in the airways and lungs contribute to optimisation of the breathing pattern, by providing local pulmonary information to the central nervous system. Airway sensory nerve terminals are consequently tailored to detect changes readily in the physical and chemical environment, thereby leading to a variety of respiratory sensations and reflex responses.

Most intrapulmonary nerve terminals arise from fibres travelling in the vagal nerve, allowing a classification of "sensory airway receptors", based on their electrophysiologically registered action potential characteristics. Nowadays, at least six subtypes of electrophysiologically characterised vagal sensory airway receptors have been described, including the classical slowly and rapidly adapting (stretch) receptors and C-fibre receptors. The architecture of airways and lungs makes it, however, almost impossible to locate functionally the exact nerve terminals that are responsible for transduction of a particular intrapulmonary stimulus.

With the advances in immunohistochemistry in combination with confocal microscopy, airway sensory receptor end organs can now be examined and evaluated objectively. Based on their "neurochemical coding", morphology, location and origin, three sensory receptor end organs are currently morphologically well characterised: smooth muscle-associated airway receptors (SMARs), neuroepithelial bodies (NEBs) and visceral pleura receptors (VPRs). The present information on the functional, morphological and neurochemical characteristics of these sensory receptors leads to important conclusions about their (possible) function.

Currently, *ex vivo* lung models are developed that allow the selective visualisation of SMARs, NEBs and VPRs by vital staining. The described *ex vivo* models will certainly facilitate direct physiological studies of the morphologically and neurochemically identified airway receptors, thereby linking morphology to physiology by identifying *in situ* functional properties of a given receptor end organ.

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Contents

Chapter 1
Sensory Nerve Terminals in Intrapulmonary Airways and Lungs

1.1
Airway Sensory Nerves and Breathing

The generation and control of breathing patterns are governed by a multitude of complex neuronal interactions. In its most basic form, the breathing pattern of mammals is produced in the rhythmic breathing centres in the medulla of the brainstem. A permanent fine-tuning by the respiratory centres in the pons is responsible for the final output to the respiratory muscles. In addition, peripheral arterial chemoreceptors – the carotid and aortic bodies – and central respiratory chemoreceptors are crucial for the maintenance of cardiorespiratory homeostasis. Inputs from these receptors ensure adaptive changes in the respiratory and cardiovascular motor outputs under various environmental and physiological conditions.

In order to reach the optimal breathing pattern in any situation, the input of local information from the airways and lungs to the central nervous system (CNS) appears to be necessary (von Euler 1997; Ganong 1999; reviews see Duffin 2004; Canning and Spina 2009). The large-surfaced structure of the pulmonary system, which is in extensive contact with the environmental air, is equipped with sensory nerve terminals that are tailored to detect various elements of the physical and chemical environment of the airways, and to transmit this information to the CNS. In this way, a multitude of stimuli have been characterised that can cause a variety of respiratory "sensations", such as pain/ache, irritation, tightness, urge-to-cough, air-hunger, sense of effort, sense of lung volume/air flow, and temperature sense (for review, see Widdicombe 2009), yielding multiple reflex responses. Input of diverse populations of airway afferents in respiratory centres will lead to changes in the rhythmic breathing pattern and initiate body defence responses, thereby serving important roles in maintaining homeostasis and in defending airways and lungs.

The importance of lung afferents is strengthened by the observation that changes in their activation, and consequently also in the communication between the bronchopulmonary system and the brain, contribute to symptoms of pulmonary discomfort and disease, such as dyspnoea, cough, excessive mucus secretion,

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enhanced sensations and bronchospasm (Coleridge and Coleridge 1997; Sant'Ambrogio and Sant'Ambrogio 1997; Lee and Pisarri 2001; Canning et al. 2006; Widdicombe 2009; Undem and Nassenstein 2009; Burki and Lee 2010; Adriaensen and Timmermans 2011).

The afferent activities arising from sensory terminals located in the airways and lungs are believed to be mainly conducted via fibres travelling in the vagal nerve and with cell bodies in nodose and jugular ganglia (Paintal 1973; Widdicombe 1981; Sant'Ambrogio 1982; Coleridge and Coleridge 1994; Murray 2010). These vagal sensory neurons innervate the entire respiratory tract, ranging from the larynx and trachea to the lung parenchyma, and project to the nucleus of the solitary tract in the medulla (Agostoni et al. 1957; Jammes et al. 1982). Regarding the regulation of respiratory function, and generation of sensations, sympathetic afferents with cell bodies in the thoracic dorsal root ganglia (DRG), are believed to be less important than the vagal pathways, and have not been investigated as extensively (Widdicombe 1986, 2009; Kummer et al. 1992b; Plato et al. 2006). Traditionally, vagal afferents have been considered as the sensory pathways that collect physiological information from the airways and lungs, while spinal afferents would be involved in the detection of "noxious" information. Recent data, however, clearly suggest that certain populations of vagal airway afferents may also be implicated in processing potentially harmful stimuli (nociceptors) (Kollarik et al. 2010).

1.2 Activation of Airway Sensory Nerves

Mechanical perturbation, inflammatory mediators, pH, chemicals, temperature, and osmolarity are stimuli that are able to trigger activation in airway sensory nerves (Sant'Ambrogio 1982; Mazzone 2004, 2005; for reviews see Taylor-Clark and Undem 2006; Kollarik and Undem 2006; Canning et al. 2006). It is therefore not surprising that the sensory nerves most often studied in the airways have been subdivided into two general categories: (1) Mechanoreceptors, which respond to the mechanical forces caused by inflation and deflation of breathing (stretch receptors), including high-threshold mechanosensors (extensive stretch), and (2) Chemosensors, including so-called nociceptors, which are adapted to respond to chemicals and the potential threat of tissue damage (e.g., trauma, inflammation).

Airway sensory neurons are activated by physical and/or chemical stimulation by converting the analogue sensory stimuli into changes in ionic conductance across the membrane, leading to depolarisation of the plasma membrane. This initial depolarisation of the membrane of the afferent terminal is referred to as the "generator potential". If the generator potential is of sufficient magnitude, it leads to the formation of the all-or-nothing action potential that travels along the fibre to the central neuronal terminals. Some chemicals and mediators can also cause "neuromodulation" of peripheral nerves, either by changing the excitability in

such a way that it alters its response to subsequent activating stimuli, or by leading to changes in gene expression at the cell body.

Peripheral processes of primary afferents also transport and release transmitters, thus subserving a potential local effector function. These processes have generally been considered as dendrites, because they harbour the "sensing" function of primary afferents, but it became clear that they also display all structural and functional features of an axon, except for the specialised peripheral terminals where sensory transduction takes place (Raab and Neuhuber 2007).

Although some possible working mechanisms are starting to be elucidated, it is clear that much remains to be learned before we will fully understand how the airway environment is recognised and transduced to meaningful information by the sensory nervous system.

1.2.1 Mechanical Stimuli

Many airway afferent nerves are reproducibly responsive to some kind of mechanical stimulation, although the sensitivity substantially varies between different subtypes. The response to mechanical stimuli is mediated by specific ion channels and accessory proteins in the peripheral nerve terminals (for review, see Brierley 2010).

The "physical hypothesis" for mechanical nerve activation is that the stimulus physically alters a "mechanogating protein" leading to the opening of a cation channel in the membrane of the terminal. Well-known superfamilies of mechanogated ion channels are the epithelial sodium channels (ENaCs), "two-pore domain" K^+ channels (K_{2P}) and the "transient receptor potential (TRP)" non-selective cation channels (for review, see Brierley 2010).

The "chemical hypothesis" of mechanotransduction considers that mechanical perturbation of tissues causes the release of substances from non-neuronal cells such as epithelial cells or smooth muscle cells, which subsequently activate the "mechanosensitive" nerve (Taylor-Clark and Undem 2006).

A defining feature of mechanoreceptors is their rate of adaptation. Vagal airway receptors are no exception, since both "rapidly" and "slowly" adapting receptors can readily be distinguished.

1.2.2 Chemical Stimuli

Many different chemical mediators delivered to the lungs can influence action potential discharge at afferent nerve terminals. Whereas the action of chemical mediators often involves more complex mechanisms (e.g., chemicals leading to bronchospasm may activate mechanoreceptors), a growing number of mediators

is known to directly and overtly activate afferents in the respiratory system. The nociceptive nerves are more responsive to chemical mediators than the mechanosensitive fibres. Because they respond to a broad range of stimuli, pulmonary nociceptors are often referred to as "polymodal" fibres (Taylor-Clark and Undem 2006; Kollarik et al. 2010).

Chemical mediators that directly activate bronchopulmonary afferent nerves often interact with ionotropic receptors. Binding of ligands to ionotropic receptors induces receptor protein deformation, resulting in the formation (or closure) of a pore through which ions more or less selectively pass. Changes in cation flux through the pore may depolarise the membrane, causing a generator potential.

Agonists that interact with G-protein-coupled receptors (GPCRs) can also lead to generator potentials, although the mechanism by which ligand binding to a GPCR is transduced to membrane depolarisation is more complex and the details are not always well understood. At least some of the activating GPRCs secondarily induce the opening of ion channels (e.g., TRP channels and certain calcium-gated chloride channels) through the action of phospholipase C and increase in intracellular free calcium.

Water, hypotonic and hypertonic solutions are powerful activators of airway afferent nerves (Taylor-Clark and Undem 2006). Whether sensory nerve terminals are directly activated (e.g., through the presence of osmosensitive ion channels), or the activation is due to mechanical forces of tissue swelling or shrinkage and subsequent release of chemical mediators, is less clear.

1.3 Studying Airway Afferents

Basically, the afferent pathways of sensory information from within the lung to the central nervous system have been outlined, being predominantly vagal in most animal species studied, with a more limited sympathetic/spinal component. However, the architecture of airways and lungs, in which sensory nerve terminals are deeply embedded in the extensively branching airway labyrinth (for review, see Murray 2010), has made it almost impossible to exactly locate the nerve terminals within the lung that are responsible for transduction of the electrophysiologically identified functional vagal afferent activity induced by a variety of local stimuli.

Working with "respiratory sensations" in humans allowed to conclude that vagotomy and vagal or bronchopulmonary local anaesthesia abolished or ameliorated some of these sensations (Widdicombe 2009). Vagotomy in humans was in the past sometimes used as part of a (therapeutic) treatment (Morton et al. 1951), but these surgical procedures are for obvious reasons very rare nowadays. On the contrary, vagotomy can more easily be carried out in experimental mammals, leaving the problem that "sensation" can only be studied imprecisely in these animals. Chemical or physical denervation in experimental animals allows us to study degeneration patterns of the peripheral terminals and therefore to determine

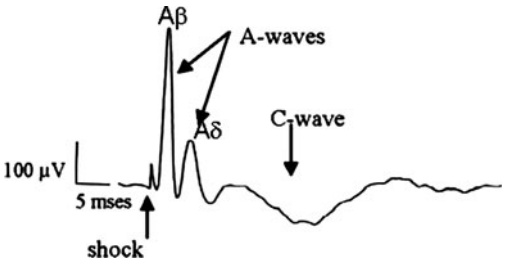

Fig. 1.1 Recording of a compound action potential in a guinea-pig isolated vagus nerve. Based on their specific conduction velocities typically three distinct waves can be recorded. The first wave reflects fibres that conduct action potentials in the Aβ range, the second wave reflects fibres in the Aδ range, and the third and slowest wave reflects fibres that conduct action potentials in the C-fibre range. The C wave is typically much broader and less defined than the A waves (from Carr and Undem 2003)

the origin of nerve terminals that innervate the different structural components of the airways.

The origin of sensory nerve terminals within the respiratory tract can also be determined in detail by retrograde tracing. Tracer substances (Fast blue, fluorogold, DiI,...) are injected into the airways and/or lungs of experimental animals. Retrogradely labelled neuronal perikarya can then be detected in vagal sensory and thoracic dorsal root ganglia (Springall et al. 1987; Kummer et al. 1992b; Kwong and Lee 2002; Dinh et al. 2005; Plato et al. 2006; Groth et al. 2006). The method further allows us to elucidate the neurochemical coding of the neuronal cell bodies of airway afferents.

Anterograde tracing studies, in which tracer substances (e.g., DiI) are injected directly in the neuronal ganglia or sectioned nerves, on the other hand, allow the examination of intrapulmonary nerve fibres. It remains, however, necessary to localise the labelled terminals in association with the visualisation of specific structures (Adriaensen et al. 1998; Van Lommel et al. 1998; Yu et al. 2003).

Another approach to get more insight into the putative functional significance of nerve terminals that reside in the airways and lungs is studying their characteristics (e.g., neurotransmitter content, molecular receptors...) using a morphological approach. Whereas a lot of information can be gathered in this way, direct proof of functional nerve terminal activation cannot be provided.

Because depolarisation of afferent nerve terminals induces discharges that are directed towards the CNS, the activity of airway sensory nerves has mainly been examined by extracellular recordings of action potentials travelling the vagal nerve from the lungs towards the CNS (Fig. 1.1) (Adcock 2002; Carr and Undem 2003; Widdicombe 2006). Studying the functional properties of these airway afferents has led to the classification of electrophysiologically identified "sensory airway receptors" (for review, see Widdicombe 2001; Canning and Mazzone 2005; Lee and Undem 2005; Widdicombe 2009).

Chapter 2
Electrophysiologically Identified Airway Receptors: Main Characteristics

The most generally accepted classification discriminates mechanosensory "slowly adapting (stretch) receptors (SARs)", "rapidly adapting stretch receptors (RARs)" and chemosensitive "C-fibre receptors (CFRs)" (Sant'Ambrogio 1982; Widdicombe 2001; Carr and Undem 2003; Canning and Mazzone 2005; Yu 2009).

Mechanoreceptive stretch-sensitive airway afferents have been classified, based on their adaptation properties to sustained inflation, into SARs (slowly adapting) and RARs that discharge action potentials upon lung inflation but then rapidly adapt to the stimulus (Sant'Ambrogio and Widdicombe 2001; Schelegle and Green 2001; Schelegle 2003; Widdicombe 2003a). The mechanoreceptors are categorised as myelinated "A-fibres", conducting action potentials at a fast rate along their axons (about 10–50 m/s).

Chemosensors in the lungs are commonly referred to as "C-fibres", conducting action potentials at a slow velocity (about 0.3–2 m/s). CFRs can be further subdivided into those situated deep in the lung (pulmonary C-fibres) and those in the conducting airways (bronchial C-fibres). Regardless of their location, nociceptive C-fibres in the airways can typically be stimulated by capsaicin, a pungent ingredient of red hot chilli peppers (Lee and Pisarri 2001; Lee et al. 2003; Lee 2009).

Electrophysiologically identified RARs, SARs and CFRs have been discriminated based on their physical and chemical sensitivity, adaptation to mechanical stimulation, neurochemistry, origin, myelinisation, conduction velocity, activity during tidal breathing, reflexes associated with their activity, ganglionic location of the neuronal cell body, possible sites of termination in the airways and lungs, and their involvement in expiratory sensations. Extensive studies have resulted in several classification schemes, none of which is able to take all of these features into account (Sant'Ambrogio 1982; Carr and Undem 2003; Widdicombe 2009). Because of the large variety of electrophysiologically identified airway receptors, especially when species differences are taken into account, several receptor subtypes do not seem to readily fit the classical classification scheme.

RARs and SARs, the two primary classes of mainly mechanosensitive airway receptors are differentiated by their adaptation to sustained lung inflation. However, a considerable number of lung mechanoreceptors represent intermediate

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types, based on their responses to lung inflation and adaptation indexes (Bergren and Peterson 1993; Burnet and Hilaire 1999; Carr and Undem 2003; Canning and Mazzone 2005; Lee and Undem 2005; Widdicombe 2006). Sensitivity to capsaicin is considered to be the prototypical feature of chemosensitive C-fibre endings, but some airway CFRs are not activated by capsaicin, even at relatively high doses (Ho et al. 2001; Kollarik et al. 2003). In addition, another type of vagal chemosensory nociceptive nerve fibres, originating in the jugular ganglia and conducting action potentials in the A-fibre range, has been identified. Although these receptors show some resemblance to SARs (albeit with a high mechanical threshold) and CFRs, this group of chemosensitive A-fibres does not correspond to any of the well-defined electrophysiological airway receptors. These sensors have been referred to as "high threshold A-receptors (HTARs)" and have been identified *in vivo* and *in vitro* (Widdicombe 2003a; Yu et al. 2006).

The cough reflex is one of the most effective defence functions against inhaled irritants and is induced exclusively via activation of vagal afferents. "Urge-to-cough" is one of the most common respiratory discomforts and symptoms found in airway disease patients (Lee 2009). However, the questions which airway afferents are responsible for eliciting the cough reflex, and whether a true "cough receptor" that initiates these reflex actions actually exists, have been debated for a long time (Undem et al. 2002; Canning et al. 2004, 2006; Mazzone 2004, 2005; Lee and Undem 2005). Today, substantial evidence supports the hypothesis that at least two separate vagal sensory populations of airway afferents are responsible for initiating cough reflexes (Undem and Carr 2010). One is a capsaicin-insensitive, acid-sensitive, myelinated type of nerve that conducts action potentials in the A-range, is limited to the extrapulmonary airways and large bronchi, and mediates cough evoked by aspiration in both conscious and anesthetised experimental animals and humans. The other is a C-fibre population that, when selectively stimulated, causes an irritating, itchy urge-to-cough sensation in conscious animals and humans only, mimicking sensations associated with inflammatory airways diseases (Undem and Carr 2010; Canning 2011). All of these imply the existence of two parallel pathways for cough, one essential and homeostatic, the other nonessential and pathophysiological (Canning 2011).

The classification of vagal airway afferents is further complicated by substantial species differences in the afferent properties of some airway receptors, as well as by phenotypic switches that these sensory receptors may undergo in certain pathophysiological conditions, such as inflammatory airway disease (Barnes 2001; Schelegle and Green 2001; Lee and Undem 2005; Undem and Kollarik 2005; Undem and Nassenstein 2009; Lieu and Undem 2011).

The present review certainly does not have the intention to reinvent or reorganise the categorising system of the physiologically identified airway receptors, and we will, as much as possible, further on stick to the classical well-accepted divisions based on stereotypical characteristics discriminating RARs, SARs and CFRs.

2.1 Slowly Adapting (Stretch) Receptors

The original designation of slowly adapting receptors or SARs was based on the observation that inflation of the lung increases receptor discharge, and that this increase in discharge essentially does not adapt when lung inflation is maintained (Fig. 2.1) (Knowlton and Larrabee 1946; Widdicombe 1954). Nerve fibres that give rise to SARs correspond to myelinated fibres that conduct action potentials in the A-fibre range (mainly Aβ and Aγ) and have their origin in the nodose ganglion (Jordan 2001; Kubin et al. 2006). Based on the indirect functional evidence, it is believed that SARs are anatomically located within the airway smooth muscle layer in several mammalian species, including humans (Larsell 1921; Larsell and Dow 1933; von Düring et al. 1974; Bartlett et al. 1976). Although SARs can be found throughout the entire tracheobronchial tree, considerable variation in the distribution of SARs exists between mammalian species. In cats, guinea-pigs and rats, SARs are more abundant in the intrapulmonary airways, whereas dogs tend to have more SARs in the extrapulmonary airways (Widdicombe 1954; Miserocchi et al. 1973; Bartlett et al. 1976; Schelegle and Green 2001). SARs outside the lungs

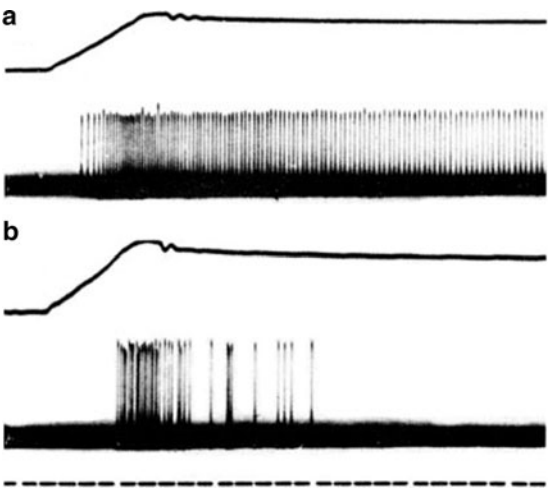

Fig. 2.1 Discharge pattern of two types of afferent nerve fibres in the vagus nerve of the cat, as recorded under maintained lung inflation, discriminating the two predominant types of airway mechanoreceptors. *Upper traces* represent the intratracheal pressure, *lower traces* the recorded action potentials. (**a**) This first receptor adapts slowly to maintained lung inflation and can be identified as a slowly adapting receptor (SAR). (**b**) The second receptor adapts much more rapidly to sustained lung inflation compared to the SAR and is therefore designated as a rapidly adapting receptor (RAR). From Knowlton and Larrabee (1946)

may exert different features compared to those in the intrapulmonary bronchi (Schelegle and Green 2001).

Different types of SARs have been described based on adaptation rate, volume threshold and sensitivity to inflation (Paintal 1973; Sant'Ambrogio 1982; Bergren and Peterson 1993; Schelegle and Green 2001; Schelegle 2003). A commonly used classification subdivides SARs into "high and low threshold" receptors. "High threshold" receptors generate action potentials when high tensions are reached during inspiration, these types of SARs are mainly found in the intrapulmonary airways. "Low threshold (type II)" receptors are activated at much lower excitation levels and are also active during expiration. Type II receptors are predominantly present in the extrapulmonary airways. Although the different subtypes of SARs may exhibit diverse patterns of discharge, it is not known whether they give rise to different reflexes and/or sensations (Paintal 1973; Schelegle and Green 2001; Schelegle 2003; Widdicombe 2009).

SARs, predominantly activated by mechanical stimuli, are the vagal airway afferents that are responsible for eliciting reflexes evoked by moderate lung inflation. Activation of SARs leads to reflexes that are known to play an important role in regulating the pattern of breathing (Schelegle and Green 2001; Schelegle 2003). In contrast to the observations in animal models, the role of SARs in controlling the pattern of breathing in humans seems to be minor, at least during quiet breathing (Sant'Ambrogio and Sant'Ambrogio 1997). Nevertheless, it is generally agreed that SAR activity regulates the Hering-Breuer reflex, which terminates inspiration and initiates expiration when the lungs are adequately inflated. Once a certain volume threshold is achieved, the inspiratory off-switch provided by the SARs input results in a termination of inspiration (Hering 1868; Adrian 1933; Sant'Ambrogio 1982). It has been proposed that a certain pattern of breathing corresponds with a certain demand of the respiratory muscles. SARs appear to be responsible for the volume feedback necessary to adjust the breathing pattern to the optimal load: once a threshold volume is reached, SARs control in a reflex manner the tidal volume and respiratory rate (Widdicombe and Nadel 1963b; Widdicombe 2006). SAR activation also initiates reflex bronchodilatation by inhibiting tonic parasympathetic cholinergic drive to the airway smooth muscle, resulting in a reduction in baseline cholinergic airway tone (Widdicombe and Nadel 1963a, b), but the role that SARs play in determining normal airway tone is poorly understood. At low inflation pressures, SARs are also believed to be the vagal afferents that are responsible for initiating a cardiovascular reflex that results in tachycardia (Agostoni et al. 1957; Daly 1986; Kaufman and Cassidy 1987; Looga 1997). Although SARs are generally considered to be pure mechanoreceptors and relatively insensitive to chemical stimuli (compared with other lung receptors), their activity during eupneic breathing has been shown to be inhibited by increasing CO_2 partial pressure in the pulmonary arterial blood (Coleridge and Coleridge 1978; Sheldon and Green 1982, 2001).

2.2 Rapidly Adapting (Stretch) Receptors

An important feature to discriminate RARs from the slowly adapting mechanoreceptors in the airways is their rapid adaptation to maintained lung inflation (Fig. 2.1) (Keller and Loeser 1929; Adrian 1933; Knowlton and Larrabee 1946; Widdicombe 1954). RARs have thin myelinated nerve fibres that conduct action potentials in the A-fibre range (mainly Aδ) exhibit an irregular discharge and respond typically to the dynamic phase of a mechanical stimulus (Widdicombe 1954; Sant'Ambrogio 1982; Sant'Ambrogio and Widdicombe 2001; Widdicombe 2003a). These receptors are found throughout the respiratory tract from nasopharynx and larynx to bronchi, but are scanty in the smaller bronchi and none have been identified in the bronchioles and alveoli (Widdicombe 1954; Sant'Ambrogio 1982; Lee et al. 1992). RARs are usually not very active during quiet breathing but do react to rapid changes in airway volume, discrete probing of the airway mucosa or gross deformation of the airways, such as those induced by a large distension or collapse of the lung. In addition to these mechanical modalities, RARs can readily be recruited by inhalation of irritants (e.g., ammonia, ethyl ether, sulphur dioxide and cigarette smoke) and the release of inflammatory or immunological mediators (Sant'Ambrogio and Widdicombe 2001; Widdicombe 2001, 2003a). Several investigators, however, have suggested that RARs are relatively insensitive to "direct" chemical stimuli (Coleridge and Coleridge 1984; Joad et al. 1997; Ho et al. 2001; Belvisi 2003). The responses of RARs to inhaled substances would then result from secondary effects initiated by the action of these irritants on the mechanical properties of the airways and lungs. Stimuli-dependent release of secretion products of unmyelinated C-fibre receptors may evoke bronchospasm and mucus-secretion-induced airway obstruction, mimicking the mechanical consequences of lung inflation and deflation, and thus increase RAR activity. Nevertheless, there is also convincing evidence for "true" chemosensitive properties of RARs (Joad et al. 1997; Sant'Ambrogio and Widdicombe 2001; Widdicombe 2001, 2003a; Canning 2006). Cigarette smoke, for example, exerts an immediate stimulatory effect on RARs, before any change of the bronchomotor tone can take place (Kou and Lee 1990, 1991).

The high sensitivity of RARs to inhaled irritants and mechanical probing suggests a role in the airway defence mechanism. An intraepithelial location of at least part of the terminals of RARs has been proposed in agreement with this function (Riccio et al. 1996; Widdicombe 2001, 2003a). However, little is known about the membrane receptor properties of RARs that allow them to be activated by a variety of mechanical and chemical stimuli (Widdicombe 2001, 2009).

RARs have different reflex actions on breathing and bronchomotor tone, and discharge during lung inflation, or sometimes during the deflation phase of a respiratory cycle, and become more active as the rate and volume of lung inflation are elevated. Activation of RARs generally leads to increased inspiratory

effort, augmented breaths, a rise in parasympathetic outflow (i.e., bronchoconstriction and mucus secretion). While SARs prolong expiration, RARs have the opposite effect and shorten expiration (Sant'Ambrogio and Widdicombe 2001; Widdicombe 2003a; Lee and Undem 2005).

RARs appear to form a heterogeneous collection of sensory receptors, and their properties, both in relation to their sensitivity to various stimuli and the reflexes they induce, largely depend on the characteristics of the individual receptor and on its location in the airways (Sant'Ambrogio and Widdicombe 2001). RARs in the larynx, trachea and large bronchi are highly mechanosensitive and often referred to as "irritant receptors" (Knowlton and Larrabee 1946; Widdicombe 1954). At least subpopulations of laryngeal and tracheal RARs cause cough (Undem and Carr 2010; Canning 2011), the expiration reflex, and other laryngeal reflexes such as mucus secretion and broncho- and laryngoconstriction, but also stimulate hyperventilation and augmented breaths (Karlsson et al. 1988; Widdicombe 1998, 2003a; Sant'Ambrogio and Widdicombe 2001). RARs occurring in the lung are more sensitive to chemical irritant stimuli, and less to mechanical stimuli. A large body of evidence suggests that the latter receptors stimulate breathing, including augmented breaths, and cause reflex tracheo- and bronchoconstriction, airway mucus secretion and laryngeal closure (Sant'Ambrogio and Widdicombe 2001; Widdicombe 2003a).

As mentioned above, RAR activation may evoke cough, and these receptors indeed fulfil many of the accepted criteria for mediating the defensive cough reflex (Sant'Ambrogio et al. 1984; Widdicombe 1998; Canning 2011). However, for a considerable part of the RARs, it is unlikely that they would initiate the cough reflex, because many of the stimuli that robustly activate RARs (e.g., prostaglandins, histamine, neurokinins, metacholine) are often ineffective or only modestly effective in evoking cough (Barnes et al. 1984; Joos et al. 1987; Fujimura et al. 1992; Sant'Ambrogio and Widdicombe 2001; Widdicombe 2001). Although it is clear that the general population of RARs may not be the primary afferent nerve fibres to evoke cough, RARs throughout the airways are believed to act synergistically with other afferent nerve types to induce coughing (Mazzone 2004, 2005; Canning et al. 2006). Recently, selective subtypes of RAR-like A-fibres have been identified as real cough receptors (Undem and Carr 2010; Canning 2011). These receptors are highly sensitive to punctuate mechanical stimulation, acids and low chloride solutions but are, unlike typical RARs and SARs, not activated by airway distension or collapse. They are likely quiescent during tidal breathing, only becoming active in the presence of stimuli evoking cough.

2.3 C-Fibre Receptors

In addition to the myelinated RARs and SARs, the respiratory tract is also abundantly innervated by a large population of thin unmyelinated afferent

nerve fibres (Agostoni et al. 1957), which conduct action potentials in the C-fibre range and are therefore termed C-fibre receptors (CFRs). Vagal afferent C-fibres comprise a majority of the vagal airway afferents, innervate multiple targets over the entire respiratory tract and project to the nucleus of the solitary tract. Their activity is now believed to play essential roles in the physiological regulation of cardiopulmonary function (Lee 2009). Vagal CFRs are generally quiescent in the healthy, non-inflamed airways but are readily activated by noxious chemicals, including cigarette smoke and inflammatory mediators, and may therefore play a critical role in regulating and/or initiating airway defence reflexes (Paintal 1973; Coleridge and Coleridge 1984; Riccio et al. 1996; Ho et al. 2001; Lee and Pisarri 2001; Undem et al. 2004; Lee and Undem 2005; Kollarik et al. 2010; Lee et al. 2010). CFRs are polymodal, responding to both mechanical and chemical stimuli but are relatively unresponsive to lung inflation, collapse or stretch (Coleridge and Coleridge 1984; Ho et al. 2001). Because CFRs are much more readily activated by chemical stimulants than vagal mechanoreceptors, i.e., RARs and SARs, C-fibres may represent the primary type of chemosensitive or nociceptive afferents in the lung (Lee et al. 2003; Lee 2009).

With few exceptions – i.e., in humans it still remains a matter of debate (Lee 2009) – CFRs in mammalian lungs are robustly activated by capsaicin. Capsaicin has been widely used to stereotypically identify C-fibre afferent endings and for assessing their physiological effects (Ho et al. 2001; Lee and Pisarri 2001; Lee et al. 2003; Undem et al. 2004). The vanilloid receptor subtype 1 (VR1 or transient receptor potential vanilloid, TRPV1), a ligand-gated, non-selective cation channel, has been recognised as the "capsaicin receptor" on CFRs (Caterina et al. 1997; Helliwell et al. 1998). However, the suggestion of a strict relation of TRPV1 and CFRs is not entirely accurate because it has also been demonstrated that capsaicin can have a stimulatory effect on some thin myelinated Aδ afferents, found in various visceral organ systems, including the lung (Riccio et al. 1996; Kajekar et al. 1999; Takemura et al. 2008).

The majority of CFRs, particularly in rats and guinea-pigs, synthesise neuropeptides such as tachykinins and calcitonin gene-related peptide (CGRP), which are located in synaptic vesicles in their central and peripheral terminals (Lundberg et al. 1984; Springall et al. 1987; Baluk et al. 1992; Kummer et al. 1992b; Riccio et al. 1996; Myers et al. 2002; Undem et al. 2004). This neurochemical property has been exploited to describe the distribution of the peripheral terminals of the unmyelinated C-fibre afferents in the airway epithelium. In large airways, CFRs form a dense nerve plexus just beneath the epithelium (Lundberg et al. 1984; Dey et al. 1990; Baluk et al. 1992; Hunter and Undem 1999; Canning et al. 2002). In the gas exchange area, C-fibres have been reported in close apposition of the capillaries, resulting in the term "juxtacapillary receptors" or "J-receptors" (Paintal 1995). Based on these variable anatomical locations, C-fibres have been subclassified into bronchial and pulmonary C-fibres (Coleridge and Coleridge 1984).

Bronchial C-fibres are experimentally identified as those most accessible for manipulation via the bronchial circulation, whereas pulmonary C-fibres are more readily accessed from the pulmonary circulation (Coleridge and Coleridge 1984). The subclassification of bronchopulmonary C-fibres has been extended, at least for mice and guinea-pigs, by considering the ganglionic location, coinciding with their embryonic origin, of the cell somata (Undem et al. 2004; Nassenstein et al. 2010). The jugular (neural crest) C-fibres innervate the larynx, trachea, main bronchi, and intrapulmonary airways (Riccio et al. 1996; Undem et al. 2004; Nassenstein et al. 2010). The nodose (placodal) C-fibres innervate intrapulmonary tissues but are rarely found in extrapulmonary airways. It would seem that the nodose fibres at least loosely correspond to pulmonary C-fibres and that jugular fibres correspond to bronchial C-fibres (Nassenstein et al. 2010). In addition to the different location and neuronal origin, subtypes of C-fibres appear to have different sensitivities to chemical and mechanical stimuli (Coleridge and Coleridge 1994; Ho et al. 2001; Lee and Pisarri 2001; Undem et al. 2004; Chuaychoo et al. 2005; Lee 2009). However, the distinction based on circulatory accessibility is less clear in smaller animals, e.g., rodents, as compared to cats and dogs (Coleridge and Coleridge 1994).

C-fibres are activated by a wide range and variety of exogenous and endogenous substances. The stimulatory effect of a number of inhaled chemicals (e.g., capsaicin, bradykinin, citric acid, ozone, ammonia) and volatile anaesthetics on bronchopulmonary CFRs has been extensively described (Paintal 1973; Coleridge and Coleridge 1984, 1994; Lee and Pisarri 2001; Lee et al. 2003; Lee and Undem 2005; Lee 2009). One important example is cigarette smoke, which has a pronounced stimulatory effect on CFRs and may have special significance in the pathogenic effects of smoke on normal airway function (Bonham et al. 2001; Lee et al. 2007, 2010). Endogenous inflammatory and immunological mediators, such as histamine, bradykinin and prostaglandins, are also known to activate C-fibre reflexes (Paintal 1973; Coleridge and Coleridge 1984; Undem and Nassenstein 2009; Lee 2009). Activation of airway C-fibres may cause local neurogenic inflammation. CFRs could be regarded as biosensors that monitor the inflammatory status of the lung by directly interacting with inflammatory mediators (Lee et al. 2007; Yu 2009).

When stimulated, pulmonary and bronchial CFRs can trigger profound airway/respiratory and cardiovascular responses, mediated via both peripheral/local axon reflexes and/or central/CNS reflex pathways. Central respiratory reflexes include airway constriction, mucus secretion, cough, tachypnea and rapid shallow breathing (Coleridge and Coleridge 1984; Lee 2009), while cardiovascular effects of pulmonary C-fibre stimulation include bradycardia, and systemic and pulmonary hypertension (Paintal 1973; Coleridge and Coleridge 1984; Ho et al. 2001; Lee and Pisarri 2001; Lee et al. 2003; Lee and Undem 2005; Lee 2009). Reflex bronchoconstriction and reduced tidal volume, which limits the inhalation of irritants into the lungs, together with the increase in bronchial blood flow to remove the irritants, are consistent with the role of CFRs in the pulmonary defence reflex (Lee and Pisarri 2001). Local actions are mediated by the release

of neuropeptides from C-fibre endings, which probably results from propagation of sensory impulses via the axonal ramifications to other branches of the sensory terminals (Lundberg and Saria 1987). The release of tachykinins and CGRP, which can act on a number of effector cells, may produce potent local effects, such as bronchoconstriction, chemotaxis of inflammatory cells, extravasation of macromolecules and oedema of the airway mucosa (Paintal 1973; Coleridge and Coleridge 1984; Ho et al. 2001; Lee and Pisarri 2001; Lee et al. 2003; Lee and Undem 2005). The great variability may reflect differences in stimuli and/or the possibility that several subtypes of CFRs are involved (Lee 2009). Recently, it has been suggested that the considerable neurochemical and molecular variety of CFRs in rodents may hold the key for the observed variability in sensations evoked by peripheral stimulation (Nassenstein et al. 2010; Adriaensen and Timmermans 2011).

The sensitivity of CFRs to a potential stimulus can be increased by several substances. This process is called sensitisation and can be mediated by certain autocoids (e.g., histamine, prostaglandins) and agents such as ozone, cigarette smoke, adrenaline and adenosine (Coleridge and Coleridge 1984; Ho and Lee 1998; Ho et al. 2000; Lee and Pisarri 2001; Kwong and Lee 2002; Lee et al. 2002; Lee and Undem 2005). The inappropriate and exaggerated activation of the C-fibres may contribute to the airway hyper-responsiveness seen, for example, in asthma patients.

Evidence also supports a role for CFRs in the cough reflex. Capsaicin, bradykinin and citric acid, selective stimulants of airway C-fibres are also potent mediators of cough (Coleridge and Coleridge 1984; Karlsson 1996; Mazzone et al. 2002; Canning et al. 2004). However, these substances only evoke cough in conscious animals and humans, but not in anaesthetised subjects, leading to the suggestion that cough evoked by airway irritation and mediated by CFRs may induce a conscious/voluntary urge-to-cough (Hutchings et al. 1984; Tatar et al. 1988, 1994; Canning et al. 2004; Undem and Carr 2010; Canning 2011), making cough not necessarily completely reflexive in nature. In view of the recent identification of RAR-like “true” cough receptors (see higher Undem and Carr 2010; Canning 2011), C-fibres are believed to be activated by chemical irritation and may be responsible for the recruitment of secondary pathways that evoke or modify cough responses via interactions with the primary cough pathway (Mazzone 2004, 2005; Canning et al. 2006; Canning 2011).

Chapter 3
Morphology and Location of Electrophysiologically Identified Sensory Airway Receptors

Technical and methodological limitations prevent direct measurements and manipulation of individual intrapulmonary sensory airway receptors, forcing lung physiologists to make indirect interpretations of the events that occur at the level of the (sensory) receptor endings. Recent reviews described at least six subtypes of vagal sensory airway receptors, including SARs, RARs, bronchial and pulmonary CFRs, HTARs and cough receptors (Widdicombe 2009). Knowledge of the characteristics of intrapulmonary sensory receptor morphology is, however, crucial because the behaviour of sensory units largely depends on it. To allow for reproducible location and morphological identification of the distinct populations of electrophysiologically identified airway receptors, the neurochemical characteristics of afferent nerve terminals need to be determined, and likely will turn out to be essential to (finally) define the actual targets of the many subpopulations of airway-related vagal afferents (Yu 2009).

In contrast to the multiplicity of sensory airway receptors that have been identified by physiologists, descriptions of morphologically well-defined airway receptors are still rare, implying that many of the pulmonary receptors have a so far poorly identified morphology (Carr and Undem 2003).

Although it has always been somewhat controversial (Bitensky et al. 1975; for review, see Sant'Ambrogio 1982; Widdicombe 2001; Schelegle 2003), it is generally believed that the predominantly mechanosensory SARs are located in airway smooth muscle bundles (Widdicombe 2001; Yu 2009). The latter was partly confirmed by rare but elegantly combined morphological and electrophysiological studies (Yu et al. 2003; Yu 2009). Especially for the trachea and extrapulmonary bronchi, evidence based on direct functional dissection of tissues provided convincing evidence for the location of a SAR population in the smooth muscle layer (Bartlett et al. 1976).

Based on their location, appearance and sometimes a presumed relationship to physiologically characterised receptors, mechanoreceptor-like structures believed to be associated with airway smooth muscle have been described under different names [e.g. "smooth muscle nerve spindles" (Larsell 1922); "pulmonary stretch receptors" (von Düring et al. 1974); "slowly adapting stretch receptors" (Yamamoto et al. 1999; Yu et al. 2004)]. Using classic light microscopic methylene blue,

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silver or osmium tetroxide staining, nerve fibres that give rise to complex terminals (considered sensory) have been described in airway smooth muscle bands (Larsell 1922; Larsell and Dow 1933; Elftman 1943; Baluk and Gabella 1991). Myelinated afferents with terminals integrated in the "myoelastic system" of bronchi were also seen in a combined conventional light and electron microscopic study in rats (von Düring et al. 1974). More recent immunocytochemical studies have reported the presence of branching receptor-like nerve complexes associated with the tracheal and bronchial wall in different animal species (Yamamoto et al. 1995, 1998, 1999; Bak and Panos 1997; Yu et al. 2003, 2004; Yu 2009). However, in none of these studies co-staining was performed to locate airway smooth muscle effectively.

Except for the fact that they are undoubtedly myelinated (mainly Aδ range), the location and morphology of RARs have so far not been determined with certainty in most species (Sant'Ambrogio 1982; Sant'Ambrogio and Widdicombe 2001; Widdicombe 2003b). A mucosal location seems consistent with the high sensitivity of RARs to inhaled irritants and intraluminal mechanical stimuli. Considerable efforts have been made to identify RAR-like sensors in guinea pig airways (for review see Fox 1996; Undem et al. 2002) and suggest that RARs tend to have nerve terminals under, but not in, the epithelium (Kappagoda et al. 1990).

Unmyelinated CFR terminals in the airways and lungs have mostly been characterised via immunohistochemical staining for the tachykinins and CGRP they produce (Lundberg et al. 1984; Springall et al. 1987; Baluk et al. 1992; Kummer et al. 1992a; Riccio et al. 1996; Myers et al. 2002; Undem et al. 2004; Lee 2009). C-fibre endings were found to be located between the airway epithelial cells or to form poorly organised plexuses immediately beneath the airway epithelium (Lundberg et al. 1984; Dey et al. 1990; Baluk et al. 1992; Hunter and Undem 1999; Canning et al. 2002; Lee 2009). Given our lack of knowledge about the terminations of many types of airway afferents, including other chemosensory subtypes, it seems logic to speculate that at least part of the extensive neuropeptide-immunoreactive (ir) varicose nerve fibres that are found intraepithelially represent C-fibre subpopulations (Canning and Mazzone 2005; Lee and Undem 2005).

It is clear that data on the morphology and especially the neurochemical characteristics of putative airway mechanoreceptors have remained limited for long time, hampering a scientifically justified correlation between the multiplicity of physiologically identified receptor types and subtypes, and the so far limited number of morphologically well-defined populations of sensory lung receptors.

Chapter 4
The Neurochemical Coding of Airway Afferents

The "neurochemical coding" is the combination of chemical features (e.g., structural proteins, molecular receptors, neurotransmitter content) that defines a neuronal cell type or certain nerve fibre population. With the advances of immunohistochemistry, in combination with confocal microscopy, intrapulmonary airway sensory nerve fibres and the concomitant airway receptor end-structures can now be examined in detail and evaluated objectively (Yu 2009). Moreover, application of these techniques potentially allows to clearly evaluate the relationship of primary afferents with associated tissues and cells, and to get more profound insights into their potential functions.

Nowadays, an enormous variety of antibodies is available to explore nerve fibre characteristics, ranging from neurotransmitter content to the presence of ion channels and neurotransmitter receptors at synaptic sites.

To visualise nerve terminals in lungs, general neuronal immunohistochemical markers such as protein-gene-product 9.5 (PGP9.5) (for review, see Day and Thompson 2010), neuron specific enolase (NSE), neurofilament proteins or synaptic vesicle proteins [e.g., SV2 (Pan et al. 2004, 2006a); synaptophysin (SYN) (Lee et al. 1987)] have been widely used. Immunolabelling with any of these pan-neuronal markers reveals a high number of nerve fibres and terminals with varying intensities, mostly in the vicinity of blood vessels, airway smooth muscle and the epithelium. Evidently, only a subpopulation of these labelled nerves concerns sensory fibres and terminals, exemplifying the necessity of additional specific markers for identification of the latter.

As already mentioned in Sect. 2.3, at least part of the unmyelinated C-fibre population constitutively expresses CGRP and SP in many species, allowing to use antibodies against these peptides to visualise CFRs. Because the vast majority of these terminals are capsaicin-sensitive, antibodies against the capsaicin receptor TRPV1 can also be utilised for their identification. However, it cannot be excluded that other C-fibre populations are present in the airways.

Electrophysiological characterisation of airway receptors, however, proved the presence of a large number of myelinated mechanosensitive nerve fibre populations. Myelin sheaths of these fibres can be visualised using antibodies against myelin proteins (e.g., myelin basic protein; MBP). However, because of the

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lack of CGRP and SP in these mechanosensory nerve endings in the healthy airways of most animal species, their full exploration in lungs started only about a decade ago, when new marker molecules were reported that appeared to selectively label known mechanosensory nerve terminals in other organs.

4.1 Antibody Markers for Potential Pulmonary Mechanosensory Nerve Terminals

Mechanosensory airway receptors have been massively studied by "single fibre" electrophysiological recordings in the vagal nerve. For neurochemical studies, infranodosal vagotomy in combination with selective markers is most often used to study the degeneration of vagal afferent mechanosensory receptor terminals in lungs. In this chapter, an overview is given of the more recently discovered antibody markers that were known to label mechanoreceptor terminals in other organs, and subsequently have been tested in the airways and lungs to label potential mechanoreceptor terminals. Whereas in the airways these nerve terminals are most often vagal in origin, terminals in the visceral pleura, which have a strikingly similar neurochemical coding, seem to have a predominant spinal origin. Section 5.3 will further elaborate on the receptor terminals present in the visceral pleura.

Calbindin D-28k (CB) and calretinin (CRT) have been widely used to label mechanoreceptor terminals in e.g. muscle spindles (Duc et al. 1994), intraganglionic laminar endings (IGLEs; (Kuramoto and Kuwano 1994; Dütsch et al. 1998), and Ruffini endings (Ochi et al. 1997a, b). Both molecules belong to the family of EF-hand calcium-binding proteins, usually referred to as "Ca^{2+}-buffers", although data is accumulating for CB also serving a role as calcium sensor (Schwaller 2009). In rodent airways and lungs, CRT-ir nerve terminals have been identified in the tracheal and bronchial wall (Yamamoto et al. 1999). In rats, intrapulmonary CB-ir nerve terminals are typically distinct from the CGRP-ir C-fibres. Because all CB-expressing nerve terminals in the airways disappear after infranodosal vagotomy (rat, mouse), CB could be put forward as a good candidate marker for vagal mechanosensory afferent nerve terminals in rodent lungs (Brouns et al. 2000, 2009b).

It is known that a considerable number of primary afferent neurons can be excited by ATP via binding to the P2X receptor family of ligand-gated ion channels (Taylor-Clark and Undem 2006). $P2X_3$ receptors may function as homomeric and heteromeric ($P2X_{2/3}$) channels, and have been detected on sensory neuronal somata in DRGs and vagal sensory ganglia (Brouns et al. 2000; Kwong et al. 2008; Nassenstein et al. 2010), as well as in peripheral nerve terminals in rodent lungs (Brouns et al. 2000, 2006b, 2009b; Pintelon et al. 2007; Lembrechts et al. 2011). In rodents, $P2X_{2/3}$ receptors and CGRP are not co-expressed in airway sensory nerve fibres, indicating that different nerve fibre populations are

concerned. The disappearance of characteristic intrapulmonary $P2X_{2/3}$-positive nerve terminals in the ipsilateral, but not the contralateral lung after unilateral infranodosal denervation, implies that $P2X_{2/3}$ receptors are expressed on vagal sensory nerve endings (Brouns et al. 2000, 2006b, 2009b). In lungs and many other organs, it has been proposed that $P2X_3$ or $P2X_{2/3}$ receptors are involved in mechanosensory transduction (for review, see Burnstock 2009).

The excitatory amino acid glutamate is a well-known neurotransmitter in the brainstem projections of airway-related sensory neurons (Lawrence 1995; Haxhiu et al. 2005; Lachamp et al. 2006). A few years ago, also the peripheral nerve terminals of sensory neurons residing in the lungs were shown to have the capacity to store glutamate in secretory granules. At first sight, the presence of synaptic vesicles that contain a motor transmitter in sensory nerve endings seems contradictory, but the occurrence of glutamate in peripheral terminals suggests that glutamate may have peripheral effector functions influencing the activity of neighbouring tissues and/or cells (Raab and Neuhuber 2003; Bewick et al. 2005), or may play an important role in modulating the excitability of the afferents (Banks et al. 2002). "Glutamatergic" nerve terminals can nowadays be characterised very selectively by the expression of vesicular glutamate transporters (VGLUTs), which are transmembrane transporter proteins that are involved in accumulating glutamate in synaptic vesicles (Takamori 2006). At least in rodent lungs, glutamatergic nerve fibres visualised by VGLUT1 and/or VGLUT2 immunostaining appear to be exclusively sensory, and nicely revealed specialised pulmonary receptor structures at various locations in the airways and lungs (see further on). The observation of VGLUTs in mechanosensory IGLEs in the gastrointestinal tract (Raab and Neuhuber 2003) and in the sensory terminals of muscle spindles (Banks et al. 2002) argues for the pulmonary VGLUT-labelled nerve terminals serving a mechanosensory role.

Na^+/K^+-ATPase is a complex of integral membrane proteins that is able to carry out a fast and continuous active exchange of sodium and potassium across the cell plasma membrane, and in that way to maintain chemical gradients of these ions. These gradients are of fundamental importance for signalling and secondary transport, control of cell volume and osmotic activity, and for keeping the resting potential in certain types of neurons. The $\alpha 3$ subunit of Na^+/K^+-ATPase is believed to be predominantly expressed in primary afferent neurons with mechanosensory abilities that are capable of continuously firing over longer periods (i.e., slowly adapting) (Dobretsov et al. 2003; Dobretsov and Stimers 2005). Antibodies against Na^+/K^+-ATPase $\alpha 3$ have been used to label mechanosensory nerve terminals in airways and lungs of different animal species (Wang and Yu 2002, 2004; Brouns et al. 2006a, b).

Chapter 5
Morphologically Identified Sensory Receptor End-Organs in the Airways, Lungs and Visceral Pleura

Immunohistochemistry, chemical or mechanical denervation, and neuronal tracing, in combination with confocal microscopy have proven to be valuable tools to study the overall sensory innervation of the airways, with special reference to airway sensory receptor morphology. The current overview focuses on the morphology, location, origin and neurochemical coding of intrapulmonary sensory receptor end-organs that are morphologically well characterised: smooth muscle-associated airway receptors (SMARs), neuroepithelial bodies (NEBs) and visceral pleura receptors (VPRs).

SMARs, VPRs and the vagal sensory nerve terminals in NEBs reveal a somewhat different morphology, location and origin. However, the typical "laminar" [descriptive name because of the strong resemblance of the nerve endings to the well-established IGLEs in enteric ganglia (Rodrigo et al. 1975)] nerve terminals of these lung receptors share a nearly identical neurochemical coding, and originate from myelinated fibres, therefore potentially fulfilling complementary mechanosensory roles in lung function. The similar neurochemical and morphological characteristics of, e.g., vagal SMAR endings and vagal nodose nerve terminals in NEBs, perfectly illustrates the difficulty to functionally discriminate between these terminals, and hence to make straightforward correlations between morphologically identified intrapulmonary sensory airway receptor candidates and electrophysiologically identified receptor activity.

Usage of the terms dedicated to describe morphologically characterised sensory airway receptors seems, therefore, at this point more appropriate, and allows the uncompromised combination of knowledge about the neurochemical coding of morphologically identified sensory airway receptors and physiological recordings.

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5.1 Smooth Muscle-Associated Airway Receptors

5.1.1 General Morphology and Origin of SMARs

SMARs have been identified as branching nerve terminals with well-delineated laminar end-organs that are typically found in the smooth muscle layer of intrapulmonary conducting airways (Figs. 5.1 and 5.2) [rats: (Brouns et al. 2006a, b); mice: (De Proost et al. 2007a; Lembrechts et al. 2011)]. The consistent association of the terminals of these receptor-like structures with airway smooth muscle, and the present lack of direct functional correlation, prompted us to suggest the term "smooth muscle-associated airway receptors (SMARs)" a few years ago.

Immunostaining of airway whole-mount preparations (Fig. 5.1a, d–f) or lung cryostat sections (Fig. 5.1b, c) with PGP9.5 as a pan-neuronal marker revealed that SMARs in rodents are composed of branching laminar nerve endings, although PGP9.5 IR often appeared to be rather weak in SMARs. In addition, immunolabelling for PGP9.5 revealed also a high number of morphologically less well-characterised nerve fibres and terminals with variable staining intensities in the vicinity of airway smooth muscle and epithelium (Fig. 5.1), and in the wall of blood vessels. Also immunostaining with other pan-neuronal markers, such as neuron-specific enolase (NSE), SV2 or SYN, on cryostat sections of rodent lungs (own unpublished observations, Fig. 5.2f, g) revealed SMAR-like endings that are present in the subepithelial region of bronchioles. Since general neuronal markers are not able to discriminate motor and sensory terminals, SMARs apparently have not been specifically recognised in earlier works that reported on PGP9.5, NSE, SV2 or SYN immunostaining of airways and lungs.

Immunohistochemical staining of airway whole mounts (i.e., airway "trees" after careful removal of the pleura, alveolar tissue and blood vessels) revealed that nerve bundles traverse the airways, split off smaller bundles and eventually single nerve fibres that can be followed over long distances, and subsequently branch and give rise to SMAR terminals (De Proost et al. 2007a). In this way, the majority of SMARs can be observed in the wall of large-diameter intrapulmonary airways, but some are also present in more distal bronchioles. Moreover, the explicit preferential location of SMARs near airway bifurcations, noted in both rats and mice (De Proost et al. 2007a), may hold clues for their physiological role.

The receptor fields of SMARs, visualised in airway whole mounts, appear to be more expanded in rats than in mice. In rats, single nerve fibres are regularly observed that branch into two to four fibres over a short distance, each of the branches then giving rise to a separate complex of laminar nerve endings (Yu et al. 2004; Brouns et al. 2006a, b). In mice, the receptor-like structures are generally smaller and less complex, often being composed of just one modestly branching terminal (De Proost et al. 2007a; Lembrechts et al. 2011).

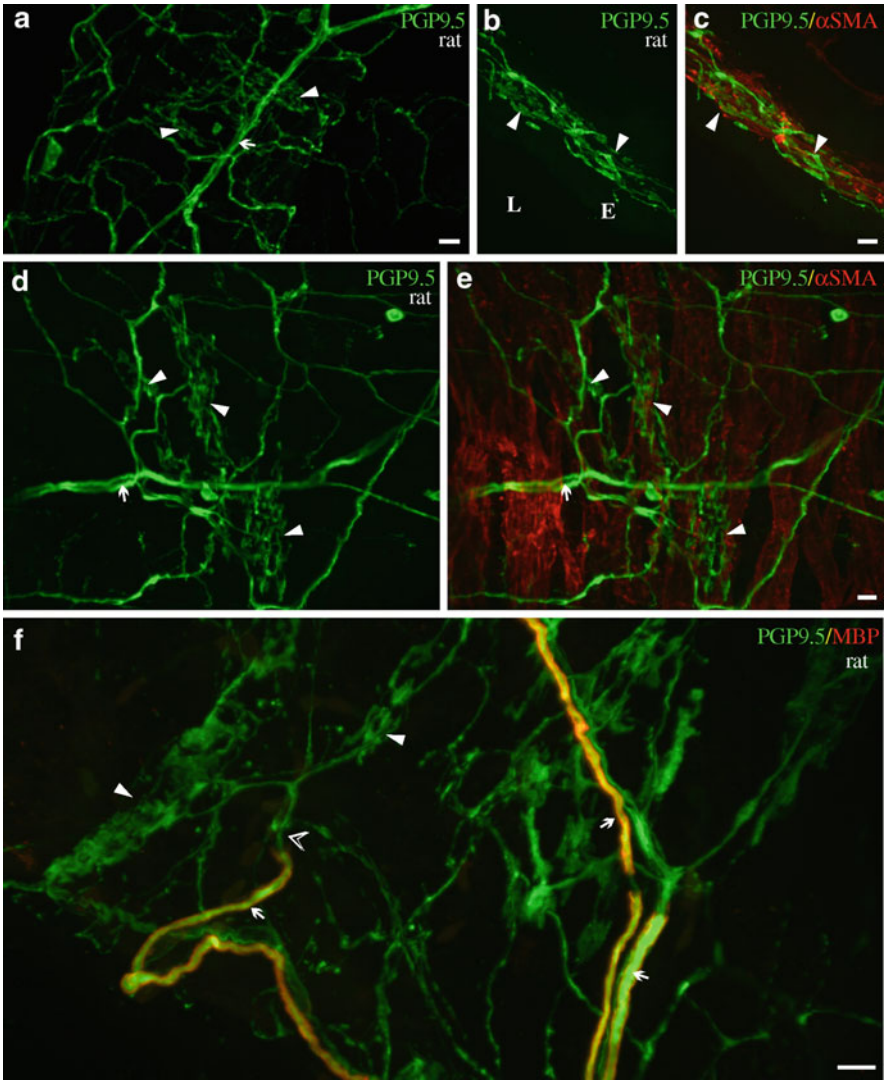

Fig. 5.1 Immunocytochemical staining of smooth muscle-associated airway receptors (SMARs) in rat airways. Confocal images of airway whole-mounts (**a, d–f**) and lung cryostat sections (**b, c**), immunostained for protein gene product 9.5 (PGP9.5). Branching nerve bundles (*arrows*) give rise to several receptor-like end-organs with laminar nerve endings (*arrowheads*). Double labelling with α smooth muscle actin (αSMA; **c, e**), as a marker of smooth muscle cells, reveals that the PGP9.5-ir nerve terminals are located between αSMA-ir smooth muscle cells. (**b, c**) Note that none of the laminar nerve endings of the terminal complexes seems to protrude between epithelial cells (*E*). (**f**) In a bundle of PGP9.5-ir nerve fibres, some fibres are surrounded by a myelin basic protein (MBP)-ir myelin sheath (*arrows*), which is lost (*open arrowhead*) when the nerve fibre branches and forms SMAR-endings (*arrowheads*). *L*: airway lumen. *Scale bars* = 10 μm

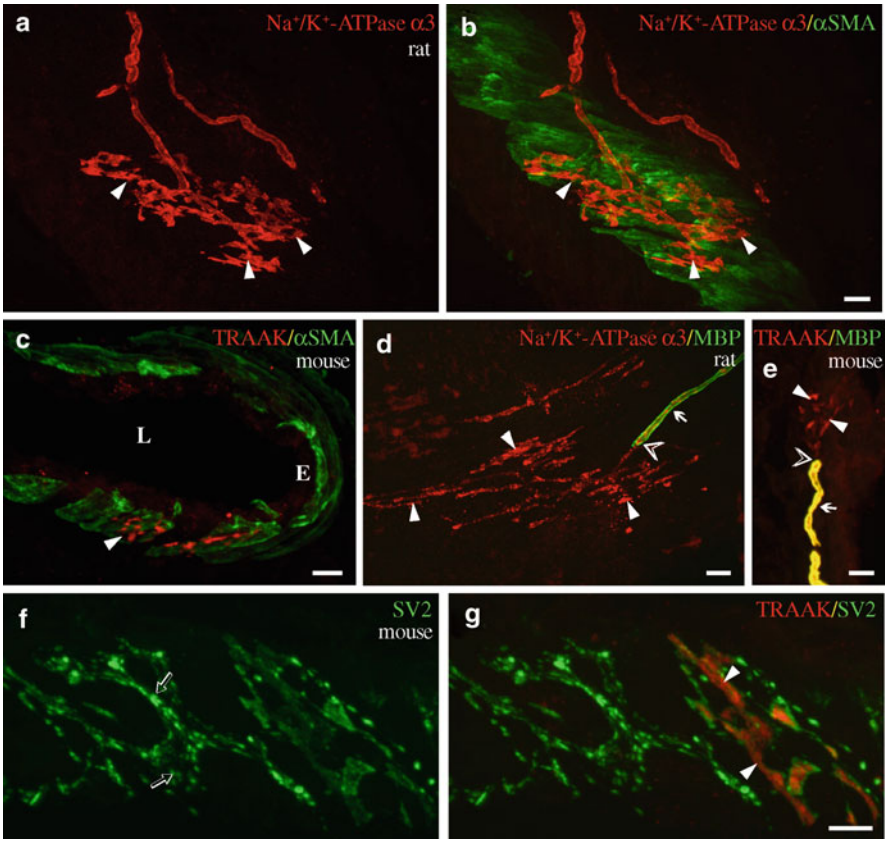

Fig. 5.2 Overview of SMAR-markers and of the morphological features of SMARs in rodents. (**a**, **b**) Rat lung cryostat section stained for Na^+/K^+-ATPase α3 and for αSMA. The membrane-bound location of Na^+/K^+-ATPase α3 clearly illustrates the laminar appearance of the SMAR-terminals (*arrowheads*) that intercalate between the αSMA-ir smooth muscle cells. (**c**) Mouse bronchiole double immunostained for the mechano-gated K^+ channel TRAAK and for αSMA. TRAAK-ir SMAR terminals (*arrowhead*) are located in the airway smooth muscle. *L*: airway lumen; *E*: epithelium. (**d**, **e**) Rat (**d**) and mouse (**e**) bronchioles double stained for SMAR markers and for MBP, showing that the myelin sheath disappears (*open arrowhead*) just before branching of the nerve fibre (*arrow*) that gives rise to the SMAR endings (*arrowheads*). (**f**, **g**) Double immunocytochemical staining for the mouse SMAR marker TRAAK and for SV2 showing an abundance of SV2-ir nerve fibres (*open arrows*) in the airway wall. Obviously, the presence of a SMAR (*arrowheads*) can only clearly be determined by its TRAAK IR. *Scale bars* = 10 μm

In both rats and mice, the specific location of these nerve terminals in smooth muscle bands has been confirmed by double immunostaining of lung sections for "SMAR markers" and α-smooth muscle actin (αSMA), which labels smooth muscle cells. The laminar nerve endings of SMARs are seen to run parallel to

the long axis of smooth muscle bundles, while intercalating between the smooth muscle cells. Similar receptor-like nerve endings have not been observed in the smooth muscle layer that surrounds arterial blood vessels in intrapulmonary airways (Brouns et al. 2006a, b).

The vagal origin of SMARs has been proven by unilateral cervical vagal denervation. SMARs then can no longer be detected in airways ipsilateral to the denervated side, while the side contralateral to the denervation still harbours SMAR terminals.

Of significant functional importance was the observation that nerve fibres giving rise to the complexes of SMAR endings are invariably myelinated. MBP immunostaining visualised the myelin sheath of the nerve fibres that approach airway smooth muscle, and unravelled that myelin sheaths are lost just prior to branching of the nerve fibres into one or more SMARs (Figs. 5.1f and 5.2d, e). Myelinated nerve fibres that give rise to SMARs were found to have diameters ranging from 1 to 3.5 μm [rats: (Brouns et al. 2006a, b); mice: (De Proost et al. 2007a; Lembrechts et al. 2011)].

The combined observation that SMARs originate from myelinated fibres, and disappear after vagal denervation, suggests that SMARs should certainly be considered a good candidate for being the morphological counterparts of at least a subgroup of the physiologically characterised myelinated vagal airway mechanoreceptors.

5.1.2 Functional Morphological Characteristics of SMARs

5.1.2.1 Transmitters and Bioactive Substances

Airway smooth muscle is extensively innervated by postganglionic parasympathetic cholinergic motor fibres that originate from ganglia in the airway wall (Racké and Matthiesen 2004; Canning 2006) and can be visualised using immunohistochemistry for cholinergic markers, e.g., the vesicular acetylcholine transporter (VAChT) (Schäfer et al. 1994). VAChT-ir nerve terminals in airway smooth muscle appear as free endings that never co-localise with SMAR terminals in rodent lungs (Fig. 5.3a–f). Sympathetic adrenergic motor fibres, detected by using antibodies against tyrosine hydroxylase (TH), an enzyme involved in catecholamine synthesis, were found mainly running along blood vessels. No VAChT- or TH-ir was observed in morphologically typical SMAR endings, suggesting that sensory nerve terminals are concerned (Brouns et al. 2006a, b; Lembrechts et al. 2011).

Further screening for the expression of specific markers for peripheral sensory nerve fibres showed that thin varicose CGRP-ir nerve fibres pass the airway smooth muscle bundles, often parallel to the laminar SMAR endings, if present,

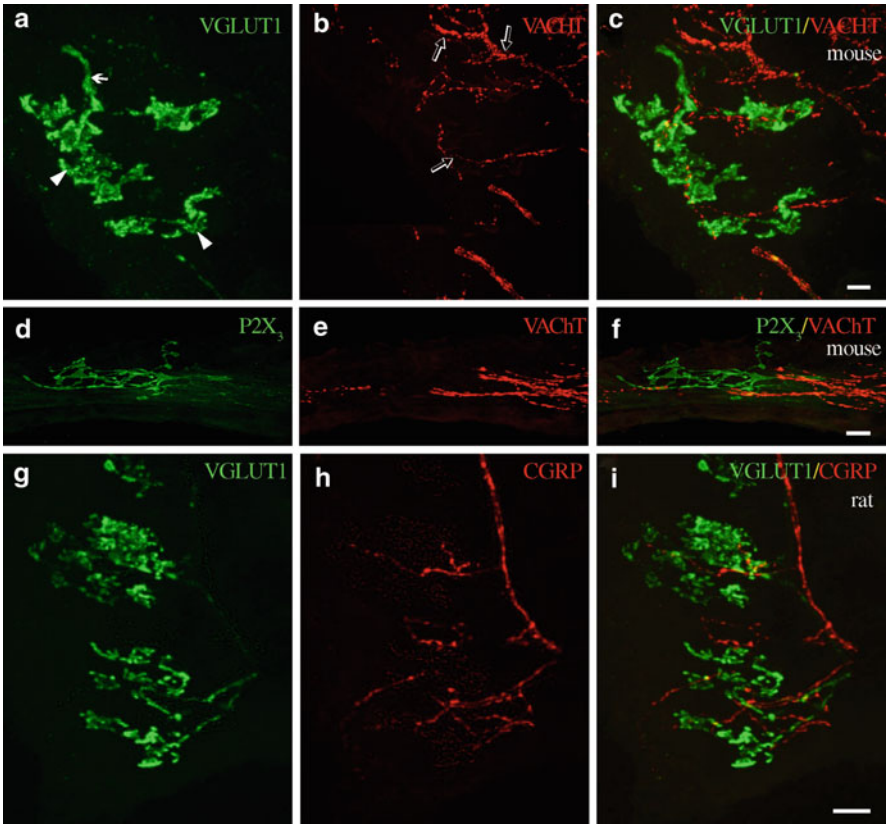

Fig. 5.3 Neurochemical characterisation of rodent SMARs. (**a–c**) A VGLUT1-ir nerve fibre (*arrow*) gives rise to a bronchial SMAR (*arrowheads* in **a**). Although cholinergic VAChT-ir nerve fibres (*open arrows* in **b**) are also abundant in airway smooth muscle, VAChT-ir fibres do not co-localise with the SMAR terminals. (**d–f**) A $P2X_3$-receptor-ir SMAR, the laminar endings of which are intermingled with separate VAChT-ir cholinergic nerve fibres. (**g–i**) Rat bronchiole showing laminar nerve terminals of a VGLUT1-ir SMAR that are present in the neighbourhood of subepithelial CGRP-ir nerve fibres. No co-localisation or specific interaction between both populations of nerve terminals is observed. *Scale bars* = 10 μm

but apparently are not specifically related to the latter (Fig. 5.3g–i). None of the SMARs was observed to express CGRP (Fig. 5.3g–i). Most likely, the observed CGRP-ir fibres do not represent sensory terminals that are selectively related to airway smooth muscle, but are C-fibres that are just passing by.

Immunostaining for the calcium-binding protein CRT in rat lungs revealed that many but not all SMARs were CRT-ir (Fig. 5.4a–c). CRT-ir has been described before in branching nerve endings in smooth muscle of the trachea, principal and lobar bronchi, but not in smaller bronchi in rat lungs (Yamamoto et al. 1999).

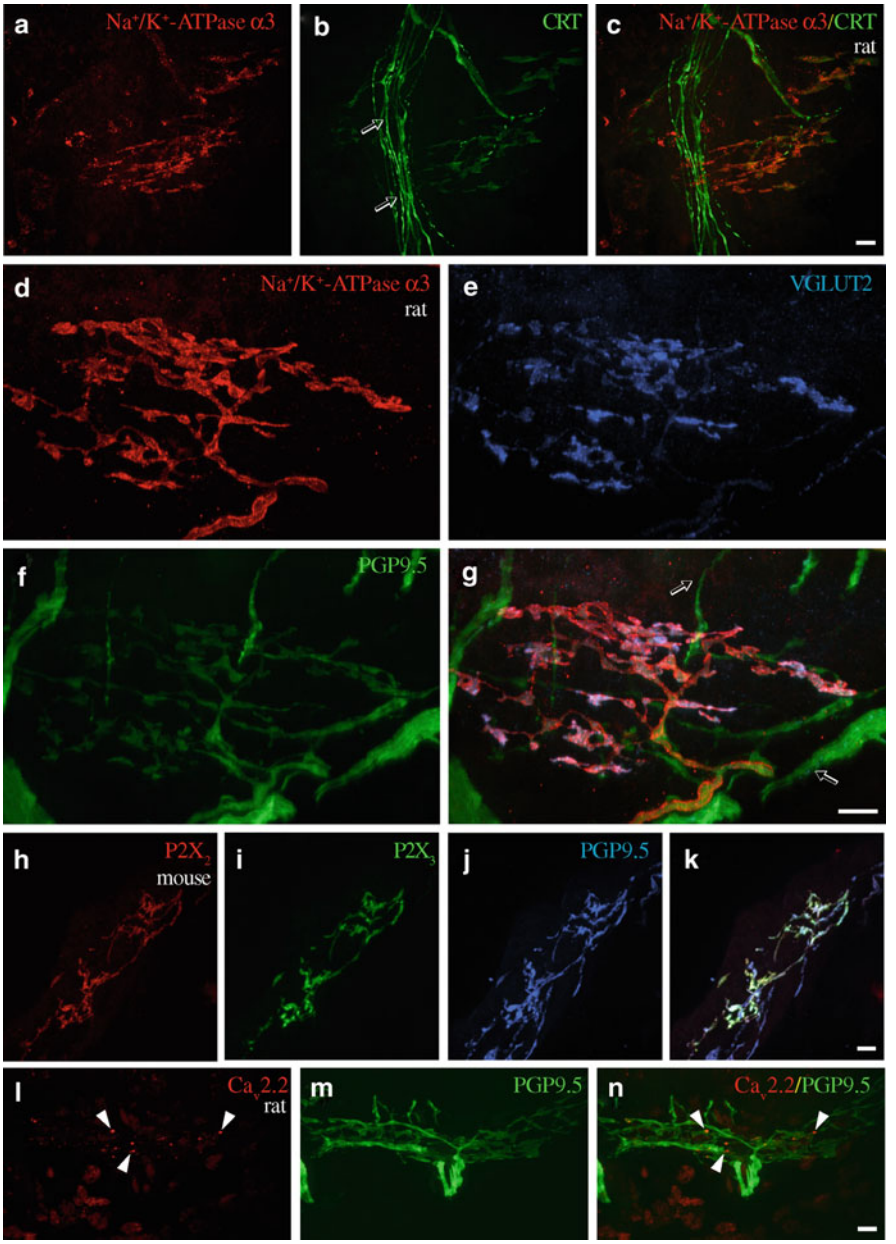

Fig. 5.4 Neurochemical characterisation of rodent SMARs. (**a–c**) Rat bronchiole. Double immunocytochemical labelling for Na^+/K^+-ATPase α3 and the calcium-binding protein calretinin (CRT), reveals a Na^+/K^+-ATPase α3-ir SMAR. CRT IR is present in the SMAR and in some additional Na^+/K^+-ATPase α3-negative nerve fibres (*open arrows*). (**d–g**) SMAR-terminals are selectively ir for Na^+/K^+-ATPase α3 and VGLUT2, while PGP9.5 IR

Therefore, calcium-binding protein immunostaining may not be a sufficiently reliable marker for SMARs.

Immunostaining for VGLUTs, as outstanding marker molecules to reveal glutamatergic nerve fibres and terminals, showed that in rodent lungs both VGLUT1 and VGLUT2 are excellent tools to specifically visualise SMARs (Figs. 5.3a–c, g–i and 5.4d–g) (Brouns et al. 2006a, b; Lembrechts et al. 2011). Whereas the staining intensity for VGLUTs of the approaching nerve fibre shows considerable variation, the cytoplasm of the laminar endings of all SMARs is consistently clearly VGLUT-ir. The expression of VGLUTs in the SMAR endings indicates their ability to locally store glutamate in secretory vesicles and to release it by exocytosis (Takamori 2006). This suggests either autocrine actions of glutamate in regulating sensory excitability and/or transduction processes, or the ability of these afferent nerve terminals to directly influence the associated smooth muscle cells.

5.1.2.2 Molecular Receptors and Ion Channels

Na^+/K^+-ATPase activity is important for the maintenance of membrane potential in excitable cells such as neurons. In rodent lungs, the peripheral sensory nerve marker Na^+/K^+-ATPase α3, appeared to be an excellent marker to label all SMARs (Figs. 5.2a, b, d and 5.4a–g). In accordance with its function as a fast plasma membrane sodium/potassium exchanger, Na^+/K^+-ATPase α3 appeared to be predominantly expressed on the surface membrane of the nerve terminals, even more accentuating the laminar appearance of SMAR endings (Brouns et al. 2006a, b; Lembrechts et al. 2011).

Immunostaining unravelled that the ATP receptor $P2X_3$ can be expressed on SMAR terminals (Figs. 5.3d–f and 5.4h–k). However, while some of the SMARs exhibited clear $P2X_3$ receptor IR, others appeared to be negative, indicating that SMAR populations with a differential expression of purinergic receptors may be involved (Brouns et al. 2006a, b; Lembrechts et al. 2011).

Voltage-gated calcium channels (VGCCs) play a crucial role in cell signalling as mediators of membrane depolarisation-induced calcium entry. The calcium channels partially control intracellular calcium concentrations and regulate processes, such as secretion, neurotransmission, muscle contraction and gene expression (Hille 1986). The channels consist of an α1 subunit, which forms the core of the channel, and auxillary subunits, which modulate the functional properties of the α1 subunit. In rat lungs, the laminar endings of SMARs revealed IR for both $Ca_v2.1$ (P/Q-type) and $Ca_v2.2$ (N-type) (Fig. 5.4l–n) channels (De Proost et al. 2007b), suggesting that depolarisation of the SMAR terminals

Fig. 5.4 (continued) is also present in other nerve fibres (*open arrows*) in the neighbourhood. (**h–k**) Triple immunocytochemical staining revealing that at least some SMAR in mouse lungs express $P2X_{2/3}$ ATP receptors. (**l–n**) $Ca_v2.2$ channels are expressed in focal spots (*arrowheads*) on the surface membranes of the laminar nerve endings of a PGP9.5-ir SMAR. *Scale bars* = 10 μm

may not only result in action potential discharge, but also in opening of Ca_v channels, causing calcium influx and subsequent neurotransmitter release.

Recently, it has been shown that the mechanogated "two-pore domain" potassium (K_{2P}) channel TRAAK is expressed on the laminar nerve terminals of SMARs in mice (Fig. 5.2c, e–g) (Lembrechts et al. 2011). Although other stimuli are able to activate TRAAK K^+ channels, mechanical stimuli such as membrane stretch, shear stress or cell swelling have been described to open the channels (Maingret et al. 1999; Kim 2003; Bayliss and Barret 2008), suggesting that SMAR terminals are involved in mechanosensation.

5.1.3 Functional Implications

The combined observation that all SMAR fibres are myelinated, disappear after vagal denervation and do not present characteristics of the motor innervation of airway smooth muscle, confirms that SMARs belong to the group of physiologically characterised myelinated vagal airway receptors.

From a morphological point of view, SMARs share distinct characteristics with established or presumed low-threshold slowly adapting mechanosensors with myelinated axons in other systems [e.g., "Ruffini-like endings" (Kannari et al. 1991); IGLEs and "intramuscular arrays" (IMAs) in the gastrointestinal tract (Berthoud et al. 1997; Phillips and Powley 2000; Powley and Phillips 2011)], contributing to the suggestion that SMARs represent at least a subpopulation of the well-known group of electrophysiologically characterised vagal airway mechanoreceptors, i.e., SARs (Adriaensen et al. 2006). Comparison of the morphology of SMARs with the earlier morphologically described "smooth muscle nerve spindles" (Larsell 1922), "pulmonary stretch receptors" (von Düring et al. 1974) and "slowly adapting stretch receptors" (SARs) (Yamamoto et al. 1999; Yu et al. 2004; Wang and Yu 2004) suggests that most likely the same structures are concerned.

It has been presumed that some SAR populations play a role in the negative feedback mechanism that inhibits increases in parasympathetic airway smooth muscle tone (Widdicombe and Nadel 1963a, b), thereby optimising the mutual relationship between dead space and airway resistance. These SARs probably sense tension within the myoelastic components of the airways caused by lung inflation, smooth muscle contraction and/or tethering of small intrapulmonary airways. Evidently, SMARs with terminals located between and parallel with the smooth muscle cells, seem to be perfectly positioned to perform such a function.

Based on the origin of SAR activity arising from the terminals of myelinated vagal sensory nerve fibres residing in airways and lungs, and on the detailed localisation and expression of Na^+/K^+-ATPase $\alpha 3$ of these terminals, it has been suggested that in rodents the observed SAR activity may potentially have been recorded from at least subpopulations of SMARs (Adriaensen et al. 2006).

Thereby, the sodium pump isozyme Na^+/K^+-ATPase $\alpha 3$, postulated as a unique marker for receptor terminals that are believed to represent the group of airway SARs (Yu et al. 2003, 2004), would be essential for the ability of SARs to continuously fire for the full duration of inspiration. More recently, Na^+/K^+-ATPase $\alpha 3$ has also been found in another group of mechanosensitive airway afferents, i.e., cough receptors, and has been suggested to be critical to the high-frequency activation required to encode coughing (Mazzone et al. 2009).

The mechanogated ion channel TRAAK is responsible for background or "leak" K^+ currents that are critical for the excitability of neurons and are broadly expressed in the nervous system, including vagal afferents (Zhao et al. 2010). Mechanical stimuli, such as membrane stretch, shear stress or cell swelling have been reported to open TRAAK channels (Maingret et al. 1999; Kim 2003; Bayliss and Barret 2008). Because TRAAK is responsible for background or "leak" K^+ currents, TRAAK K^+ channel opening drives the cell membrane towards hyperpolarising potentials, thereby leading to decreased cell activity and responsiveness (Bayliss and Barret 2008). The observation that TRAAK is expressed on SMARs implies that mechanical activation of TRAAK may modulate the excitability of these nerve terminals.

It is generally accepted that airway SARs mediate the Hering–Breuer reflex (Hering 1868; for review see Widdicombe 2006), a reflex that has also been registered in mice (Zhang et al. 2006). In this Hering–Breuer reflex the achievement of a certain lung volume threshold, registered by SAR activation, induces an inspiratory off-switch, resulting in termination of inspiration and initiation of expiration (Zhang et al. 2006). The observation that inhibitory stretch-activated TRAAK channels are expressed on the SMAR terminals is suggestive of a possible involvement of TRAAK in the inspiratory off-switch mechanism of the activity of airway SARs. During inhalation, vagal mechanosensory SAR terminals appear to be continuously activated via a so-far unknown mechanism. At depolarising potentials, the opening of TRAAK is facilitated, the threshold for mechanical activation is lowered and the maximal TRAAK channel activity increased (Maingret et al. 1999), eventually resulting in a hyperpolarising effect. At a specific point, the hyperpolarising effect of TRAAK may be sufficiently strong to prevent the nerve fibre from firing, thereby inhibiting inspiratory activation.

On the other hand, an increasing volume of air in the lungs with constant airway volume, which is today believed to mainly result in an enhanced pressure over the airway wall (called "cyclic compressive stress"), would potentially stimulate "mucosal sensors" at least as effectively as receptors located in the muscle layer, suggesting that "tension sensors" do not necessarily need to be located in the muscle layer. A similar discussion about the correlation between electrophysiologically and morphologically characterised receptors has been going on in the gastrointestinal tract, where it is now becoming increasingly clear that there are two types of vagal mechanosensors involved: (1) the IMAs, located between smooth muscle cells and most likely representing stretch receptors and (2) the IGLEs, located in enteric ganglia and regarded as tension receptors.

A full neurochemical characterisation of all morphologically characterised airway receptors therefore seems to be crucial for further interpretation.

5.2 Pulmonary Neuroepithelial Bodies

Although still neglected in discussions about the regulation of lung function (Kubin et al. 2006; Murray 2010), pulmonary NEBs are prime candidates to serve as sensory end-organs in the airways. Their organisation as clusters of pulmonary neuroendocrine cells (PNECs), closely associated with many different populations of nerve terminals, and the localisation of NEBs as an integrated component of the epithelial lining of the airways, principally make them ideal to sense changes in the airway environment and transduce this information to the CNS.

The current knowledge about their innervation patterns allows to state that NEBs represent an extensive population of very complex intraepithelial receptor structures. Although the exact physiological significance of most of the components of the innervation of NEBs is still an enigma, their connections with sensory nerve terminals, and therefore the intrinsic sensory receptor capacities of NEBs have been beyond dispute for some time now (Widdicombe 2001; Linnoila 2006; Cutz et al. 2008).

The traditionally ascribed functions of NEBs include chemoreception – especially hypoxia sensing (see further on) – and regulation of lung maturation and growth (Sorokin and Hoyt 1990, 1993; Sorokin et al. 1997). Consequently, many articles have focused on their role during embryonic and neonatal periods. Postnatally, the close association of NEB cells with nerve terminals clearly argues for a receptor function, as was already suggested more than 60 years ago (Fröhlich 1949). Whereas pulmonary NEBs are airway receptors that are morphologically well-characterised (Adriaensen et al. 2003, 2006; Brouns et al. 2009b), it is still not clear what the link is between the vagal sensory innervation of pulmonary NEBs and the numerous electrophysiological recordings of vagal afferents after intrapulmonary stimulation.

The current review summarises the present knowledge of the nervous connections of pulmonary NEBs. Because most data were obtained in rats and mice, the chemical coding, exact location and origin of the nerve terminals in contact with pulmonary NEBs will mainly be outlined in these species.

Although the NEB-associated nerve terminals on their own could perform distinct functions, it remains challenging to find out what may be the meaning and possible input of the neuroendocrine NEB cells and the covering specialised Clara-like cells (CLCs), in the NEB complex. After a short literature review on the general morphological aspects of pulmonary NEBs, their nervous connections will be outlined. A full review on the neurochemical coding of the neuroendocrine

cells will, evidently, be helpful for speculation on and interpretation of the role of NEBs in pulmonary functioning.

5.2.1 General Morphological Aspects of Pulmonary NEBs

The first observations of organised "neuroepithelial" structures within the airway epithelium can be traced back to the turn of the nineteenth century (Berkley 1894). Later on, the presence of irregularly distributed clusters of intraepithelial nerve terminals in lungs were described in many animal species (Larsell 1921; Larsell and Dow 1933; Elftman 1943; Honjin 1956; Spencer and Leof 1964). Most likely, these reported terminals constituted the intraepithelial innervation of pulmonary NEBs, at present unquestionably observed in both light and electron microscopic investigations (Scheuermann 1987; methodological overview see Adriaensen et al. 2003).

The first descriptions of groups of neuroendocrine cells, on the other hand, may be credited to Feyrter (1938, 1953) and Fröhlich (1949), who recognised clusters of "clear cells" ("helle Zellen") in light microscopic sections of mammals after routine light microscopic staining, and found that nerve endings were in close contact with these clear cells. At the time, data were limited as a consequence of the small number of PNECs present in the lung, their highly scattered appearance between the bulk of other pulmonary epithelial cells and the lack of reliable methods for their selective demonstration.

Whereas solitary PNECs are mainly present in extrapulmonary airways and in intrapulmonary bronchi, groups of neuroendocrine cells (NEBs) are found in bronchi, bronchioles and respiratory areas. In several species, NEBs were reported to be preferentially located at intrapulmonary airway bifurcations (Wasano 1977; Wasano and Yamamoto 1981; Cho et al. 1989; Montuenga et al. 2003), although this location is never limiting. In other species or even other strains of the same species such a favourite position is less obvious (Carabba et al. 1985; Gomez-Pascual et al. 1990; De Proost et al. 2007b).

There exists a general impression that the number of NEBs declines shortly after birth. However, studies in mammals (Cutz et al. 1984; Redick and Hung 1984; Van Lommel and Lauweryns 1997) showed that the absolute number of NEB cells in adult lungs appears to be comparable to foetal or neonatal lungs. The apparent decrease in the NEB population is now believed to be mainly caused by a "dilution effect" of the population of NEBs, which is established before birth, and the postnatal expansion of the lungs, in which the non-endocrine tissue is highly amplified compared to the endocrine tissue (Gosney 1993). In rat lungs, the total number of NEBs has been estimated to be around 3,500 (Van Genechten et al. 2004), resulting in the same number of possible receptor points.

The most significant ultrastructural feature for the indisputable identification of PNECs is the presence of numerous typical dense-cored vesicles (DCVs), small

endocrine-like secretory granules mainly situated at the basal cell pole, ranging from 60 to 200 nm in diameter and showing a characteristic electron-dense core that is separated from the limiting membrane by a clear "halo" (for review see Scheuermann 1987). Number, size and electron density of DCVs may vary between species, within one animal, and often from one PNEC to another. Two or more signalling molecules have been demonstrated in the same DCVs (Dey and Hoffpauir 1986; Stahlman et al. 1987) and transmission electron microscopic studies were even able to provide indirect evidence for the release of the content of DCVs by exocytosis (Lauweryns et al. 1985; Scheuermann 1987; Van Lommel and Lauweryns 1993a). The released transmitters might bind to structures in very close proximity to NEBs, e.g., smooth muscle cells and NEB-associated nerve terminals, or might be taken up in the blood stream by thin-walled fenestrated capillaries that are sometimes found within <1 μm from the base of NEBs (Van Lommel and Lauweryns 1993a), suggesting an endocrine function (Adriaensen and Scheuermann 1993). Because of the presence of characteristic endocrine-like DCVs, neuroendocrine cells of the airway epithelium belong to the "diffuse neuroendocrine system (DNES)" (Pearse 1977), a system that is known to play an important role in the local control of various organs.

In NEBs, all constituent NEB cells seem to rest on the basal membrane. Interestingly, in the NEBs of most animal species, the apical poles of PNECs are largely covered by a unicellular layer of a special type of Clara cells, the so-called Clara-like epithelial cells (Cutz et al. 1974; Hung 1982; Stahlman and Gray 1984; Pearsall et al. 1985; Haller 1994), that are sealed with tight junctions (De Proost et al. 2008). Only a few narrow pores between the CLCs allow direct access of NEB cells to the airspace via slender apical processes that bear long microvilli. As an exception, in late foetal/neonatal rabbit lungs, NEBs show most of their apical membrane surfaces to be widely exposed to the airway lumen (Cutz et al. 1978). NEB cells, the covering CLCs and the network of multiple nerve fibre populations that contacts and protrudes between the NEB cells, form a very specialised organoid structure today referred to as the "NEB microenvironment".

5.2.2
Selective Innervation of Neuroepithelial Bodies

5.2.2.1

Observations of the Innervation of NEBs at the Electron Microscopic Level

Using transmission electron microscopy (TEM), different types of morphologically characterised nerve terminals have been described in contact with NEBs, but a majority of them appeared to have afferent (sensory) features (Cook and King 1969; Wasano and Yamamoto 1978; Lauweryns et al. 1985; Lauweryns and Van Lommel 1987; Adriaensen and Scheuermann 1993; Van Lommel and Lauweryns 1993b; Adriaensen et al. 2006).

The most often reported type of innervation of NEBs consists of nerve fibres that are observed to penetrate the basement membrane as unmyelinated processes that widen and enter into the intercellular space between the NEB cells. The terminals of these nerve fibres are packed with small mitochondria, suggesting their afferent nature. Locally, the nerve endings are seen to form synapses with NEB cells (for review see Adriaensen et al. 2006). At the level of these asymmetric synaptic contacts, DCVs accumulate near electron-dense cone-shaped thickenings of the surface membrane of the NEB cells. This type of synapse is indicative of signals passing from NEB cell to nerve ending, implying afferent signalling to the CNS. Frequently, these nerve endings can be found between the apex of NEB cells and the covering CLCs (for review see Adriaensen et al. 2006). No direct contacts have been observed between the nerve endings in NEBs and the airway lumen. In favourable sections, nerve endings were seen to form "loops" over some NEB cells (for review see Adriaensen et al. 2003). The intraepithelial nerve terminals were reported to disappear after infranodosal, but not supranodosal vagotomy [rabbits: (Lauweryns et al. 1985; Lauweryns and Van Lommel 1986); rats: (Van Lommel and Lauweryns 1993a)]. In only very few cases, it has been reported on EM level that these sensory nerve terminals originate from myelinated nerve fibres, that lose their myelin sheath before entering the pulmonary NEB (Van Lommel and Lauweryns 1993a).

Much more rare, intraepithelial nerve endings with small clear cholinergic-type synaptic vesicles that accumulate near synaptic specialisations, and sometimes a few larger DCVs and a mitochondrion could be observed (Walsh and McLelland 1978; Sonstegard et al. 1982; Stahlman and Gray 1984; Adriaensen and Scheuermann 1993). These morphologically efferent-like nerve endings were regarded as axon collaterals of the intraepithelial sensory nerve endings (Scheuermann 1987; Van Lommel and Lauweryns 1993a). The two kinds of synaptic regions could be seen side by side in a single nerve terminal in some lower vertebrates and were referred to as reciprocal synapses (Scheuermann 1987).

Without any doubt, TEM has offered a good morphological characterisation of the direct innervation of pulmonary NEBs. However, due to the preparation techniques intrinsic to TEM, only a limited number of NEBs has been examined, giving rise to incomplete and conflicting data and interpretations.

5.2.2.2

Observations of the Innervation of NEBs at the Light Microscopic Level

To be able to reveal their overall innervation pattern, it is important to allow the simultaneous observation of numerous NEBs. Currently, combinations of chemical or mechanical denervation, ultrasensitive immunohistochemistry, tracing studies and confocal microscopy are used to elucidate the neurochemical coding and origin of the different nerve fibre populations that selectively innervate NEBs.

To obtain general information about the distribution of NEBs and their connecting nerve terminals, unspecific neuronal and neuroendocrine markers

like PGP9.5 (Lauweryns and Van Ranst 1988a; Larson et al. 2003), NSE (Adriaensen and Scheuermann 1993), SV2 (Pan et al. 2004, 2006a) and synaptophysin (SYN) (Lee et al. 1987), have been shown to be very useful. It is now clear that pulmonary NEBs become gradually innervated, with the density of nerve fibres increasing with progressing gestation, and peaking postnatally, consistent with the process of maturation (rat, rabbit, mouse) (Sorokin et al. 1997). One of the limitations of using immunolabelling with general neuronal and neuroendocrine markers is that those markers do not discriminate between different nerve subtypes or their origin, which are valuable features in view of functional interpretations.

Although multiple immunohistochemical staining has revealed that the innervation of pulmonary NEBs is subject to species variation, it is obvious that in all species examined so far NEBs are contacted by vagal sensory nerve terminals (Lauweryns et al. 1987; Van Lommel et al. 1995; Cutz and Jackson 1999; Van Lommel et al. 1999; Bollé et al. 2000; Adriaensen et al. 2003, 2006; Widdicombe 2009; Brouns et al. 2009a, b). Simultaneously, the use of new marker molecules for nerve terminals unravelled an intricate innervation pattern of this receptor end-organ that is still revealing its hidden complexities. Because most studies about the neurochemical coding of the innervation of NEBs have been carried out in rats and mice, an overview of the selective innervation of pulmonary NEBs in these species will be outlined in the next sections.

Selective Innervation of Neuroepithelial Bodies in Rat Lungs (For a Summary, See Fig. 5.5)

Vagal Sensory Connections

Although it was suggested in the earlier literature for many years, direct proof for the vagal nodose sensory innervation of pulmonary NEBs came from tracing experiments in rats. A few weeks after injection of the red fluorescent lipophilic carbocyanine tracer DiI (Honig 1993) in the nodose ganglion, DiI-traced nerve endings were seen in the lungs, ramifying in the epithelium between NEB cells in a candelabrum-like pattern (Fig. 5.6a, b) (Van Lommel et al. 1998; Adriaensen et al. 1998). These vagal nodose intraepithelial nerve terminals could be correlated to the intraepithelial mitochondria-rich afferent nerve terminals (Van Lommel et al. 1998), the most constantly reported innervation of NEBs.

Because neuronal tracing is a labour-intensive and time-consuming technique, and hardly compatible with the routine identification of nerve fibre populations, the neurochemical coding of this vagal nodose population of nerve terminals in NEBs needed to be further characterised.

Multiple immunostaining with antibodies against marker molecules for glutamatergic nerve terminals revealed that intraepithelial sensory nerve terminals in pulmonary NEBs invariably express VGLUTs (Brouns et al. 2004, 2006a, 2006b).

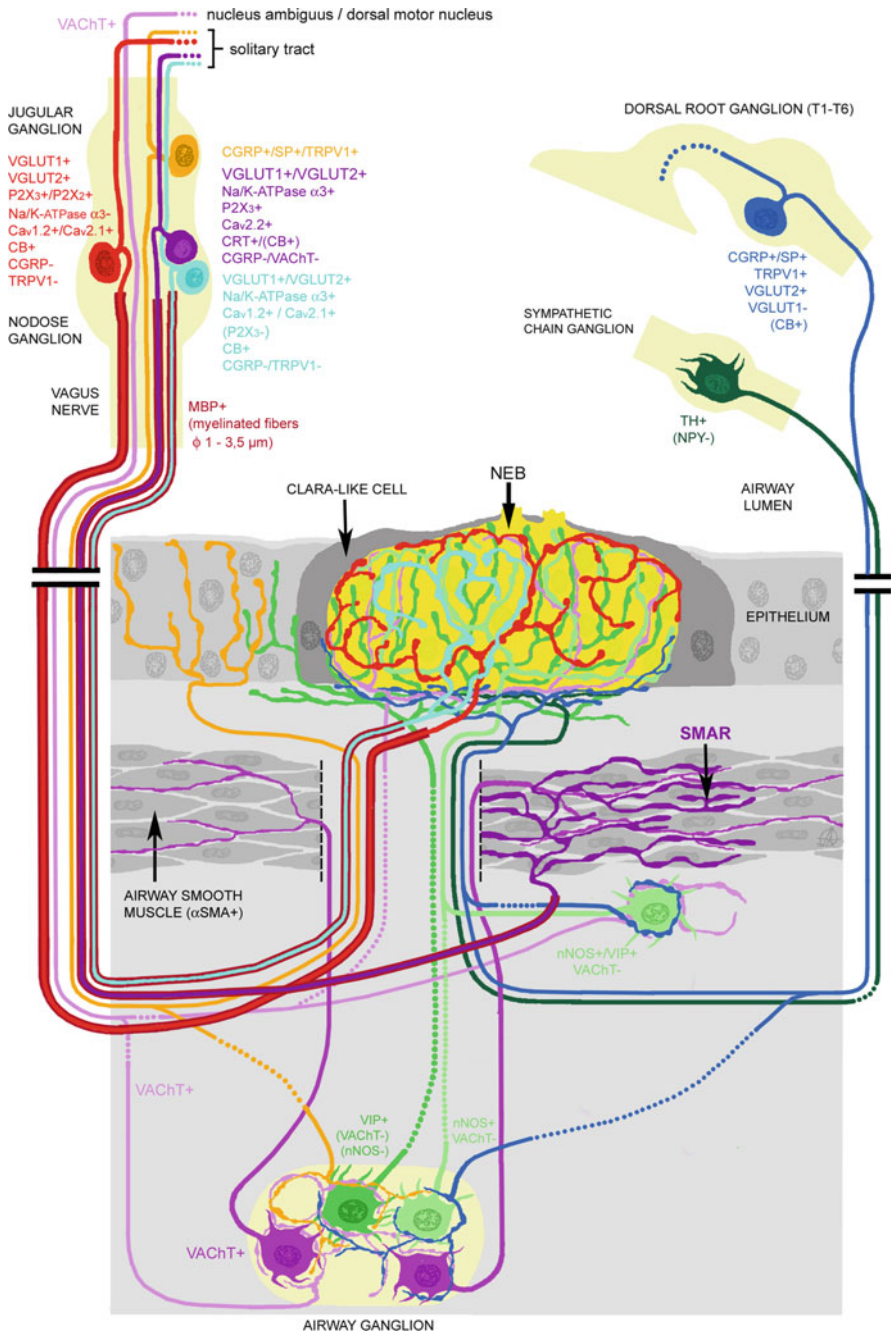

Fig. 5.5 Schematic representation of the main innervation of *airway smooth muscle* and of the sensory innervation of complex *NEB* receptors in rat lungs. Known characteristics of

Both VGLUT1 (Fig. 5.6e, f) and VGLUT2 IR (Fig. 5.6c, d, g–m) were detected in intraepithelial nerve terminals that make extensive contact with most cells in a NEB, and with almost all of the NEBs (Brouns et al. 2004, 2006a, b). Combinations of VGLUT1 and VGLUT2 immunostaining have, however, been technically difficult. For indisputable staining of intraepithelial sensory nerve terminals in rat pulmonary NEBs, VGLUT2 appeared to be the marker of choice. Since the expression of VGLUTs is, at present, regarded as an unequivocal identification of the glutamatergic nature of neurons (Takamori 2006), the detection of VGLUT1 or VGLUT2 in profuse nerve fibre populations that selectively contact pulmonary NEBs in rats, should be considered to reflect the capacity of these terminals to store and release glutamate as a neurotransmitter at the level of NEB cells. The vagal nature of the intraepithelial nerve terminals was demonstrated by the loss of intraepithelial VGLUT-ir nerve terminals in NEBs in the ipsilateral lung after unilateral infranodosal vagotomy. Apart from a very small population, all vagal sensory VGLUT-ir nerve fibres also expressed CB IR (Fig. 5.6c–m). CB IR of the vagal sensory innervation of NEBs was also proven by combinations of unilateral infranodosal vagal denervation and immunohistochemistry experiments (Brouns et al. 2000).

$P2X_3$ purinoreceptors (belonging to the family of ligand-gated ATP receptors) are present on the vagal nodose nerve fibre population that selectively contacts NEBs (Fig. 5.7a–i) (Brouns et al. 2000; Van Genechten et al. 2004). Combination of quinacrine histochemistry, to label ATP-storing cells, and $P2X_3$ receptor staining showed that the ATP receptor-expressing vagal sensory nerve terminals in rat lungs are specifically associated with quinacrine-stained NEB cells (Brouns et al. 2000). As has already been suggested (Adriaensen and Timmermans 2004), $P2X_3$ receptors on vagal sensory nerve terminals in NEBs seem to form heteromultimers with $P2X_2$ receptors (Fig. 5.7d–f; own unpublished observations).

Intraepithelial vagal sensory nerve terminals expressing the plasma membrane sodium/potassium exchanging protein subunit Na^+/K^+-ATPase α3, can be double stained with antibodies against VGLUTs. However not all VGLUT-ir nerve terminals express Na^+/K^+-ATPase α3. Na^+/K^+-ATPase α3-ir nerve endings appear to form unique "caps" over the apical pole of NEB cells.

Further neurochemical identification of the vagal nodose nerve fibre population showed that two different VGLUT/CB-ir nerve endings have to be concerned, which both reveal extensive intraepithelial terminals in NEBs: (1) those additionally expressing $P2X_{2/3}$ receptors; (2) those additionally expressing Na^+/K^+-ATPase α3 (Figs. 5.7g–i and 5.8a, b). When both types of terminals are present in the same pulmonary NEB, they seem to typically innervate separate groups of NEB cells.

Fig. 5.5 (continued) the represented neuronal populations are included in the scheme in the same colour as the respective structures. The pulmonary NEB cells (*yellow*) express ATP, CGRP, CB, 5HT, calcitonin, VAChT, $P2X_2$ (ATP) receptors and $Ca_v2.1$. Clara-like cells (*dark grey*) can be distinguished by their location and their IR for CCSP, $P2Y_2$ and $Ca_v2.3$

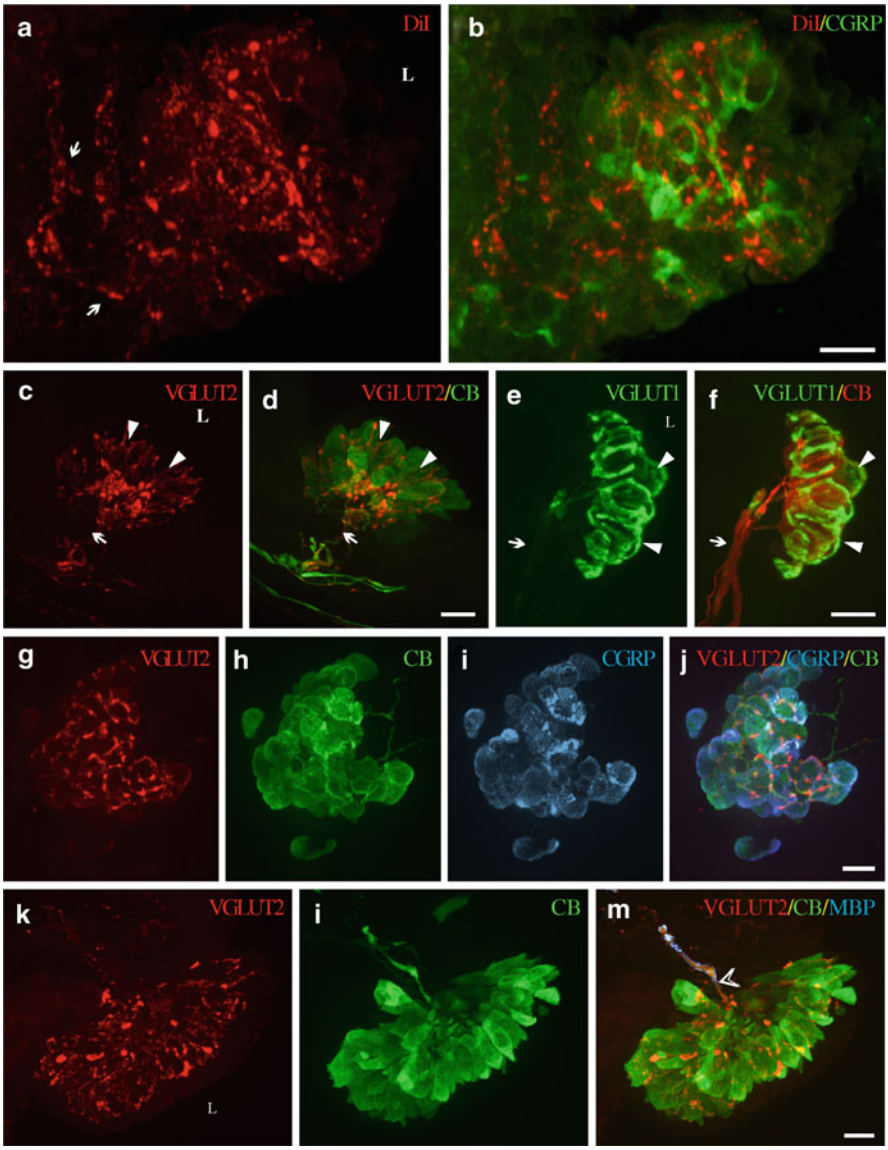

Fig. 5.6 Main characteristics of the vagal nodose sensory innervation in rat lungs. (**a**, **b**) A *red*-fluorescent DiI-containing nerve fibre (*arrows*), traced from the vagal nodose ganglion in a rat, approaches the airway epithelium, branches and gives rise to an abundance of traced nerve terminals in the epithelium. Subsequent immunostaining with CGRP (*green*; as a marker of rat NEBs) shows that the DiI-traced vagal sensory nerve terminals run between the NEB cells. (**c**, **d**) Vagal sensory VGLUT2+/CB+-ir nerve fibres (*arrow*) approach the airway epithelium, branch and form an extensive intraepithelial arborisation of nerve terminals (*arrowheads*) that innervate a CB-ir pulmonary NEB. It is clear that

Vagal sensory nerve fibre populations that selectively contact pulmonary NEBs revealed Ca_v 1.2 (Fig. 5.8c, d) and Ca_v2.1 IR (Fig. 5.8e–g), invariably co-localised with VGLUT2 IR, but not all VGLUT2-ir nerve fibres appeared to express Ca_v channels, confirming that different VGLUT2-ir nerve fibre populations contact NEBs (De Proost et al. 2007b).

A functionally very important feature of the sensory component of the innervation of NEBs is the presence of myelin sheaths. MBP immunostaining showed that in adult rat lungs the vagal nodose sensory nerve fibres that contact NEBs are surrounded by myelin sheaths that are lost in the immediate neighbourhood of the NEB, just before branching of the nerve terminals (Figs. 5.6k–m and 5.7a–c) (Brouns et al. 2000, 2003b, 2004, 2006a, b). The latter myelinated vagal sensory nerve fibres have diameters ranging from 1 to 3.5 μm Although myelinated nerve fibres had been reported in the vicinity of NEBs using TEM (Van Lommel and Lauweryns 1993a), MBP immunostaining provided the first evidence for a direct link between the myelinated fibres and the two populations of vagal nodose intraepithelial nerve terminals in NEBs.

The vanilloid capsaicin is known to establish a long-lasting depletion of certain populations of afferent nerve fibres (see Sect. 2.3: C-fibre receptors; for review see Holzer 1991), also in rat lungs (Cadieux et al. 1986; Martling et al. 1988; Shimosegawa and Said 1991a). Systemic treatment of rats with capsaicin revealed no changes in the CB-ir vagal nodose innervation of NEBs as compared to control rats, strongly suggesting that a capsaicin-insensitive population is concerned (Brouns et al. 2003b). Double staining for the capsaicin receptor TRPV1 and CB confirmed that the vagal nodose component of the innervation of NEBs in rats does not express the capsaicin receptor (Brouns et al. 2003b).

Spinal Sensory Connections

For many years now, CGRP-positive nerve fibres have been reported to contact CGRP-ir NEBs at all levels of rat intrapulmonary airways (Fig. 5.9a, b) (Cadieux et al. 1986; Shimosegawa and Said 1991b; Terada et al. 1992; Sorokin et al. 1997). Retrograde tracing from the lungs (Springall et al. 1987) and denervation studies revealed that the CGRP-ir nerve fibre population of rat pulmonary NEBs is

Fig. 5.6 (continued) especially VGLUT2 is an excellent marker for the vagal sensory innervation of rat NEBs. (**e**, **f**) CB-ir NEB cells are in abundant contact with laminar VGLUT1-ir vagal sensory nerve terminals (*arrowheads*). VGLUT1 fluorescence is very intense in the nerve terminals, but less in the approaching nerve fibre (*arrow*), which can be visualised by CB IR. (**g**–**j**) Triple immunocytochemical staining for VGLUT2, CB and CGRP. Pulmonary CB+/CGRP+ NEB cells are extensively innervated by VGLUT2+/CB+-ir vagal sensory nerve terminals. (**k**–**m**) Pulmonary CB-ir NEB innervated by CB+/VGLUT2+ nerve terminals. Note that the vagal sensory nerve fibre that approaches the airway epithelium is surrounded by an MBP-ir myelin sheath that disappears (*open arrowhead*) right before branching of the nerve fibre. *Scale bars* = 10 μm

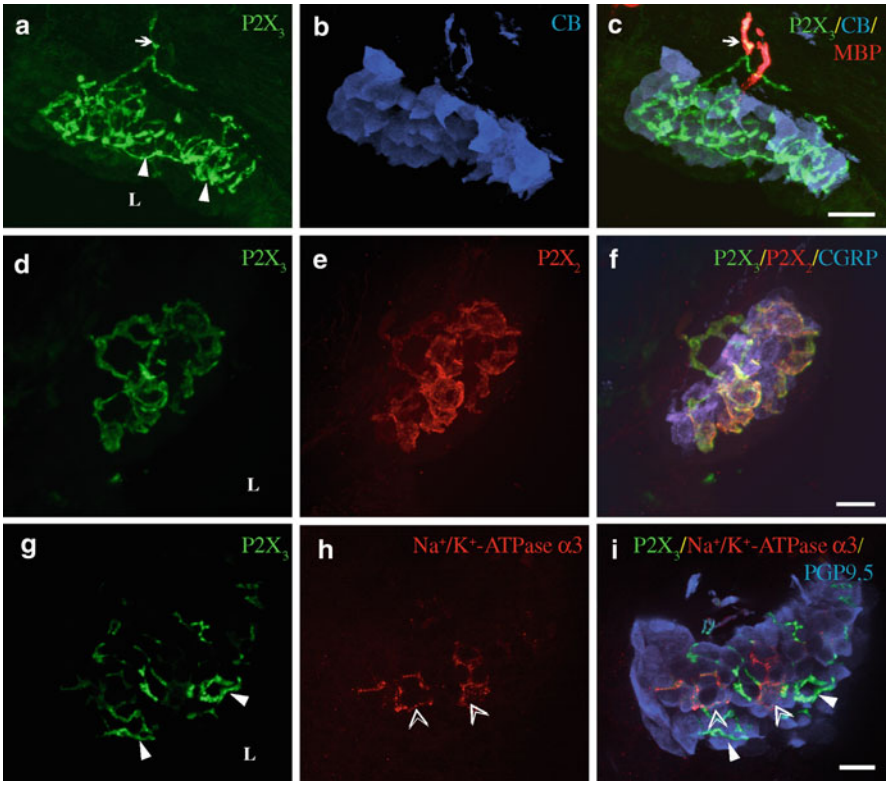

Fig. 5.7 Characterisation of the vagal sensory $P2X_3$-ir innervation of rat pulmonary NEBs. *L*: airway lumen. (**a–c**) A $P2X_3$-ir nerve fibre, surrounded by an MBP-ir myelin sheath (*arrow*), approaches the airway epithelium and forms abundant intraepithelial terminals (*arrowheads*) between CB-ir NEB cells. (**d–f**) CGRP-ir pulmonary NEB innervated by $P2X_{2/3}$-ir nerve terminals. Although $P2X_3$ and $P2X_2$ IR are expressed in the same intraepithelial nerve terminals, the staining intensity for both ATP receptors may vary from terminal to terminal. (**g–i**) PGP9.5-ir pulmonary NEB contacted by vagal sensory nerve fibres that give rise to separate intraepithelial $P2X_3$-ir (*arrowheads*) and Na^+/K^+ ATP-ase α3 (*open arrowheads*) nerve endings that innervate different NEB cells. *Scale bars* = 10 μm

non-vagal (Adriaensen et al. 1998; Brouns et al. 2003b). CGRP-ir nerve fibres that selectively contact NEBs in rat lungs belong to a spinal sensory population that originates from thoracic DRGs. Double immunocytochemical staining for CGRP and the above-mentioned markers for the vagal sensory connections of NEBs have proven that the CGRP-ir nerve endings are clearly different from the vagal nodose sensory component of the innervation of NEBs (Adriaensen et al. 1998, 2006; Brouns et al. 2000, 2004).

CGRP-positive nerve terminals in contact with rat pulmonary NEBs, appear as thin varicose nerve endings. One of the important features of these CGRP-ir nerve endings is the observation that they all seem to co-localise SP, making CGRP/SP

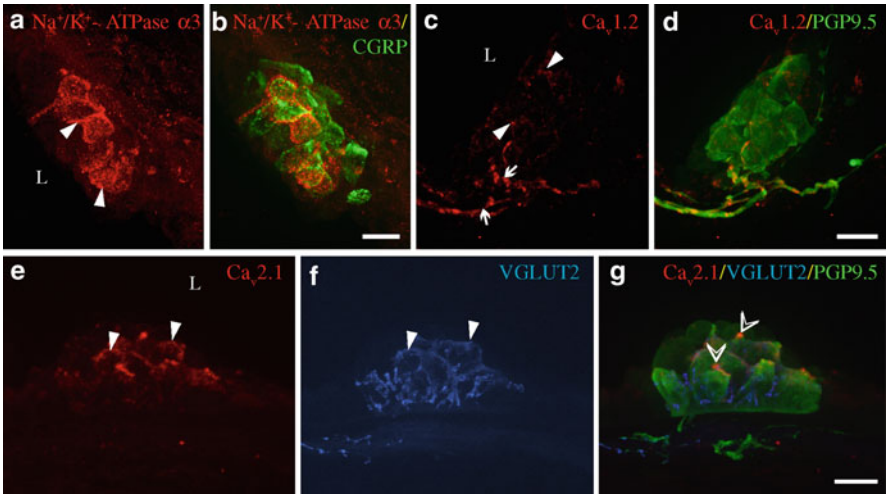

Fig. 5.8 Functional morphological characteristics of the vagal nodose sensory innervation of rat NEBs. (**a**, **b**) Immunocytochemical staining for Na^+/K^+-ATPase α3 and CGRP shows that some, but not all, CGRP-ir NEB-cells are surrounded by Na^+/K^+-ATPase α3-ir nerve terminals. (**c**, **d**) Double immunolabelling for $Ca_v1.2$ and PGP9.5, showing that $Ca_v1.2$ is expressed on nerve fibres (*arrows*) that approach a NEB, branch and form a complex of intraepithelial terminals(*arrowheads*) between the NEB cells. (**e**–**g**) Triple immunostaining for $Ca_v2.1$,VGLUT2 and PGP9.5 reveals that the $Ca_v2.1$-ir spots that are not in contact with the airway lumen (*L*) co-localise with intraepithelial VGLUT2-ir terminals of the vagal sensory innervation. The *open arrowheads* point to the spot-like staining of $Ca_v2.1$ in the tip of the apical processes of NEB cells that reach the luminal surface. *Scale bars* = 10 μm

double labelling a valid tool to differentiate between the individual CGRP+/SP+ nerve terminals and the NEB cells, which in rats store CGRP, but not SP (Polak et al. 1993). Confocal microscopy revealed that in rats the CGRP+/SP+ nerve terminals do not penetrate the epithelium, but rather form a nerve plexus at the basal pole of the NEBs (Brouns et al. 2003b; Van Genechten et al. 2004).

The thin, varicose spinal CGRP+/SP+ nerve fibres that selectively contact NEBs were further shown to express VGLUT2 IR (Brouns et al. 2004), but with varying intensities both within single fibres and between fibres. Also a very weak CB IR was observed in the CGRP+/SP+ nerve fibres. Since very sensitive immunolabelling procedures are required for the detection of VGLUT2 and CB, CGRP and/or SP immunostaining appears to be the method of choice for visualising the spinal sensory connections of rat NEBs.

After capsaicin treatment, the percentage of NEBs contacted by CGRP/SP-positive nerve terminals was dramatically reduced compared to control lungs, while the number of CGRP-ir NEBs remained unchanged (Brouns et al. 2003b). All CGRP-ir nerve fibres in the vicinity of and contacting NEBs appeared to express TRPV1 (Fig. 5.9a, b) and may therefore be considered capsaicin-sensitive. In

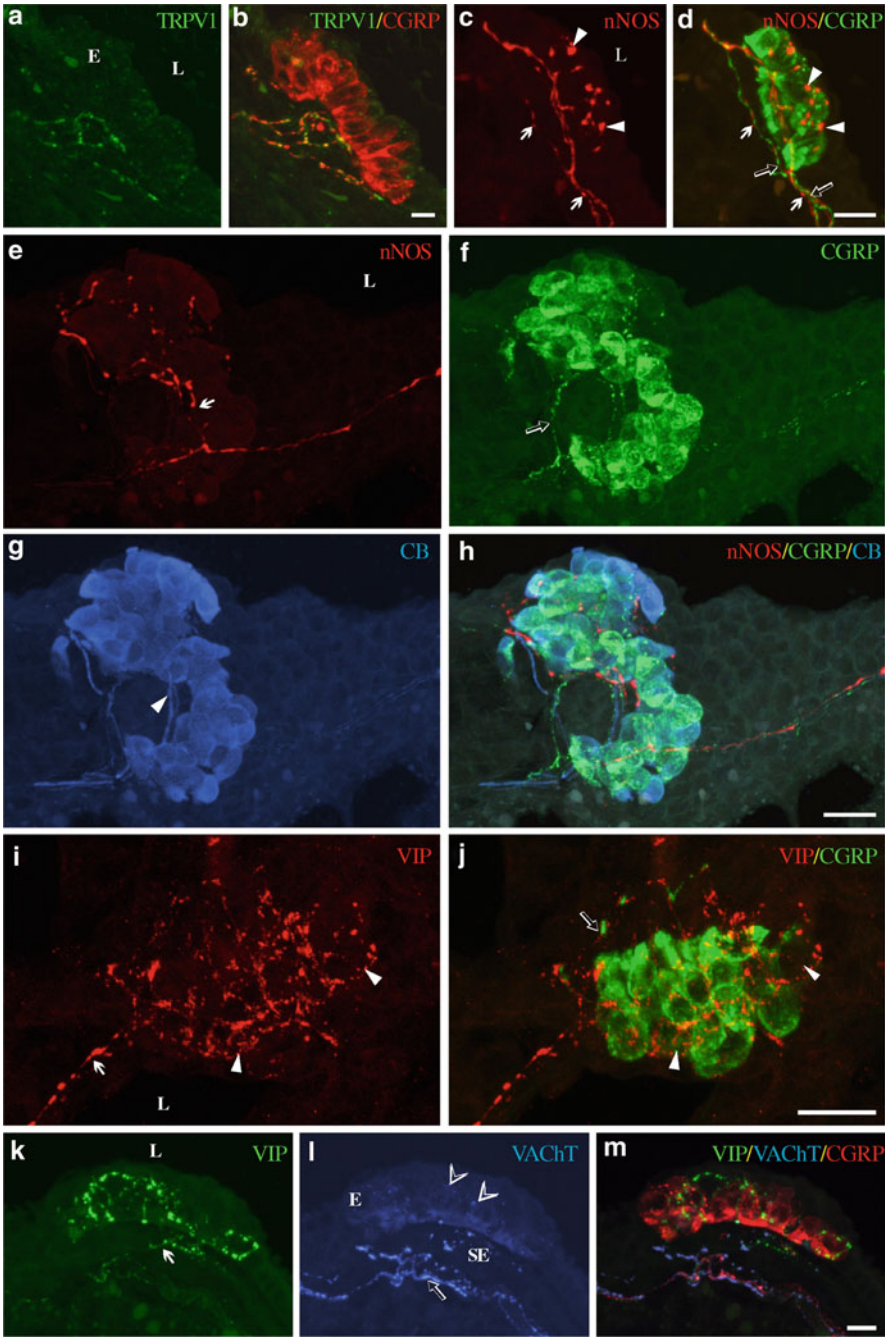

Fig. 5.9 Overview of some of the different nerve fibre populations that are associated with rat pulmonary NEBs. *L*: airway lumen (**a, b**) CGRP immunostaining labels NEB cells, as well

capsaicin-treated lungs, NEB cells, which do not express TRPV1 did not show obvious differences to control lungs (Shimosegawa and Said 1991b; Brouns et al. 2003b).

All thin varicose CGRP+/SP+-fibres contacting NEBs were found to be unmyelinated. Considering all the above-mentioned features, the spinal sensory nerve fibres in contact with pulmonary NEBs display obvious C-fibre characteristics (Adriaensen et al. 2003).

Efferent Innervation

The functional morphology of rat pulmonary NEBs clearly appears to be much more complex than could ever be predicted by EM studies alone (Van Lommel and Lauweryns 1993a). Detailed studies have indicated that several other nerve fibre populations provide additional nervous connections to NEBs.

Immunodetection of the NO-synthesising enzyme neuronal nitric oxide synthase (nNOS) revealed that part of the pulmonary NEBs in rats are selectively innervated by nNOS-ir (nitrergic) nerve fibres (Fig. 5.9c–h) (Brouns et al. 2002a, b). The latter were observed to give rise to extensive intraepithelial nerve terminals in pulmonary NEBs. Apparently, these nerve terminals originate from a non-

Fig. 5.9 (continued) as the spinal sensory innervation of rat NEBs. Double immunostaining for transient receptor potential vanilloid 1 (TRPV1), the capsaicin receptor, shows that the spinal sensory nerve terminals in contact with the base of the NEB cells express both CGRP and TRPV1. No TRPV1 IR is seen in NEB cells (*E:* epithelium). (**c**, **d**) CGRP-ir NEB contacted by neuronal nitric oxide synthase (nNOS) – and CGRP-ir nerve terminals. In the subepithelial region spinal sensory CGRP-ir nerve fibres (*open arrows*) run in the very close proximity to the nNOS-ir nerve fibres (*arrows*). While the CGRP-ir terminals contact the base of the NEB, the nNOS-ir nerve fibres form an extensive intraepithelial arborisation (*arrowheads*). (**e–h**) (**e**) An nNOS-ir intraepithelial arborisation originates from a subepithelial nerve fibre (*arrow*). (**f**) CGRP-IR NEB cells contacted by CGRP-IR nerve fibres (*open arrow*). (**g**) A bundle of CB-ir nerve fibres (*arrowhead*) contacting CB-IR NEB cells. (**h**) nNOS, CGRP and CB are located in separate nerve fibre populations. The nerve fibres of the different populations are in many places so close to one another that they could only be distinguished in single confocal optical sections. (**i, j**) Pulmonary CGRP-ir NEB innervated by varicose vasoactive intestinal polypeptide (VIP)-ir nerve terminals (*arrowheads*) that ramify between the neuroendocrine cells. The extensive VIP-ir nerve endings arise from a VIP-ir nerve fibre (*arrow*) that does not co-localise with the spinal sensory CGRP-ir innervation of the NEB. (**k–m**) Pulmonary NEB triple immunostained for VIP, VAChT and CGRP. (**k**) A VIP-ir intraepithelial arborisation originates from a subepithelial nerve fibre (*arrow*). (**l**) Extensive VAChT-ir nerve fibres (*open arrow*) in the subepithelial area (*SE*). Weak VAChT IR is present in the epithelium (*E*), as well as in some intraepithelial VAChT-IR "dots" (*open arrowheads*). (**m**) Combination of the three channels reveals that VIP, VAChT and CGRP are located in separate nerve fibre populations, only to be distinguished by confocal microscopy. *Scale bars* = 10 μm

cholinergic population of nitrergic neurons, located in the lamina propria of intrapulmonary airways (Brouns et al. 2002a).

These intrinsic nNOS-ir neurons were shown to co-localise vasoactive intestinal polypeptide (VIP) (Adriaensen et al. 2003). The nNOS+ neuronal cell bodies appeared to be invariably surrounded by a basket of CGRP-ir nerve terminals that seemed to originate from CGRP-ir fibres that, starting from the NEB area, spiral around the axons of the nitrergic neurons (Brouns et al. 2002a; Van Genechten et al. 2004).

nNOS IR was absent from the spinal afferent and from the vagal nodose afferent nerve fibre populations that selectively contact NEBs. Quantitative analysis revealed that all NEBs receiving nNOS-ir terminals were also contacted by spinal sensory CGRP-ir nerve fibres, while only about half of them was additionally contacted by vagal nodose fibres (Brouns et al. 2002a). Since nitrergic neuronal cell bodies are always surrounded by a basket of CGRP-ir nerve terminals, presumably collaterals of the spinal afferent nerve fibres contacting NEBs, a direct relationship between the nitrergic and spinal sensory nerve fibre populations contacting NEBs has been suggested (Brouns et al. 2002a; Adriaensen et al. 2003; Van Genechten et al. 2004), whereas the correlation between nitrergic and vagal sensory nerve terminals is less obvious. In Fawn-Hooded rats, a model for primary pulmonary hypertension, the number of intrinsic pulmonary nitrergic neurons and the percentage of pulmonary NEBs revealing a nitrergic innervation have been shown to be significantly lower (Van Genechten et al. 2003, 2004).

A considerable number of NEBs appeared to be contacted by profuse beaded VIP-ir intraepithelial nerve terminals (Fig. 5.9i–m) (Adriaensen et al. 2003; Van Genechten et al. 2004). Although VIP IR has also been shown to be localised in the intrinsic nitrergic neurons that give rise to the nitrergic terminals contacting NEBs, we believe that an additional population of VIP-expressing nerve endings with a so-far unidentified origin may be involved in the selective innervation of NEBs.

As already mentioned, electron microscopy showed that NEBs in rat lungs are contacted by nerve endings that contain small electron-lucent cholinergic-like synaptic vesicles and often reveal synaptic contacts with NEB cells (Adriaensen and Scheuermann 1993). Antibodies against VAChT were, therefore, used to visualise possible cholinergic nerve terminals in direct relation to NEBs (Pintelon et al. 2003; Van Genechten et al. 2004). Weakly VAChT-ir intraepithelial cell groups, characterised as NEBs after multiple immunostaining, indeed appeared to be contacted by VAChT-ir nerve fibres (Fig. 5.9k–m). Although the latter nerve fibre population is not yet fully characterised, we have evidence (unpublished observations from tracing studies) suggesting that cholinergic motor fibres, directly originating from preganglionic parasympathetic neurons in the nucleus ambiguus, and hence evidently a so-far not reported population, may be concerned.

Preliminary data further revealed that TH-ir nerve terminals selectively contact some of the rat pulmonary NEBs at their basal pole (Van Genechten et al. 2004). These nerve fibres are likely noradrenergic and have their origin in sympathetic ganglia.

Selective Innervation of Neuroepithelial Bodies in Mouse Lungs

In view of the interesting possibilities of using genetically modified mice for functional studies [e.g., Cftr-knockout mice (Pan et al. 2006a)], detailed knowledge about sensory lung receptors in control mice is essential. Therefore, we recently characterised the complex innervation pattern of pulmonary NEBs in different mouse strains.

In mice, VGLUT1 immunostaining revealed nerve fibres that give rise to intraepithelial terminals protruding between NEB cells (Fig. 5.10a, b). The vagal nature of these terminals was demonstrated by the loss of VGLUT1 IR in NEB-related nerve endings in the ipsilateral lung after unilateral infranodosal vagotomy. VGLUT2, on the other hand, could be detected in intraepithelial nerve terminals in mouse NEBs only after highly sensitive TSA-enhanced immunostaining. VGLUT2 IR varied both between and within VGLUT1-positive nerve fibres, being most of the time very weak. It is clear that VGLUT1 immunostaining is the marker of choice for identification of the intraepithelial vagal sensory nerve terminals in mouse NEBs. Combination of VGLUT1 and CB immunostaining revealed that all VGLUT1-ir intraepithelial nerve terminals associated with mouse NEBs are also CB ir (Fig. 5.10c, d) and vice versa. In this way, both VGLUT1 and CB can be used to label this nerve fibre population. The VGLUT/CB-positive population seems to form cap-like endings over the apical pole of at least a subpopulation of the NEB cells (Fig. 5.10a–d) (Brouns et al. 2009b).

Multiple immunocytochemical staining and vagal denervation, however, disclosed another vagal sensory nerve fibre population with extensive intraepithelial nerve terminals in mouse NEBs: one that expresses $P2X_2$ and $P2X_3$ receptors (Fig. 5.10e–h), but lacks VGLUTs and CB (Fig. 5.10i–k, n). Mouse pulmonary NEBs are therefore contacted by two different intraepithelial sensory nerve fibre populations with different neurochemical characteristics, and hence potentially different functions. When terminals of both nerve fibre populations were observed in the same NEB, they typically contacted separate groups of NEB cells (Fig. 5.10i–k, n) (Brouns et al. 2009b).

Of significant functional importance is the observation that both the VGLUT/CB-ir and the $P2X_{2/3}$ receptor-ir vagal afferent fibres giving rise to nerve terminals in pulmonary NEBs, are myelinated (Fig. 5.10l–n). Upon termination of the myelin sheath, the fibres branch and give rise to extensive intraepithelial arborisations that invariably co-localise with the presence of NEBs. The vagal sensory component of the innervation of NEBs may be regarded as a “fast” connection to the CNS (Brouns et al. 2009b).

Together, the neurochemical features of both myelinated nerve fibre populations are suggestive of a mechanosensory function. It was therefore not surprising that the mechanogated K_{2P} channel TRAAK could be detected on the extensive intraepithelial terminals of both vagal sensory nerve fibres (Lembrechts et al. 2011).

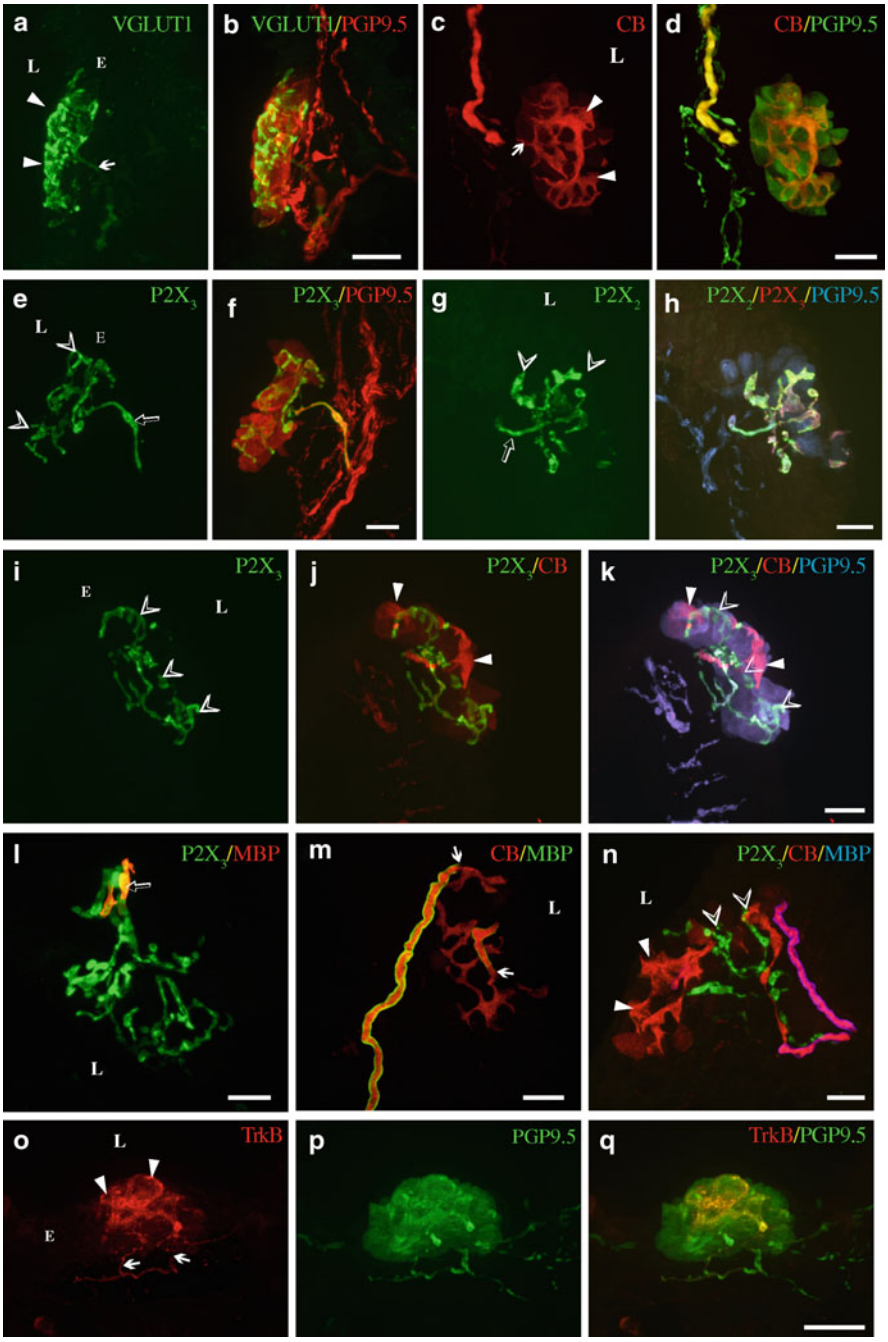

Fig. 5.10 Neurochemical characterisation of the vagal sensory innervation in mouse (PD21) pulmonary NEBs. *L* airway lumen, *E* epithelium. (**a, b**) A weakly stained *green* fluorescent

When both nerve fibre populations were tested on their dependency of neurotrophin-4 (NT-4), a neurotrophin reported to be important for the maintenance of mechanosensory nerve terminals (Fox et al. 2001; Raab et al. 2003; Perez-Pinera et al. 2008), the two different myelinated vagal sensory nerve fibre populations were found to express the NT-4 receptor TrkB (Fig. 5.10o–q). In NT-4 KO mice, however, only the $P2X_{2/3}$-ir population seemed to be dependent on NT-4 for survival (Oztay et al. 2010).

Multiple immunostainings for markers of the intraepithelial vagal sensory nerve fibre populations of mouse NEBs and for CGRP showed that they lack CGRP (Brouns et al. 2009b). CGRP IR was, however, present in C-fibre-like varicose nerve fibres close to the base of pulmonary NEBs (Fig. 5.11a), as reported before (Verstegui et al. 1997). Combined examination of SP IR unravelled for the first time that three distinct CGRP-ir nerve fibre populations have to be considered close to NEBs in mouse airways: varicose CGRP+/SP+, very thin CGRP+/SP+ and CGRP+/SP- nerve fibres (Brouns et al. 2009b). After unilateral vagal denervation, the population of varicose CGRP+/SP+ nerve terminals near NEBs seemed to disappear in the ipsilateral lung, indicative of their vagal origin.

In mouse lungs, cholinergic nerve terminals could also be identified by their VAChT IR. VAChT IR was detected in extensive varicose nerve fibres belonging to the population of postganglionic parasympathetic fibres located in subepithelial

Fig. 5.10 (continued) VGLUT1-ir nerve fibre (*arrow*) approaches the epithelium, branches and protrudes between the PGP9.5-ir NEB cells, giving rise to intensely stained intraepithelial laminar nerve endings (*arrowheads*). (**c**, **d**) A CB-ir nerve fibre (*arrow*) gives rise to an intraepithelial arborisation of laminar nerve endings (*arrowheads*). CB, as well as VGLUT1, are markers for this population of vagal sensory nerve fibres contacting mouse pulmonary NEBs. (**e**) A $P2X_3$ receptor-ir nerve fibre (*open arrow*) approaches the epithelium and protrudes between the epithelial cells, in this way forming an intraepithelial arborisation (*open arrowheads*). (**f**) The $P2X_3$-ir vagal sensory nerve fibres are in contact with a pulmonary NEB. (**g**, **h**) Triple immunocytochemical staining showing that vagal sensory nerve fibres (*open arrow*) and their terminals in pulmonary NEBs (*open arrowheads*) express both $P2X_2$ and $P2X_3$ ATP receptors. (**i–k**) Bronchiolar PGP9.5-ir NEB contacted by two separate vagal sensory nerve fibre populations. *Green* fluorescent $P2X_3$-ir nerve terminals (**i**; *open arrowheads*) are intermingled but not co-localised with CB-ir nerve endings (**j**; *arrowheads*) in a single NEB (**k**). (**l**) Double immunocytochemical staining for $P2X_3$ and MBP shows that the vagal sensory $P2X_3$-ir nerve fibre (*open arrow*) that gives rise to the intraepithelial $P2X_3$ expressing nerve terminals is myelinated. (**m**) Vagal sensory CB-ir nerve fibres that approach the airway epithelium lose their myelin sheaths (*arrows*), just before protruding between the epithelial cells. As demonstrated above, such an intraepithelial complex invariably represents a NEB. (**n**) Bronchiolar NEB contacted by separate populations of $P2X_3$-ir (*open arrowheads*), and CB-ir (*arrowheads*) nerve terminals. Note the laminar appearance of the CB-ir nerve terminals. The CB-ir nerve fibre is surrounded by an MBP-ir myelin sheath. (**o–q**) Immunohistochemical staining showing the expression of the neurotrophin 4 (NT-4) receptor TrkB on vagal sensory nerve fibres (*arrows*) and their laminar intraepithelial nerve terminals (*arrowheads*). The TrkB-ir nerve terminals surround a small group of NEB cells. *Scale bars* = 10 μm

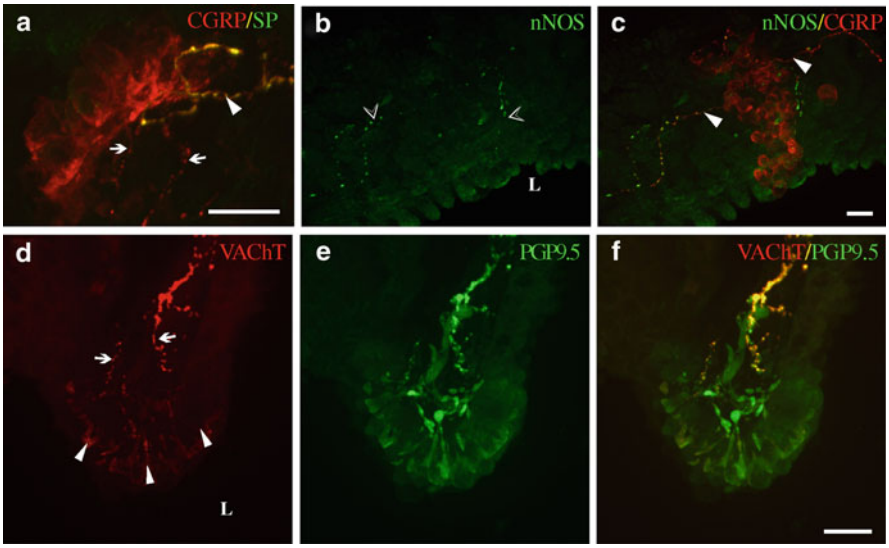

Fig. 5.11 Other nerve fibre populations in contact with mouse pulmonary NEBs. (**a**) Combination of CGRP and substance P (SP) immunostaining. Varicose CGRP+/SP+ (*arrowhead*) and delicate CGRP+/SP- fibres (*arrows*) are observed near the basal pole of CGRP+ NEB cells. (**b**, **c**) Separate nNOS-ir (*open arrowheads*) and CGRP-ir nerve fibres (*arrowheads*) are seen in the vicinity of the basal pole of the NEB. Some CGRP-ir nerve fibres run in very close proximity to the nNOS-ir terminals. No prominent intraepithelial nNOS-ir nerve endings can be observed. (**d–f**) Pulmonary PGP9.5-ir NEB with intraepithelial VAChT-ir structures, reminiscent of nerve terminals (*arrowheads*), that likely represent nerve endings that ramify between NEB cells. Some of the NEB cells also show a weak cytoplasmic VAChT staining. In addition, strongly stained subepithelial VAChT-ir nerve fibres (*arrows*) are present that do not show obvious connections with the intraepithelial VAChT-ir nerve terminal-like structures. *Scale bars* = 10 μm

airway smooth muscle bundles. Moreover, VAChT immunolabelling, combined with NEB markers, showed intraepithelial VAChT-ir nerve endings in NEBs with smooth appearance, very weak intensity, and no visible continuity with the strongly stained beaded subepithelial VAChT-ir nerve fibres (Fig. 5.11d–f). Double immunohistochemical staining provided evidence for the intraepithelial VAChT IR being located in the intraepithelial NEB-related vagal sensory nerve terminals (Brouns et al. 2009b). The faint VAChT IR, therefore, likely represents the presence of cholinergic vesicles in efferent-like synaptic areas reported to be part of the intraepithelial vagal sensory nerve terminals in NEBs in other species (Van Lommel and Lauweryns 1993a).

In mouse lungs, nitrergic nerve cell bodies were found in the adventitial layer of blood vessels, in large parasympathetic ganglia located in the hilar region, in smaller subepithelial ganglia in conducting airways and even in the alveolar interstitium (Brouns et al. 2009b). Many of the nitrergic neuronal cell bodies

appeared to be surrounded by a basket of CGRP-ir nerve endings. Nitrergic nerve terminals could be found intermingled with CGRP-ir nerve terminals forming a subepithelial nerve plexus at all levels of the intrapulmonary airways. Double labelling for VIP and nNOS revealed that some neuronal cell bodies in the hilar ganglia co-localised VIP and nNOS, while others were only VIP-positive. Labelling of NEBs disclosed nNOS-ir nerve endings (Fig. 5.11b, c) or VIP-ir nerve terminals at the base of pulmonary NEBs (Brouns et al. 2009b).

5.2.2.3

Sensory Nerve Terminals in Contact with Pulmonary NEBs Are Indicative of a Receptor-like Function

The data presented in the previous sections demonstrate that pulmonary NEBs in rodents are abundantly contacted by different populations of sensory and motor nerve terminals. Although in other mammals only sparse data on the neurochemical coding of the innervation of pulmonary NEBs are available, it is likely that also in other species pulmonary NEBs will be complexly innervated (for review see Sorokin et al. 1997).

Especially the unambiguous presence of intraepithelial vagal sensory nerve terminals forced researchers to recognise pulmonary NEBs as sensory receptor structures (Widdicombe 2001, 2009), and put them on the list of "sensory airway receptors" about 10 years ago. In the meantime it has become clear that pulmonary NEBs can simultaneously be contacted by different populations of vagal sensory nerve endings, the majority being terminals of myelinated nerve fibres. This myelinisation permits a fast transmission to the CNS.

Recent reviews still classify the myelinated vagal connections of pulmonary NEBs as "a population different from pulmonary stretch receptors" (Kollarik et al. 2010). If so, it is hard to explain the discrepancy between the observations that most "physiologically characterised" myelinated airway afferents possess mechanosensitive (SARs/RARs) features, and that "morphologically" a majority of the myelinated airway afferents appear to contact NEBs selectively (Adriaensen et al. 2006; Brouns et al. 2009b). According to some authors, notable differences may exist between vagal pulmonary receptors in rats and those known in other species (Bergren and Peterson 1993), while others reported that data for rats and mice, in general, do not appear to be all that different from other mammals (Widdicombe 2001; Ho et al. 2001; Yu et al. 2006; Zhang et al. 2006). Although efforts for matching the available physiological and morphological data may be hampered by potentially important species differences, it may be suggested that, at least in rodent lungs, discharges from NEB-related myelinated vagal afferent fibres may be part of the already characterised activities of vagal myelinated receptors in the lower airways.

Lately, more insight has been gathered into the putative functional significance of the vagal sensory innervation of pulmonary NEBs in rodents by unravelling their neurochemical features. These nerve terminals were shown to express Na^+/

K^+-ATPase α3, VGLUTs, $P2X_3$ receptors, calcium-binding proteins and TRAAK, a panel of markers that has been described to rather selectively label "mechanoreceptor terminals" in other organs (Dütsch et al. 1998; Raab and Neuhuber 2003; Dobretsov et al. 2003; Wang and Neuhuber 2003; Wu et al. 2004).

It is intriguing though that the same panel of "mechanosensory" markers can be used to reveal the presence, location, morphology and neurochemical coding of SMARs (Brouns et al. 2006a, b; Lembrechts et al. 2011). Because SMAR terminals also arise from myelinated nerve fibres (with the same average diameter) that have a vagal origin, the neurochemical coding and receptor-like appearance of at least part of the complex vagal sensory terminals in NEBs seems to be almost identical to SMARs.

The nearly identical neurochemical and morphological characteristics of SMARs and intraepithelial vagal sensory nerve terminals in pulmonary NEBs, obviously complicates the straightforward correlation of SMARs with so-called "SAR" activity. Based on inconclusive knowledge of sensory airway receptor morphology (Sant'Ambrogio 1982; Widdicombe 2001; Schelegle 2003; Yu et al. 2003, 2004; Yu 2009), SMAR-like nerve endings in the airway smooth muscle have for many years been presumed to give rise to "SAR" activity. In view of the present data, both SMARs and at least a subpopulation of the vagal nodose nerve terminals in NEBs seem to be good candidates to represent the morphological counterparts of subsets of the physiologically identified myelinated vagal airway mechanoreceptors.

The most prominent activator of electrophysiologically recorded "SAR" activity is an increase in transmural airway pressure. Although SMARs, located in airway smooth muscle, are believed to be perfectly positioned to perform such a function, an increasing intraluminal pressure could stimulate mucosal sensors (NEBs) at least as effectively as receptors located in the smooth muscle layer, which will essentially not "stretch" for most of the inflation period. Whereas vagal afferents of SMARs are located between airway smooth muscle cells, NEB-associated vagal afferents are part of the complete NEB micro-environment, in which NEB cells and other parts of the complex NEB innervation are likely involved in influencing signal transduction. The different location of vagal sensory (SAR activity producing?) nerve terminals in smooth muscle and in NEBs, respectively, may reflect the differences in low-threshold and high-threshold SAR activity that has been recorded in mice (Zhang et al. 2006).

NEBs and SMARs are located in the airway epithelium and the smooth muscle layer, respectively. Since the airways of rats and mice harbour several hundred intraepithelial vagal sensory nerve terminals connected to NEBs (Van Genechten et al. 2004), and this smooth muscle layer is invariably located adjacent to the airway mucosa in these small mammals, NEBs and SMARs are regularly found in close apposition (Fig. 5.12). This observation, together with the almost identical chemical coding of populations of vagal sensory terminals in NEBs and nearby SMARs (Brouns et al. 2006a, b; Lembrechts et al. 2011), clearly demonstrates that interpretation of electrophysiological data based on "local" stimuli should be made with great caution.

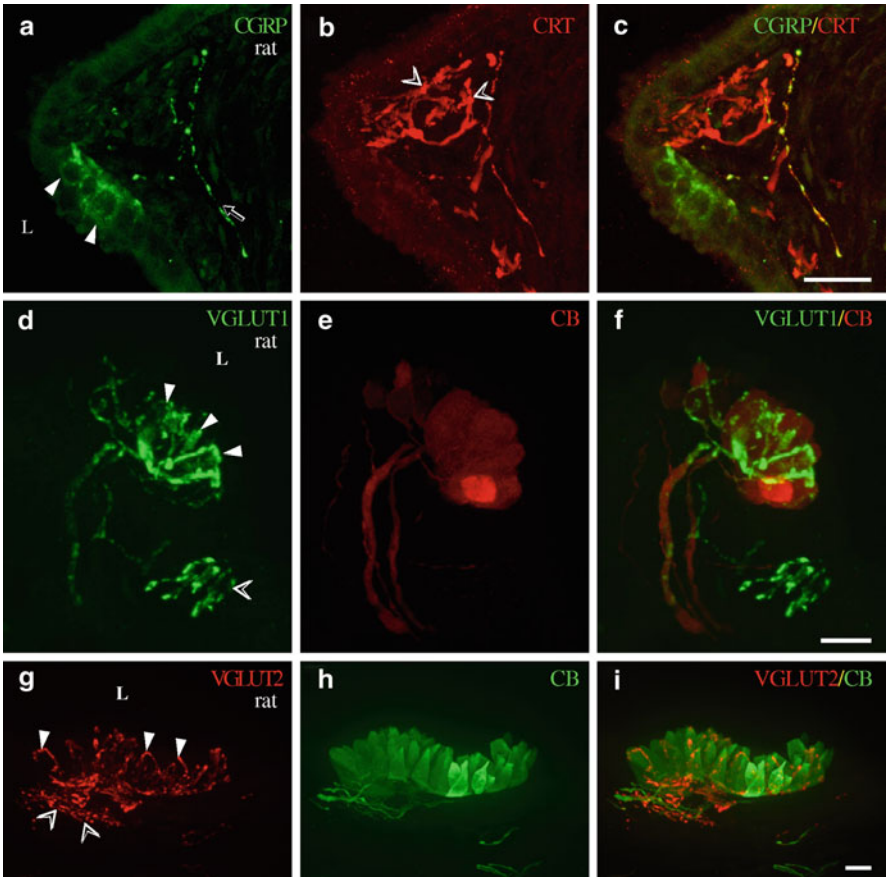

Fig. 5.12 Immunocytochemical staining of rat lungs, showing that SMARs and NEBs are often located in each other's immediate neighbourhood. *L*: airway lumen. (**a–c**) A CGRP-ir NEB (*arrowheads*) in the airway epithelium is located next to the laminar CRT-ir laminar terminals (*open arrowheads*) of a SMAR. A nerve fibre in the neighbourhood (*open arrow*) expresses both CRT and CGRP. (**d–f**) Double immunocytochemical staining for VGLUT1 and CB shows a pulmonary NEB innervated by intraepithelial VGLUT1/CB-ir nerve terminals (*arrowheads*) and a small SMAR (*open arrowhead*) visualised by its VGLUT1 IR. (**g–i**) A VGLUT2-ir SMAR (*open arrowheads*) is located in very close proximity to a pulmonary NEB, that is extensively innervated by intraepithelial VGLUT2-ir nerve endings (*arrowheads*). *Scale bars* = 10 μm

Given all the above-mentioned evidence, it may be concluded that the intraepithelial vagal sensory innervation of rodent NEBs likely belongs to the group of intrapulmonary receptor terminals that can primarily be activated by mechanical forces and, therefore, may represent the "structural" equivalent of one or more subpopulations of the electrophysiologically identified airway receptors (Adriaensen et al. 2006; Yu et al. 2006).

Apart from myelinated vagal sensory nerve fibres giving rise to intraepithelial arborisations of nerve endings, also unmyelinated nerve fibres are present that seem to contact mainly the basal side of pulmonary NEBs. These unmyelinated nerve terminals can be identified selectively by their CGRP/SP expression, a feature of the majority of peripheral C-fibre endings (Lundberg et al. 1984; Springall et al. 1987; Baluk et al. 1992; Kummer et al. 1992b; Riccio et al. 1996; Myers et al. 2002; Undem et al. 2004), which are believed to belong to the group of "chemosensors/nociceptors".

In rats, it was shown that systemic treatment with capsaicin results in a long-lasting depletion of the spinal sensory nerve terminals in contact with pulmonary NEBs (Cadieux et al. 1986; Shimosegawa and Said 1991b; Brouns et al. 2003b). Immunohistochemical staining of the spinal CGRP/SP-containing nerve fibres in very close proximity to, or even in contact with, rat pulmonary NEBs confirmed that these terminals express TRPV1 (Brouns et al. 2003b), typical of C-fibre endings (Caterina et al. 1997; Helliwell et al. 1998). Whereas TRPV ion channels are believed to be potentially chemo- and mechanosensitive, or may be involved in the transduction of noxious stimuli (Liedtke and Kim 2005), its exact function on the CGRP/SP-containing nerve terminals in contact with NEBs is still a matter of speculation. In mice, a population of DRG neurons projecting to the lung was also reported to express TRPV1 (Dinh et al. 2004; Takemura et al. 2008). In rat lungs CGRP/SP/TRPV1-ir nerve fibres constitute an unmyelinated, capsaicin-sensitive population that reveals typical C-fibre characteristics, but at least for the largest part, does not have the vagal sensory origin that was reported for bronchopulmonary CFRs. For many years, evidence has accumulated, suggesting that vagotomy abolishes all cardio-respiratory reflexes from the lungs. However, several more recent publications provided evidence for an additional spinal afferent component in the airway reflexes and/or cardio-respiratory responses to intrapulmonary chemical stimulation (Soukhova et al. 2003; Wang and Neuhuber 2003; Oh et al. 2006). In rats, for instance, the selective C-fibre-like spinal CGRP/SP-ir innervation of pulmonary NEBs may well be involved in these pathways.

Although it is clear that the sensory nerve terminals connected to pulmonary NEBs are able to carry out functions on their own, the fascinating close association of many intraepithelial and subepithelial nerve fibre populations with the specialised neuroendocrine cells in the NEB complex cannot be neglected.

5.2.3 Functional Morphological Characteristics and Neurochemical Coding of Pulmonary NEB Cells

A lot of information on the characteristics of NEB cells has been gathered from H-146 cells, an immortalised small cell lung carcinoma cell line that has been put forward as a suitable cell model for NEB cells. Given the inherent limitations of the

use of immortalised cell lines, and the isolation from the physiological environment, the current review focuses on the aspects of native NEB cells in order to be able to directly link the information to the broader NEB microenvironment.

5.2.3.1

NEB Cells Exhibit Characteristics of Excitable Cells

More than a decade ago, Youngson et al. (1993) showed in foetal rabbit lungs that NEBs express voltage-activated potassium, calcium and sodium currents, indicative of being excitable cells. Observations from live lung slices in other animal species, using patch clamp (Fu et al. 1999, 2000, 2003, 2007) or live cell imaging experiments (De Proost et al. 2008, 2009) confirmed these findings.

Voltage-activated K^+ channels (Kv) are involved in diverse physiological processes ranging from repolarisation after generation of neuronal action potentials, regulating Ca^{2+} signalling and cell volume, to driving cellular proliferation and migration (review). Recently, the presence of mRNAs for Kv1.2, Kv1.5, Kv2.1, Kv3.1, Kv3.3, Kv3.4 and Kv4.2 has been reported in laser-captured human NEB cells investigated by RT-PCR gene profiling (Cutz et al. 2009a). Functional as well as morphological studies (Fu et al. 2007; Cutz et al. 2009a), reported that NEB cells in neonatal lungs express Kv3.3a (rabbit, rat) (Wang et al. 1996b; Cutz et al. 2003), Kv3.4 and Kv4.3 (neonatal rabbit), all potentially oxygen-sensitive K^+ channels. These results confirm the findings of Youngson et al. (1993), who reported the presence of functional oxygen-sensitive channels on NEBs almost two decades ago. While Kv4.3 IR is found in the apical membrane of NEB cells in rabbit neonatal lungs (Fu et al. 2007), it is apparently lacking in human NEBs, indicative of possible species differences. RT-PCR gene profiling of laser-captured human NEBs additionally indicated the presence of the two-pore domain potassium channels TASK1-3 (Cutz et al. 2009a).

VGCCs play a crucial role in cell signalling as mediators of membrane depolarisation-induced calcium entry. VGCCs are expressed in a large variety of cell types, including neurons and neuroendocrine cells (e Silva and Lewis 1995; Overholt and Prabhakar 1997). These calcium channels partially control intracellular calcium concentrations and regulate processes such as secretion, neurotransmission and gene expression (Hille 1986). Based on their physiological and pharmacological properties, VGCC can be subdivided into low voltage-activated T-type ($Ca_V3.1$, $Ca_V3.2$, and $Ca_v3.3$) and those activated by high voltage changes, i.e., L- ($Ca_V1.1$ through $Ca_V1.4$), N-($Ca_V2.2$), P/Q-($Ca_V2.1$) and R-($Ca_V2.3$) types, depending on the channel-forming $Ca_V\alpha$ subunits (Ertel et al. 2000; Catterall et al. 2003). While initial information about the presence of Ca_V channels on NEB cells was obtained indirectly in rabbit lung slices using the whole cell patch clamp technique and amperometry (Fu et al. 2002, 2004), multiple immunohistochemical labelling in rat lungs more recently showed that the small area of surface membrane in the tip of the apical processes of NEB cells that directly contact the airway lumen, expresses $Ca_V2.1$ (P/Q-type) (Fig. 5.14j–p). This indicates that,

following depolarisation, these VGCCs may mediate a voltage-dependent calcium rise in NEB cells, potentially initiating exocytosis of DCVs (De Proost et al. 2007b).

5.2.3.2 Secretory Products and Vesicle-Associated Proteins of Dense-Cored Vesicles in NEB Cells

PNECs synthesise various bioactive substances such as amines, peptides and purines that have the potential to interact with the autonomic nervous system and to modulate cell proliferation, vascular and airway tone (for review see Sorokin and Hoyt 1989; Scheuermann et al. 1992; Adriaensen and Scheuermann 1993; Adriaensen et al. 2003). Many substances found in PNECs, even sometimes co-localised in the same vesicles, can be secreted, and thus act as neurotransmitter/neuromodulator substances. It is, however, a largely unexplained fact that there are important interspecies differences in this respect: different species may store diverse substances, and the same substances may have opposite effects. Possible postnatal changes in the substances produced by NEBs have also been reported (review see Porzionato et al. 2008). In NEBs, serotonin (5-hydroxytryptamine; 5-HT) (Lauweryns et al. 1982; review Adriaensen and Scheuermann 1993; Pan et al. 2004) and CGRP (Luts et al. 1991; Keith et al. 1991) are the amine and peptide substances, respectively, that have most frequently been reported in vesicles in the NEB cells.

Before the introduction of immunocytochemical methods, formaldehyde-induced fluorescence (FIF), a histochemical reaction that converts monoamines into fluorescent products with the aid of formaldehyde, used to be the chief method for 5-HT detection in NEB cells (for review see Adriaensen and Scheuermann 1993). Later on, specific immunolabelling for 5-HT was detected in PNECs/NEBs of nearly all species examined so far (review see Polak et al. 1993). Although 5-HT appears to be the principal monoamine produced by PNECs/NEBs, the level of expression differs between species. While rabbit, cat, monkey, pig and human NEBs reveal strong 5-HT IR, 5-HT IR in rodent NEBs can hardly be detected (Cutz et al. 1974; Wasano 1977; Van Ranst 1989; Gomez-Pascual et al. 1990). Immuno-electron microscopical studies showed that 5-HT is confined to the secretory granules of NEB cells (Dey and Hoffpauir 1986; Wang and Cutz 1993). Direct proof of quantal 5-HT release from NEB cells has been provided using carbon fibre amperometry in neonatal rabbit lung slices (Fu et al. 2002). 5-HT released from NEB cells may be important for vasomotor tone (Will et al. 1984), result in bronchoconstriction, and/or act as growth factor (Seuwen and Pouyssegur 1990).

CGRP and calcitonin, derived from a common calcitonin-CGRP gene by alternate RNA splicing (Amara et al. 1982), have been reported in solitary PNECs and in NEBs at all levels of the airways in many animal species (Figs. 5.13e–j and 5.14) (Gosney and Sissons 1985; Uddman et al. 1985; Lauweryns and Van Ranst 1987; Kasacka 2008). Because CGRP is present in DCVs (Stahlman and Gray 1997), it

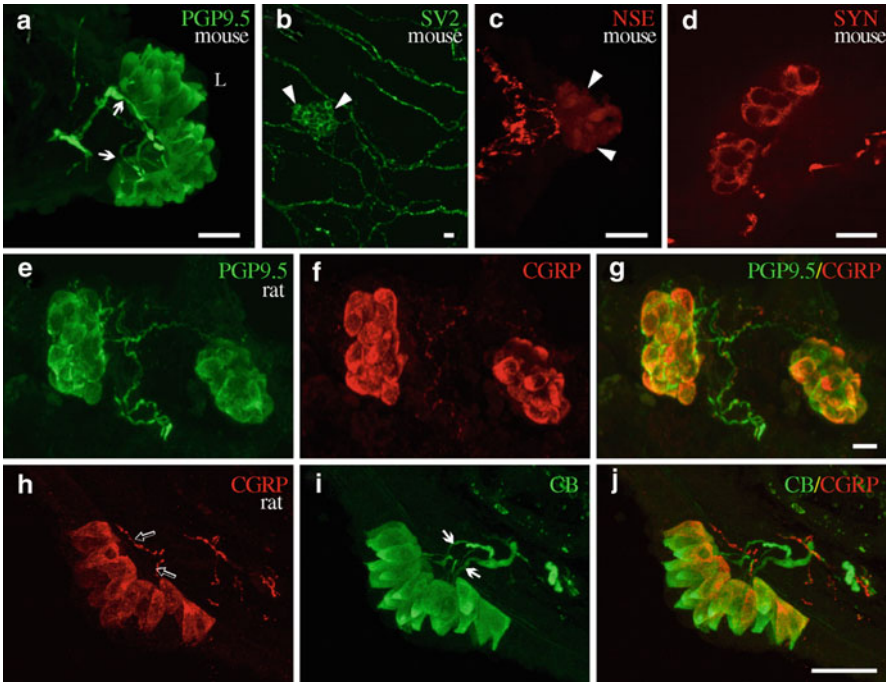

Fig. 5.13 Overview of some of the cytoplasmic contents of pulmonary NEB cells in rodents. Since pulmonary NEBs occur very dispersed in the airway epithelium, selective visualisation of these "marker molecules" is useful to unequivocally identify NEBs. Note that most markers for neuroendocrine cells also label (a subpopulation of) nerve fibres. *L*: Airway lumen. (**a**) Single immunocytochemical staining for PGP9.5 of an airway branching point in a mouse, showing a group of pulmonary NEB cells. Note several PGP9.5-ir nerve fibres (*arrows*) innervating the PGP9.5-ir NEB. (**b**) Overview of a mouse airway, showing SV2 IR in a pulmonary NEB (*arrowheads*) and in an extensive nerve fibre plexus. (**c**) Immunocytochemical staining for neuron specific enolase (NSE) in a mouse lung reveals an abundance of intensely NSE-ir nerve fibres in the lamina propria. The NEB cells (*arrowheads*) show a rather weak NSE IR. (**d**) synaptophysin (SYN)-ir NEB cells are present in a mouse bronchiole. Note the granular SYN pattern indicative of the presence of SYN on secretory vesicles. (**e–g**) Double immunocytochemical staining for PGP9.5 and CGRP revealing two NEBs in each other's immediate neighbourhood. Because of the presence of CGRP in dense-cored vesicles, CGRP IR shows a granular pattern compared to the PGP9.5 immunostaining. Note that in both NEBs a *green* intraepithelial PGP9.5+/CGRP- innervation can be distinguished from the PGP9.5+/CGRP+ NEB cells. (**h–j**) Rat lung double stained with antibodies against CGRP and CB. While the cytoplasmic marker CB stains the complete NEB cells, CGRP IR appears to be mainly located at the basal pole. All NEB cells show strong CB and CGRP IR. The CGRP+/CB+ NEB is innervated by CB-ir vagal sensory (*arrows*) and CGRP-ir spinal sensory (*open arrows*) nerve fibres. *Scale bars* = 10 μm

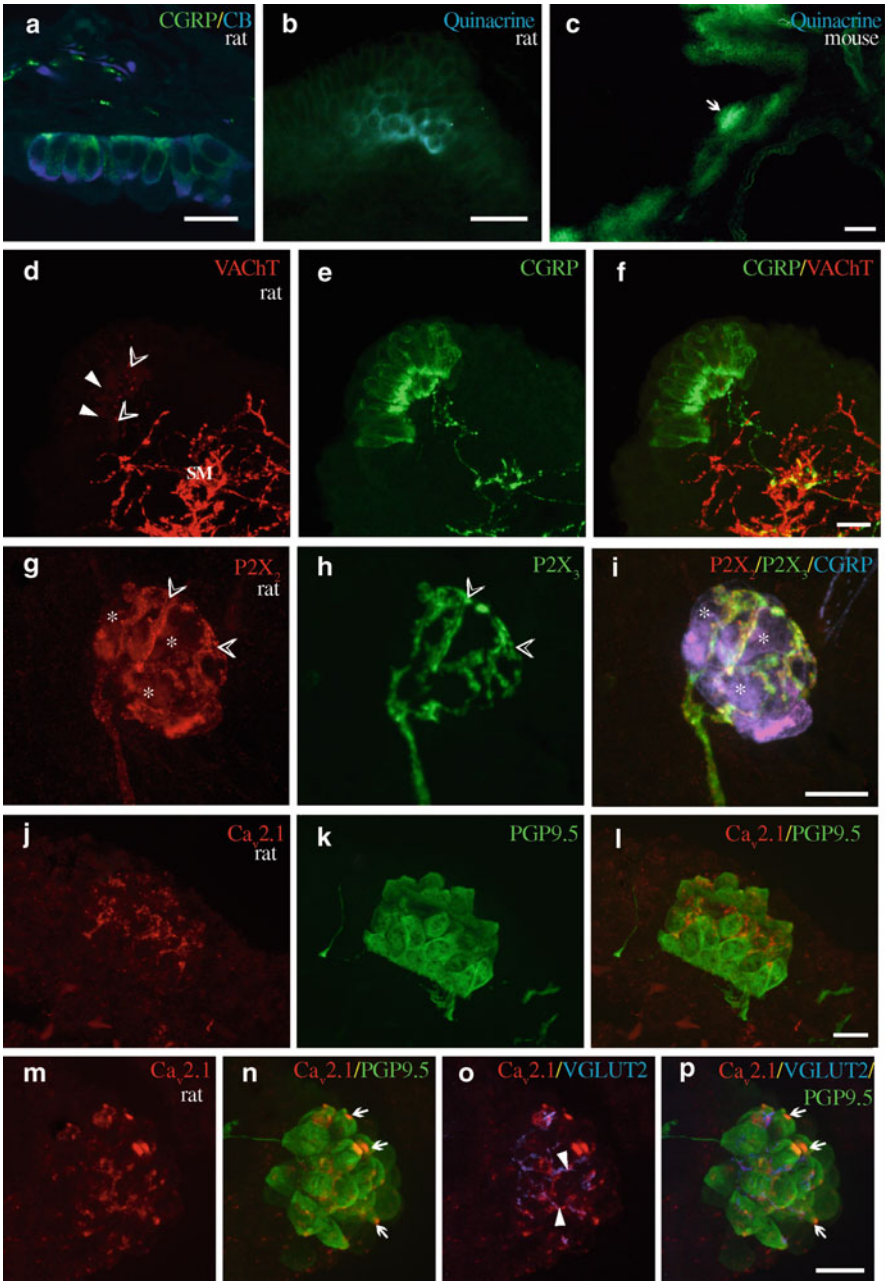

Fig. 5.14 Illustrations of the expression of some secretory products, molecular receptors and ion channels on pulmonary NEB cells in rodents. (**a**) Single confocal optical section of a rat NEB, double immunostained for CB and CGRP, revealing that CGRP is present in the basal

can be released from NEB cells by exocytosis (Dakhama et al. 2002, 2004). Although the precise role of large pools of CGRP in healthy lungs is still under debate, *in vitro* pharmacological studies have indicated that CGRP exerts a vasodilator effect in rat lungs, and that endogenous CGRP may play an important role in pulmonary pressure homeostasis by directly dilating precontracted pulmonary blood vessels (Martling et al. 1988; Tjen-A-Looi et al. 1998; Springer et al. 2004) via binding to CGRP receptors on vascular smooth muscle (Qing et al. 2001). CGRP may furthermore be involved in regulation of ciliary beat frequency (Tamaoki et al. 1989; Schuil et al. 1995) and airway mucus secretion (Kuo et al. 1990; Webber et al. 1991).

The major neuropeptide reported to be produced by PNECs and NEBs in humans and non-human primates is gastrin-releasing peptide (GRP), the mammalian homologue of the amphibian peptide bombesin (Li 1994). It may act as a regulatory hormone and as a cell type-specific growth factor, as evidenced for epithelial cells in normal and neoplastic lung tissues (Cuttitta et al. 1985). While GRP/bombesin is abundant in PNECs/NEBs in the human foetus (Wharton et al. 1978; Tsutsumi et al. 1983), it appears to be less pronounced after birth. In disease conditions it is often seen to return to very high levels of expression. The presence of GRP/bombesin in pulmonary PNECs/NEBs in man can already be detected from the eighth week of gestation. The precise mechanism of action and the target cells, however, have not yet been defined.

Another candidate transmitter in pulmonary NEBs is ATP (Linnoila 2006; Burnstock 2009). In rats (Brouns et al. 2000) and mice (De Proost et al. 2009), quinacrine staining of NEBs indicates the presence of high concentrations of ATP stored in secretory vesicles (Fig. 5.14b, c). Reporter patching, used as a uniquely

Fig. 5.14 (continued) NEB cell cytoplasm, indicative for its presence in secretory vesicles and its possible release by exocytosis. Immunoreactivity for the calcium-binding protein CB is homogeneously present in all cells of the NEB. (**b**, **c**) After systemic injection, quinacrine accumulation can be observed in pulmonary NEB cells in rat (**b**) or mouse (*arrow* in **c**), indicative of the presence of high levels of ATP in secretory vesicles. (**d**–**f**) Immunocytochemical double staining for VAChT and CGRP of a rat bronchiole, revealing very faint VAChT IR in NEB cells (*arrowheads*), suggestive of accumulation of acetycholine in secretory granules. Stronger VAChT-ir "dots" (*open arrowheads*) are present in intraepithelial nerve terminals. In the subepithelial region, the strong VAChT-ir nerve fibres are located at the airway smooth muscle (*SM*) area. (**g**–**i**) In rat airways, $P2X_2$ (ATP) receptors are expressed on NEB cells (*asterisks*) and on the $P2X_{2/3}$ receptor-ir intraepithelial vagal sensory nerve terminals (*open arrowheads*). CGRP immunostaining has been used to identify the NEB cells. (**j**–**l**) $Ca_V2.1$ IR appears to be located close to the apical and lateral surfaces of NEB cells. (**m**–**p**) Triple immunostaining for $Ca_v2.1$, VGLUT2 and PGP9.5 of a rat lung. Strong spot-like $Ca_v2.1$ IR is present in the apical membranes of the processes of NEB cells that make direct contact with the airway lumen (*arrows*). Additionally $Ca_v2.1$ IR is seen to co-localise with some VGLUT2-ir vagal sensory nerve endings (*arrowheads*) that protrude between the NEB cells. *Scale bars* = 10 μm

sensitive and selective ATP biosensor detection method, showed that depolarisation of NEB cells evokes quantal ATP release (see further De Proost et al. 2009).

Production of acetylcholine (ACh) by NEB cells in rodents is suggested by the immunohistochemical detection of VAChT (Fig. 5.14d–f) (Adriaensen et al. 2003; Brouns et al. 2009b), a transporter protein present in the membrane of cholinergic secretory vesicles. Released ACh may evoke local bronchoconstriction/vasodilatation (Coulson and Fryer 2003), stimulate ciliary beat frequency (Melville and Iravani 1975; Salathe et al. 1997) and airway mucus secretion (Coulson and Fryer 2003; Kummer et al. 2008).

Recently, it has been shown that the neurotransmitter γ-aminobutyric acid (GABA) is produced in mouse PNECs, and may likely act on nearby epithelial cells (Yabumoto et al. 2008). Other examples of reported bioactive peptides in PNECs/NEBs are enkephalin (Cutz et al. 1981), substance P (SP) (Gallego et al. 1990) and cholecystokinin (Wang and Cutz 1993). The function of these peptides in the lung remains largely unknown.

The chromogranins A and B, and secretogranin II constitute the main members of a family of secretory proteins typical of elements of the DNES (see Fujita et al. 1988). All chromogranins have comparable properties and consist of large acidic proteins that are present in neurosecretory granules. The exact function(s) of these proteins are still being unravelled (Yoo et al. 2010; Helle 2010), but they seem to be the precursors of biologically active peptides. These peptides may act as helper proteins in the packaging of peptide hormones and neuropeptides, function as high-capacity, low-affinity Ca^{2+} storage proteins, and may interact with the IP3Rs and activate IP3R/Ca^{2+} channels. PNECs/NEBs have been demonstrated in the lungs of humans and various mammals with antibodies against chromogranin A (see Adriaensen and Scheuermann 1993). Different chromogranins have been observed in human neuroendocrine lung neoplasms (Kasprzak et al. 2007).

The process of exocytosis involves fusion of secretory vesicle membranes with the plasma membrane. The various aspects of the secretory process are likely to involve specific secretory vesicle proteins that mediate membrane/membrane and membrane/cytoskeletal interactions. SYN is a major integral membrane glycoprotein that has been shown to be located specifically in neuronal vesicles with an electron-lucent content, and may therefore be involved in synaptic vesicle formation and exocytosis. SYN is a calcium-binding protein that plays an important role in Ca^{2+}-dependent transmitter release (Rehm et al. 1986). Apart from being found in the CNS and peripheral nervous system, SYN has been reported to be present in various neuroendocrine cells and neoplasms (Navone et al. 1986; Wiedenmann et al. 1986; Redecker et al. 1990; Ito et al. 1998) and is also present in NEB cells (Fig. 5.13d) (Lee et al. 1987; Castro et al. 2000). Synaptic vesicle protein 2 (SV2) proteoglycans are involved in retaining neurotransmitters in a non-diffusible form, and are a component of all vertebrate synaptic vesicles (Buckley and Kelly 1985). Although the presence of SV2 has not been explored at the ultrastructural level, its granular appearance in immunolabelling studies is indicative of a vesicle-related location. The presence of SV2 in all neuronal and neuroendocrine vesicle-

containing structures, makes it a useful pan-neuroendocrine marker, including in the lungs (Fig. 5.13b) (Weichselbaum et al. 1996; Pan et al. 2004, 2006a; Lembrechts et al. 2011).

5.2.3.3

Cytoplasmic Contents of Pulmonary NEB Cells

The cytoplasm of NEB cells harbours molecules that are functionally related to their role as excitable transmitter-releasing cells, and as receptor cells able to react to specific stimuli.

The cytoplasmic calcium-binding protein calbindin-D28k (CB) has been detected in a broad range of tissues including diffuse neuroendocrine and nervous tissues. CB has been observed mainly in the cytoplasm, and partly in the nuclei of single and grouped hamster PNECs (Ito et al. 1998), has proven to be a good marker for all neuroendocrine cells in rat NEBs (Figs. 5.13h–j and 5.14a) (Brouns et al. 2000), and labelled only a minor part of the constituent cells in mouse NEBs (Brouns et al. 2009b). Although immunoreactivity for CB has been described in lung neuroendocrine cells, the biological role of this Ca^{2+}-binding protein is still poorly understood. Recently, it has been suggested that CB functions as Ca^{2+} sensor, in addition to its Ca^{2+} buffering capacities (Schwaller 2010).

Because of their ability of amine precursor uptake and decarboxylation, NEB cells belong to the so-called APUD system (amine precursor uptake and decarboxylase system), a name originally used to describe the DNES. The enzyme responsible for decarboxylation of amine precursors is aromatic L-amino acid decarboxylase (AADC), which has been immunohistochemically demonstrated in NEBs of the human, rat and mouse respiratory mucosa (Lauweryns and Van Ranst 1988b).

The decarboxylation of glutamate to gamma-aminobutyric acid (GABA) is performed by glutamate decarboxylase (GAD). In mammals, GAD exists in two isoforms: GAD65 and GAD67. Immunohistochemical studies using antibodies against GAD65/67, localised this enzyme in NEB cells of monkeys (Fu and Spindel 2009) and mice (own unpublished observations; Yabumoto et al. 2008). A very useful model for studying the distribution of GABAergic cells in many tissues and organs, are GAD-GFP knock-in mice, in which the presence of GAD-producing NEB cells has been suggested (Yabumoto et al. 2008).

Using enzyme histochemistry, cholinesterase activity was observed in the cytoplasm of NEE cells in the foetal rabbit (Lauweryns and Cokelaere 1973a; Hung 1980; Sonstegard et al. 1982) and rat lung (Morikawa et al. 1978a, b). The enzyme may be transported to the cell surface and subsequently released, thereby involved in ACh hydrolysis, thus probably modifying the responsiveness of the NEB cells to ACh (Adriaensen and Scheuermann 1993).

PGP9.5, also now known as ubiquitin carboxyl-terminal hydrolase L1 (UCHL1) is a highly conserved protein in neurons and neuroendocrine cells in vertebrates, belonging to the carboxyl-terminal ubiquitin hydrolase family (Day and

Thompson 2010). PGP9.5, first detected as a neuronal and neuroendocrine specific marker, is an abundant (5–10%) cytoplasmic protein. Using antibodies against PGP9.5, the pulmonary neuroendocrine system and its innervation could be demonstrated in the respiratory tract of various mammals (Figs. 5.13a, e, f and 5.14j–p) (for review see Adriaensen and Scheuermann 1993), including humans (Lauweryns and Van Ranst 1988a).

NSE is a glycolytic enzyme, localised primarily to the neuronal cytoplasm. In neuronal tissue, the appearance of NSE seems to coincide with the initiation of synaptic contacts (Marangos et al. 1979). Therefore, the presence of NSE in PNECs (Fig. 5.13c) (for review see Adriaensen and Scheuermann 1993) may be indicative of the onset of their functional activity.

Among the major modulators or transducers of information from the extracellular environment to intracellular processes are the heterotrimeric guanine nucleotide-binding proteins (G proteins). G proteins act as switches that regulate information-processing circuits connecting cell surface receptors to a variety of intracellular effectors. The alpha subunit of the G_o protein has been immunodetected in PNECs of mice, rats and hamsters (Ito et al. 1999).

5.2.3.4

Receptors, Channels and Other Surface Membrane Molecules of Pulmonary Neuroepithelial Bodies

By far the most constantly proposed function of NEB cells is their capability of sensing hypoxia in the airway lumen (Youngson et al. 1993; Linnoila 2006; Cutz et al. 2008; Domnik and Cutz 2011). Central to this O_2 sensing is the hypoxic inhibition of K^+ channels, which subsequently causes membrane depolarisation, Ca^{2+}-influx – P/Q-Ca_v channels ($Ca_v2.1$) have been reported on the apical membrane of NEB cells (De Proost et al. 2007b) – and neurotransmitter release. Considerable efforts have been made to localise the O_2-sensing molecular complex, composed of O_2-sensing proteins coupled to O_2-sensitive K^+ channels (Youngson et al. 1993; Wang et al. 1996b; Peers and Kemp 2001; Kemp et al. 2003). The main candidate as an O_2-sensing protein is the reduced heme-linked nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase; NOX2) (for review see Kemp et al. 2009), which consists of a catalytic membrane-bound subunit, cytochrome b558, an integral membrane heterodimer containing $gp91^{phox}$ and $p22^{phox}$, and the regulatory subunits $p47^{phox}$, $p67^{phox}$ and rac2 that constitute the functional NADPH oxidase. Evidence in support of NADPH oxidase as a principal O_2 sensor in NEB cells includes gene expression profiling experiments (Cutz et al. 2009a), *in situ* hybridisation (Wang et al. 1996a) and immunohistochemical localisation of $gp91^{phox}$ and/or $p22^{phox}$ in mouse, neonatal rat (Cutz et al. 2009a), foetal rabbit (Youngson et al. 1993) and neonatal human NEB cells (Wang et al. 1996b; Youngson et al. 1997). Immunolocalisation of NOX2 (gp91 phox) in neonatal rat lung has been reported to be restricted to the apical surface facing the airway lumen, as would be expected of an airway sensor monitoring

intraluminal O_2 concentration (Cutz et al. 2009a). In NADPH oxidase-deficient mice, NEBs failed to respond to hypoxia, whereas in control wild-type mice this response was intact (Fu et al. 2000; for review see Cutz et al. 2009a). Although NADPH oxidase has generally been the centre of studies on oxygen sensing compounds in pulmonary NEBs (review see Kemp and Peers 2009; Cutz et al. 2009a), also other players may be involved in this crucial pulmonary sensory mechanism.

Smoking or inhalation of cigarette smoke is known to have a profound effect on the diffuse pulmonary neuroendocrine system, as apparent from both indirect evidence in cigarette smokers and animal models (Chen et al. 1987; Tabassian et al. 1988, 1993; Schuller et al. 1990, 2003; Aguayo 1993). Administration of nicotine or the carcinogenic nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanon (NNK) to isolated foetal hamster PNECs in culture is believed to initiate the release of 5-HT (Fu et al. 2003). Furthermore, electrophysiological experiments performed on NEBs in live hamster lung slices have indicated that NEB cells *in situ* may depolarise after stimulation with nicotine and ACh, the endogenous ligand of nicotinic ACh receptors (nAChRs) (Fu et al. 2003). Immunocytochemical staining designated α7, α4 en β2 nAChR as the receptor subunits involved. Literature data also suggest the expression of α7 nAChRs in monkey (Sekhon et al. 1999; Schuller et al. 2000) and human NEBs (Sartelet et al. 2008).

The effects of GABA are mediated through ionotropic $GABA_A$ and $GABA_C$ receptors, and metabotropic $GABA_B$ receptors. Recent RT-PCR analyses in mouse lungs mainly revealed mRNA encoding the $GABA_B$ receptor subunits necessary for the assembly of functional receptor R1 and R2, which were immunohistochemically localised to non-endocrine airway epithelium (Yabumoto et al. 2008). $GABA_A$ β3 receptors were reported to be expressed in monkey NEB cells (Fu and Spindel 2009).

A study in hamster suggested that ATP may modulate the synaptic output of NEB cells by binding to heteromeric $P2X_{2/3}$ ATP receptors expressed in the plasma membrane of NEB cells (Fu et al. 2004). In contrast, NEB cells in mice completely lack $P2X_{2/3}$ IR (Brouns et al. 2009b), whereas rat NEB cells express $P2X_2$, but not $P2X_3$ receptors (own unpublished observations, Fig. 5.14g–i).

The presence of functional 5-HT_3 receptors, belonging to a family of ligand-gated ion channels, has been reported on pulmonary NEB cells (Fu et al. 2001). Using the non-isotopic *in situ* hybridisation method, 5-HT_3 mRNA transcripts were detected in NEB cells from different animal species and humans (Fu et al. 2001). Expression of 5-HT_3 receptors was immunolocalised to the plasma membrane of cultured rabbit NEBs.

A single report showed the presence of VIP receptor IR on human NEBs (Fischer et al. 1992).

The neural cell adhesion molecule (NCAM), a membrane glycoprotein involved in cell–cell adhesion within the CNS and peripheral nervous system, has been observed in the surface membrane of cat (Seldeslagh and Lauweryns

1997), neonatal rabbit (Yeger et al. 2001) and hamster (Ito et al. 1995) NEB cells, but a variation in NCAM density was observed during postnatal development. It was proposed that the presence of NCAM on NEB cells may be a prerequisite for the formation of neuroendocrine cell clusters and for a coordinated NEB cell function in vivo. Moreover, the confined presence of intraepithelial nerve endings among the corpuscular NEB cells suggests involvement of NCAM in adhesive interactions between NEB cells and nerve terminals. Indeed, cell surface molecules may play a significant role in various developmental events including the outgrowth and guidance of axons and dendrites towards their target epithelial cells, and thus precede or participate in synapse formation (Seldeslagh and Lauweryns 1997). MOC-1 antigen (belonging to the class of NCAMs) has been used to isolate PNECs from rabbit foetal lung by means of immunomagnetic separation (Speirs et al. 1992).

5.2.3.5

Selective Identification of NEB Cells

Because of their widespread distribution in the airway epithelium, the main prerequisite to performing large-scale functional morphological investigations on NEB cells is their clear identification and visualisation in the microscope. Early observations of NEB cells were based on their ultrastructural characteristics (Hung et al. 1973; Cutz et al. 1974; Hung and Loosli 1974; Jeffery and Reid 1975), on silver staining (Wasano 1977; Hung 1984) and FIF (Hage 1976). Historically, numerous methods have been developed to selectively identify PNECs and NEB cells [extensive reviews: (Scheuermann 1987; Sorokin and Hoyt 1989; Adriaensen and Scheuermann 1993; Adriaensen et al. 2003)]. Whereas in bright field microscopy, NEB cells are hardly recognisable after routine fixation and classic light microscopic staining, immunohistochemical methods are excellent to unambiguously identify NEBs. Today, combination of immunofluorescent labelling with visualisation by fluorescence or confocal microscopy seems to be a valuable tool to study the overall features of pulmonary NEBs.

Using immunohistochemistry, it is possible to determine which transmitters are stored in secretory granules in NEB cells (Figs. 5.13 and 5.14a). Additionally, because of the presence of certain amines and peptides (e.g., 5-HT and CGRP) in DCVs of most NEB cells, it is possible to discriminate NEB cells from other airway epithelial cells, making these subcellular molecules major "marker substances". The presence of DCVs in all NEB cells also makes vesicle-associated proteins, such as SV-2 (Fig. 5.13b), SYN (Fig. 5.13d) and chromogranins, suited to identify NEBs.

More reliable parameters for the identification of PNECs/NEB cells, other than the occurrence of certain bioactive substances, are molecules that are constitutively expressed in the NEB cell cytoplasm. Because these molecules are retained after degranulation, cytoplasmic components, like NSE (Fig. 5.13c) and PGP9.5 (Fig. 5.13a, e, f), also seem to be ideal markers.

Many of the above-mentioned markers for NEB cells are able to accurately detect NEBs because of their appearance as clear epithelial cell clusters. However, if one wants to study the extensive innervation of NEBs, it is worth notifying that many of the "neuroendocrine" markers also label (part of) the nerve terminals in contact with NEBs (Fig. 5.13), making it sometimes difficult to discriminate between NEB cells and their extensive (intraepithelial) innervation. Most often, multiple immunohistochemistry, based on thorough neurochemical knowledge of the innervation pattern of NEBs in the studied species, is needed to unambiguously gather new information on the characteristics of NEB cells and/or nerve terminals.

5.2.3.6

Significance of NEB Cells in the NEB Microenvironment

Their obvious characteristics of excitable cells, their possibility to store and release secretory products and the presence of several types of molecular receptors, provide convincing evidence for NEB cells representing a distinct population of airway epithelial cells that upon appropriate stimulation can secrete substances, which may then interact with NEB-associated nerve terminals, be taken up by nearby blood vessels and exert endocrine interactions, or have paracrine effects on neighbouring non-endocrine epithelial cells, fibroblasts, immune cells, airway and vascular smooth muscle.

Over the years, several functions have been assigned to NEB cells, with a more or less general consensus about their possible dual role (Sorokin and Hoyt 1993; Linnoila 2006): (1) during early stages of lung organogenesis, NEB cells acting via their amine, peptide and purine transmitters could function as local modulators of lung growth and differentiation; and (2) later in foetal life and postnatally, NEB cells (particularly those that are innervated) could play a role as airway chemoreceptors. Given the concept of the present review, further focus will lie on the role of NEBs as transducers of environmental information and their possible role in respiratory physiology. Other functional facets of the pulmonary neuroendocrine system have been reviewed extensively a few years ago (Linnoila 2006; Giangreco et al. 2007; Cutz et al. 2008).

Nicely embedded in the airway epithelium, with slender processes that contact the airway lumen, pulmonary NEB cells are ideally located to potentially register multiple properties of the airway environment. The general belief that pulmonary NEB cells are able to monitor the concentration of airway gases, especially oxygen, stems from studies in the 1970s on neonatal rabbits, in which Lauweryns and coworkers showed – judged from TEM images – that NEB cells react to hypoxia by increasing exocytosis of their DCVs and a decrease in cytoplasmic amine content (Lauweryns and Cokelaere 1973b; Lauweryns and Van Lommel 1982). Since that time, the improvement of *in vitro* models has been very important for unravelling a functional oxygen-sensing system in NEB cells (Cutz et al. 1985; Speirs et al. 1992; Speirs and Cutz 1993; O'Kelly et al. 1998; Kemp et al. 2002; Cutz et al. 2004).

In NEB cells (Youngson et al. 1993; Fu et al. 1999) and in the immortalised small cell lung carcinoma cell line H146 (O'Kelly et al. 1998, 1999, 2000; Hartness et al. 2001), acute inhibition of K^+ channels by hypoxia seems to be central to oxygen chemosensing (Youngson et al. 1993; Cutz and Jackson 1999; O'Kelly et al. 1999; Peers and Kemp 2001; Kemp et al. 2009; Kemp and Peers 2009). The proposed signalling cascade involves closure of background K^+ channels (Fu et al. 1999), consequent membrane depolarisation and Ca^{2+} influx via Ca_v channels (De Proost et al. 2007b), eventually triggering neurotransmitter exocytosis from NEB cells (Lopez-Barneo 1994; Fu et al. 2002; Kemp et al. 2003). Both in native NEBs and in PNEC cell lines, a membrane-associated H_2O_2-producing, multicomponent NADPH oxidase has been identified as a potential molecular oxygen-sensor (Youngson et al. 1993, 1997Wang et al. 1996b; Fu et al. 2000; O'Kelly et al. 2000), although other oxygen-sensing mechanisms may be involved (O'Kelly et al. 2001; Kemp 2006). Direct proof that NEB cells are stimulated by acute hypoxia, and that this results in transmitter release, was provided by a study that used a fresh slice preparation of neonatal rabbit lungs and carbon fibre amperometry (Fu et al. 2002). The latter method is able to detect in real time the quantal release of 5-HT, and provided evidence for a dose-dependent hypoxic release of 5-HT from NEB cells within the physiological range expected in the airways (i.e., pO_2 <95 mm Hg). The release of 5-HT may result in bronchoconstriction, changes in vasomotor tone and/or growth-factor-like effects (Seuwen and Pouyssegur 1990; Vicaut et al. 2000), as well as positive feedback activation through 5-HT_3 autoreceptors residing on PNECs (Fu et al. 2001). 5-HT does not seem to be released into the pulmonary circulation during intermittent hypoxia (Sorhaug et al. 2008).

On the other hand, evidence suggests that hypoxia may lead to the inhibition of the release of NEB cell products (Springall and Polak 1993, 1997; Helset et al. 1995; Sorhaug et al. 2008), resulting in a decrease in NE products (e.g., CGRP, bombesin-like peptides) in perfusate from isolated blood-perfused lungs (Helset et al. 1995; Sorhaug et al. 2008). In rodent lungs, NEB cells could be regarded as inexhaustible local pools of the vasodilator CGRP, which under normoxic conditions may be released continuously, in this way being at least partly responsible for the homeostatic control of blood vessel relaxation. After chronic or intermittent hypoxia the number of NEB cells with a detectable immunoreactivity for CGRP is augmented (McBride et al. 1990; Springall and Polak 1993; Sorhaug et al. 2008). Apart from some contradictory findings, the current consensus is that NEB cells are activated by, and appear to adapt to chronic hypoxia (Cutz 1997). An increased number of NEBs has been reported in young rabbits kept in hypobaric chambers or breeded at high altitude, and in Sprague Dawley rats exposed to chronic normobaric hypoxia (Pack et al. 1986). In Wistar rats exposed to hypoxic conditions for 1–3 weeks, NEBs showed elevated levels of CGRP (McBride et al. 1990; Roncalli et al. 1993), without a change in CGRP mRNA levels or NEB cell numbers. Hypoxia, therefore, appears to inhibit CGRP release, rather than stimulate production or have an influence on PNEC division. The generation of reactive oxygen species

from mitochondria, having effects at the level of gene expression, has been proposed to underlie the responses of NEBs to chronic hypoxia (Peers and Kemp 2001).

In addition to 5-HT, candidate transmitters involved in chemotransduction of intrapulmonary stimuli via NEB cells include ATP and ACh. Both for ATP and ACh, at least in some species, autoreceptors on NEB cells were identified. In hamsters, pulmonary NEB cells were reported to express functional $P2X_{2/3}$ autoreceptors, which upon binding of ATP can serve as a feedback mechanism that further augments 5-HT release (Fu et al. 2004). NEB cells that harbour functional ACh receptors may, in the presence of ACh or other ACh receptor agonists play a role under both normal and pathological conditions, especially those linked to smoking (Fu et al. 2003; Schuller et al. 2003). Very recently, GABA production systems and GABA receptors have been demonstrated in mouse and monkey pulmonary NEBs, and may also function as an autoreceptor system (Yabumoto et al. 2008; Fu and Spindel 2009).

Besides hypoxia, also cigarette smoke is apparently able to induce changes in the pulmonary neuroendocrine system (Tabassian et al. 1988, 1993; Aguayo 1993). The detection of nAChRs on PNECs (Sekhon et al. 1999; Plummer et al. 2000; Fu et al. 2003), were suggestive of direct involvement of nicotine or nicotine derivates, such as nitrosamines, in changes in PNEC cells and possibly also in the development of tobacco-associated small lung cell carcinoma (Schuller et al. 2000). However, at the moment no physiological data are available on the sensitivity for, or acute activation by nicotine or ACh of NEB cells in rats or mice.

Mechanical stretch has since long been proposed as a possible stimulus for NEBs (Lauweryns and Peuskens 1972; Wasano and Yamamoto 1978) and has more recently been shown to induce release of 5-HT from NEB cells that were isolated from rabbit foetal lungs, via mechanosensitive plasma membrane channels (Pan et al. 2006b). This stretch-induced 5-HT release did not seem to involve exocytosis from DCVs.

Today, hypoxia and mechanical forces seem to be the best illustrated "natural" stimuli that are able to induce changes in the release of NEB cell secretory products. However, the complex morphological organisation of NEBs, with associated nerve terminals and covering CLCs, suggests that these may not be the only stimuli that can affect NEB cells, and that there may still be a lot to discover.

5.2.4
Neuroepithelial Bodies and the Various Associated Nerve Terminals Form Distinct Units with Great Functional Potential in the Airway Epithelium

The characteristics of the excitable NEB cells clearly allow the perception of environmental stimuli. When local stimuli activate the release of secretory products from NEB cells, afferent nerve fibres in synaptic contact are believed

to depolarise and develop a generator potential that, once a certain threshold has been reached, triggers an action potential that can reach the CNS.

So far, the best characterised possible mechanisms through which NEB cell transmitters can influence their target vagal sensory nerve terminals, relies on the expression of $P2X_{2/3}$ receptors on the vagal sensory components of the innervation of pulmonary NEBs in rodents (Brouns et al. 2000; Van Genechten et al. 2004; Brouns et al. 2009b). The extensive intraepithelial arborisations of these vagal sensory nerve terminals contact ATP-storing NEB cells, suggesting that ATP secreted by NEB cells may act as a neurotransmitter/neuromodulator in the vagal transduction pathway. In this way, the NEBs would be "functional units" that allow fast transduction of local pulmonary information to the CNS. More recently, it has been demonstrated in a lung slice preparation that NEB cells indeed release ATP when activated (De Proost et al. 2009). Similar mechanisms of functional ATP-containing receptor cells and associated ATP receptor-expressing nerve terminals have been reported for taste buds (for review see Housley et al. 2009) and carotid bodies (Rong et al. 2003).

Apart from obvious contacts with afferent nerve terminals, both light microscopical and ultrastructural observations show the existence of contacts between "efferent" nerve terminals and NEB cells, suggestive of a flexibility in neurally mediated communication and adaptation. The complex innervation pattern of pulmonary NEBs may therefore prepare NEBs for a variety of potential challenges and functions associated with their physiology. The "modulatory" roles of specific nerve fibre populations can be exerted both by motor nerve terminals and by efferent-like contents of collateral endings of sensory nerve terminals.

An interesting example of a real motor component of the innervation of pulmonary NEBs that is probably involved in modulating the NEB cell function is the population of intraepithelial nitrergic nerve terminals in rat NEBs. It has been proposed that NO, released from the terminals of intrinsic pulmonary nitrergic neurons may exert an inhibitory influence on the sensory discharge of NEB cells in response to local stimuli. Except for local actions, such a system may keep NEB receptors "quiet" as far as the CNS is concerned. In this way, signalling to the CNS via vagal or spinal afferent pathways may be limited to powerful stimuli that necessitate actions mediated by the CNS for the regulation of general lung function (Brouns et al. 2002a; Adriaensen et al. 2003).

The suggestion of a unique link of preganglionic parasympathetic motor fibres with pulmonary NEBs in rats may imply an important central control of the secretory activity of NEB cells. In both rats and mice, a considerable number of NEBs are innervated by motor components that consist of intrinsic or extrinsic populations that are clearly separate from the sensory endings. The observation that at least a subpopulation of the NEBs actually receives both intrinsic and extrinsic motor endings suggests important additional control mechanisms for the secretory activity of pulmonary NEBs.

Apart from the terminals of motor nerves, it has been postulated for many years that NEB cells may be locally modulated by efferent nerve endings that are

formed at the periphery of sensory nerve terminals. Detailed ultrastructural studies have shown that intraepithelial vagal sensory nerve fibres in (rodent) NEBs carry both afferent-like (mitochondria-rich) and efferent-like (packed with clear synaptic vesicles) nerve endings (Van Lommel and Lauweryns 1993a). The observation of vesicle-like VAChT and VGLUT IR in intraepithelial vagal sensory nerve terminals (Adriaensen et al. 2006), likely represents the light microscopical equivalents of the efferent ultrastructural characteristics. The "efferent" nerve endings of vagal sensory endings were suggested to be potential sites for a so-called "axon reflex" mechanism that could modulate the NEB receptor cells, serve to integrate sensory input or to initiate a motor response via local neurons without involvement of the CNS (Lauweryns and Van Lommel 1986, 1987; Adriaensen and Scheuermann 1993). Such systems that prevent NEB receptors from continuously transmitting information to the CNS would be crucial in avoiding exaggerated central actions that may eventually lead to airway hyperreactivity or to a general pulmonary vasoconstriction and hypertension.

Of great functional interest was the detection of VGLUTs in the majority of sensory nerve fibre populations that contact rodent pulmonary NEBs, suggesting the vesicular storage and exocytotic release of glutamate from these peripheral afferent endings (Takamori et al. 2000; Raab and Neuhuber 2007). The presence of synaptic vesicles containing the excitatory transmitter glutamate in intrapulmonary sensory nerve endings seems contradictory at first glance, but suggests that glutamatergic autocrine mechanisms regulate the transduction processes of these terminals (Raab and Neuhuber 2007), or the activity of the associated receptor cells, after or during transduction of adequate stimuli to afferent nerve discharges. Pulmonary NEBs contacted by glutamatergic vagal sensory nerve fibres may therefore be independent of additional motor fibres to regulate the excitability of the NEB receptor cells and/or control neurotransmitter release.

Since the discovery of pulmonary NEBs (Fröhlich 1949), it has been suggested that these structures might function as chemoreceptors important in sensing alterations of environmental conditions. For many decades (for review see Cutz et al. 2009b) NEBs have been proposed to be involved in oxygen sensing, and morphological, molecular and functional data provide evidence that NEB cells are able to respond to hypoxia (see Sect. 5.2.3.4). At present, however, direct evidence is still lacking for the downstream transmission of hypoxia-generated signals by NEB cells via vagal afferent fibres to the CNS. Moreover, many decades of electrophysiological experiments on vagal afferents point to the conclusion that none of the electrophysiologically characterised pulmonary sensory receptors appears to be affected by hypoxia (Widdicombe 2001; Cutz et al. 2009b). Considering, e.g., the spinal origin of CGRP/SP C-fibres in rat lungs, a possible central transduction of hypoxic stimuli may be mediated by spinal instead of vagal afferents. On the other hand, the main sensor/effector action of NEBs to hypoxia could be local. Moreover, specific populations of nerve fibres contacting the NEB cells may play a role in modulating the reactions of NEBs to hypoxia or other stimuli (Brouns et al. 2002a; Van Genechten et al. 2003, 2004). It has, for instance,

been demonstrated that the CGRP-positive C-fibre like nerve fibres in contact with NEBs are required for the modulatory effect of endogenously released CGRP on pulmonary vascular tone (Tjen-A-Looi et al. 1998). Also, the intraepithelial nitrergic nerve terminals may be involved. All NEBs that receive an intraepithelial nitrergic innervation were seen to additionally reveal basal contacts with CGRP-ir spinal sensory nerve fibres, the presumable collaterals of which form baskets around the nitrergic neurons in the lamina propria. In this way, the nitrergic innervation, together with the spinal sensory nerve fibres, may be essential components in the hypoxic inhibition of CGRP release from NEBs (Brouns et al. 2002a; Adriaensen et al. 2003). For Fawn-Hooded rats, a model for primary pulmonary hypertension, it has been proposed that the strongly reduced intrinsic pulmonary nitrergic innervation of NEBs might be involved in the mechanism of unexplained hypersensitivity of this rat strain to hypoxia (Van Genechten et al. 2003, 2004). For carotid bodies, chemosensory receptor-effector organs monitoring blood gases, it has also been proposed that inhibition of neurotransmitter release from carotid body chemoreceptor cells is mediated by the release of nitric oxide from efferent nerve terminals (Campanucci and Nurse 2007). Even during the normal pulmonary activity, parts of the lungs may be cyclically "hypoxic". A system that prevents the receptors from continuously transmitting this "redundant" information to the CNS would be crucial for avoiding exaggerated central actions that would eventually lead to a general pulmonary vasoconstriction and hypertension similar to that seen in some pathological conditions, or e.g., the Fawn-Hooded rat. In the proposed mechanism, NO released from nitrergic nerve terminals in pulmonary NEBs may inhibit the sensory discharge of NEB cells in response to mild local hypoxia. It might then be presumed that the $P2X_3$ receptor-expressing vagal sensory component of the innervation of NEBs may be involved in the central interactions, only if a certain threshold is reached.

More and more evidence appears to suggest that pulmonary NEBs may be involved in mechanosensing (earliest hypotheses: Lauweryns and Peuskens 1972; Wasano and Yamamoto 1978). Apart from their intraepithelial vagal sensory connections that express a neurochemical coding reminiscent of mechanoreceptor terminals (see Sect. 5.2.2.2), recent functional data point out that cultured primary NEB cells can be stimulated to release 5-HT upon sinusoidal cyclic stretch (Pan et al. 2006b). As proposed for many hollow organs [for review see: (Burnstock 2009)], appropriate mechanical stimuli may induce the release of ATP by exocytosis of the secretory granules from NEB cells, and may in turn bind to $P2X_{2/3}$ receptors on the myelinated vagal afferent nerve terminals located between the NEB cells (Brouns et al. 2000).

Another presumed function of NEBs that may be under control of the innervation concerns their role as "immunomodulators" (for recent review see Van Lommel et al. 2009). NEBs appear to respond to systemic sensitisation with synthesis and subepithelial secretion of messenger molecules (Bousbaa and Fleury-Feith 1991; Bousbaa et al. 1994; Van Lommel et al. 2009), suggesting a role in airway homeostasis and allergic airway disease. A prime candidate

immunomodulatory messenger molecule among several bioactive substances known to be secreted by NEBs is CGRP. The immunomodulatory functions of CGRP are well established and include induction of eosinophil chemotaxis, reduction of antigen presentation and modulation of cytokine and chemokine secretion (Tsukiji et al. 2004). It has been suggested that the extensive vagal sensory connections of NEB cells provide a potential link between inflammatory mechanisms in the airways and the induction of central hyperreactivity, and that ATP may be involved (Adriaensen and Timmermans 2004).

Besides their apparent receptor function, which is the focus of the present review, it is clear that NEBs may exert other functions (independent of their innervation?) during prenatal, perinatal and early neonatal life (for review see Sorokin and Hoyt 1989; Linnoila 2006; Cutz et al. 2008; Domnik and Cutz 2011). However, even during these crucial periods of life, NEBs are gradually contacted by different populations of nerve fibres (Brouns et al. 2002a, 2003a; Pan et al. 2004), suggesting that the formation of a functional unit including NEB cells and nerve terminals is very important for carrying out their physiological actions. Finally, not all NEBs are contacted by the same populations of nerve fibres, which probably reflect even more diverse functional capabilities of NEBs. Even considering the fact that only a subpopulation of the NEBs receives certain populations of nerve terminals, it still implies that several hundred receptor sites are involved.

5.2.5 Functional Morphological Features of Clara-like Cells: The NEB Microenvironment

Whereas the construction of NEBs as "functional units" of neuroendocrine NEB cells and nerve terminals is clear, it remains a remarkable observation that this organoid structure is in most animal species almost entirely covered by a specialised type of Clara cells, the so-called CLCs or variant Clara cells (vCE).

CLCs share most of their characteristics with Clara cells (Clara 1937), including the secretion of "Clara cell secretory protein (CCSP)". CCSP is the founder protein of the secretoglobin family [i.e., secretoglobin 1A member 1 (SCGB1A1)] and has been found in various tissues, resulting in different names [e.g., Clara Cell-Specific 10 kD Protein (CC10), uteroglobin, urine protein-1]. Because of their production of CCSP, CLCs can be identified in lungs based on the combination of antibody reagents specific to this protein (Reynolds et al. 2002) (Fig. 5.15) and their specific location in the "NEB microenvironment". Although the endogenous function of CCSP is not entirely clear, evidence points to anti-oxidant, immunomodulatory, anticarcinogenic and epithelial repair properties (Wong et al. 2009). In rats, CLCs have been distinguished from surrounding Clara cells and NEB cells by their expression of the R-type VGCC $Ca_v2.3$ in their apical membrane (De Proost et al. 2007b) (Fig. 5.15d–i).

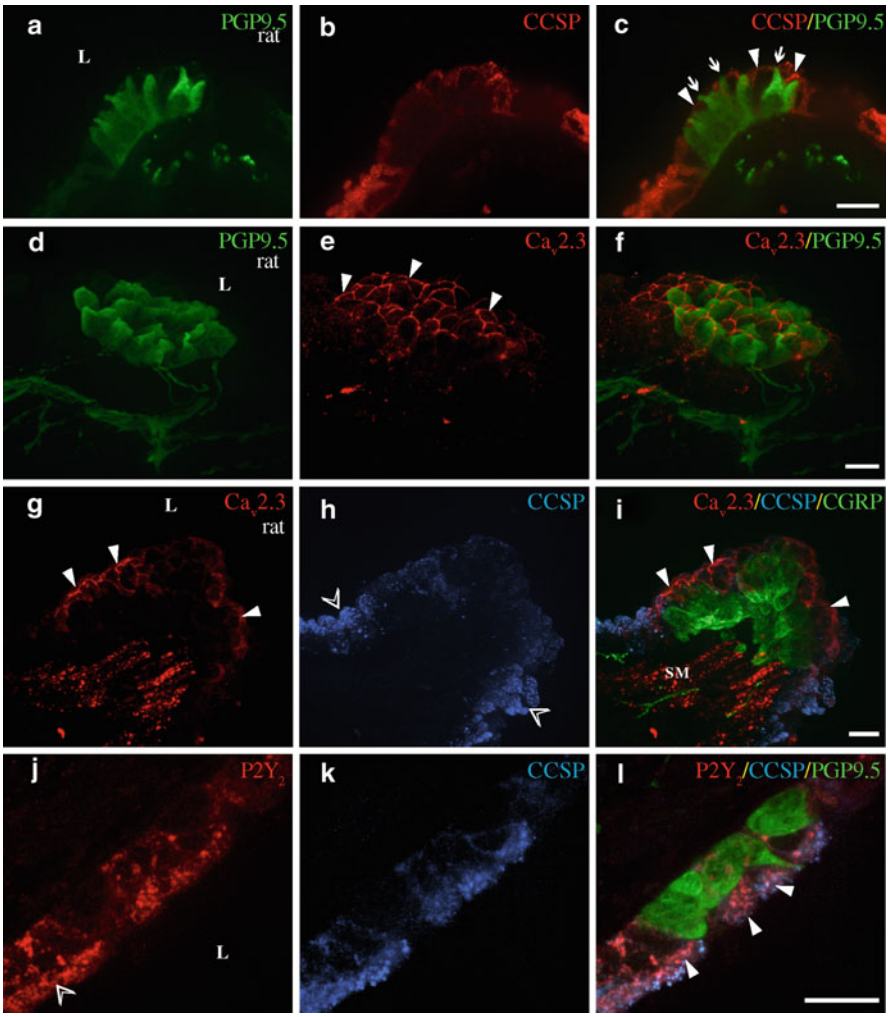

Fig. 5.15 In the NEB microenvironment the neuroendocrine NEB cells are crowned by a special type of Clara cells, termed Clara-like cells. Multiple immunocytochemical staining reveals the unique location and some specific features of this cell type. *L*: airway lumen. (**a–c**) Immunocytochemical double labelling of a rat bronchiole for PGP9.5 (**a**), and Clara cell secretory protein (CCSP; **b**), as a marker for Clara cells. (**c**) The PGP9.5-ir NEB cells are almost completely covered by Clara-like cells (*arrowheads*). Some of the NEB cells reach the airway lumen via thin apical processes only (*arrows*). (**d–f**) An extensively innervated PGP9.5-ir NEB in a rat lung is shielded from the airway lumen by a "cap" of cells that show IR for the voltage-gated calcium channel $Ca_v2.3$ in their apicolateral membranes (**e**; *arrowheads*). (**g–i**) Triple immunostaining for $Ca_v2.3$ (**g**), CCSP (**h**) and CGRP shows that only CCSP-ir Clara-like cells, overlying the CGRP-positive NEB cells, express $Ca_v2.3$ (*arrowheads*). Clara cells (*open arrowheads*) that do not belong to the NEB microenvironment lack $Ca_v2.3$ IR. Note that also the subepithelial airway smooth muscle bundle

In the bronchiolar epithelium, CLCs have been put forward as local stem/progenitor cells in specific regions of the airways (Hong et al. 2001; Meuwissen and Berns 2005; Snyder et al. 2009; Roomans 2010; Kratz et al. 2010; Sullivan et al. 2010). Based on their naphthalene resistance (Mahvi et al. 1977; Plopper et al. 1992; Stripp et al. 1995; Reynolds et al. 2000a, b; Hong et al. 2001), these CLCs have been shown to proliferate and participate in rodent airway epithelial renewal after naphthalene ablation of all other Clara cells, revealing the capacity to self-renew and proliferate, and even give rise to multipotent differentiation. The resistance of CLCs to naphthalene injury is based on their lack of expression of the enzyme CYP2F2, a member of the cytochrome 450 family that intracellularly converts naphthalene to a strongly cytotoxic product. This lack of expression may reflect the less differentiated state of the cells relative to the majority of the Clara cell population.

After lung injury, some proliferating cells in the NEB microenvironment have been reported to express both CCSP and neuroendocrine markers (Reynolds et al. 2000a; Linnoila 2006). Expansion of this dual-positive population may relate to the existence of mixed small cell and non-small cell lung carcinoma (Giangreco et al. 2007), or may reflect the ability of the CLCs to also differentiate into neuroendocrine cells.

Nowadays the "NEB microenvironment" is proposed as a niche for Clara-like stem cells (Bishop 2004; Rawlins and Hogan 2006; Liu et al. 2006; Giangreco et al. 2007; Snyder et al. 2009), similar to the situation in, e.g., the hair follicle or intestinal crypts, in which intercellular signalling between stem cells and surrounding cells has been observed. In this view, it is important to mention that we have recently demonstrated that the NEB transmitter ATP might be involved in the interaction between NEB cells and CLCs (De Proost et al. 2009). Upon NEB cell stimulation, exocytotic release of ATP was shown to activate the surrounding CLCs via functional G-protein coupled $P2Y_2$ ATP-receptors, resulting in an intracellular calcium rise in CLCs (De Proost et al. 2009) (Fig. 5.15j–l). This local purinergic signalling within the NEB microenvironment may be important in airway function, airway epithelial regeneration after injury and/or the pathogenesis of SCLCs. In other stem cell niches, it has been proposed that dysregulation of interactions between the stem cells and the niche environment may drive tumorigenesis (Kiefer 2011).

Because of the invariably observed close interaction between NEB cells, complex nerve terminals and CLCs, it is obvious that *in vitro* models for functional studies should preferentially include all components of the NEB microenvironment.

Fig. 5.15 (continued) (*SM*) expresses $Ca_v2.3$. (**j**–**l**) Triple immunocytochemical staining of the NEB microenvironment in a mouse airway, showing $P2Y_2$ (ATP) receptor IR (**j**) in CCSP expressing (**k**) Clara-like cells (*arrowheads*). Also Clara cells (**a**; *open arrowhead*) show $P2Y_2$ IR. *Scale bars* = 10 μm

5.3 Visceral Pleura Receptors

VPRs are structures with a sensory receptor-like morphology, the laminar nerve endings of which are intermingled with the elastic fibres of the visceral pleura (Pintelon et al. 2007) (Fig. 5.16a). Extensive morphological and neurochemical data about VPRs have been gathered for rats only, although literature data and preliminary studies in mice point to the existence of VPRs in other animal species too (Larsell 1921, 1922; McLaughlin 1933; Dwinnell 1966) (own unpublished observations).

In rats, the innervation of the visceral pleura can partially be studied in cryostat sections of whole lungs (Fig. 5.15a). However, only intact whole mount preparations of one or more surfaces of the visceral pleura can really reveal its complex innervation pattern (Fig. 5.15). Preparation of visceral pleura whole mounts includes removing most of the alveolar lung tissue, while carefully leaving the pleura intact (Pintelon et al. 2007). Immunocytochemical staining of rat visceral pleura whole-mount preparations with the general neuronal marker PGP9.5 revealed thick nerve bundles that enter the visceral pleura at the hilus of each lung lobe and cross the mediastinal surface. The bundles continuously branch into smaller nerve trunks that also reach the other lung surfaces, thereby giving rise to a wide-meshed network over the entire lung surface. Whereas the mediastinal side is clearly more densely innervated, more distally the nerve bundles consist of just a few fibres. At all levels, the nerve trunks appeared to repeatedly split off single PGP9.5-ir nerve fibres, which in turn revealed dichotomous branches. One of the branches terminates as a laminar end-organ with the appearance of a sensory receptor (VPR), while the other further travels along the visceral pleura, frequently forming more receptor-like VPR terminals on its way.

Double immunostaining for PGP9.5 and elastin-α revealed that the receptor-like terminals of VPRs protrude between the elastic fibres (Fig. 5.16a) and reveal no obvious contacts with blood or lymphatic vessels in the connective tissue layer of the visceral pleura, nor with specialised cells or cell groups.

Based on their morphology, rat VPRs show good resemblance with non-encapsulated nerve terminals described earlier in the pleura of rabbits, dogs and lambs using, e.g., osmium tetroxide staining (Larsell 1922; Dwinnell 1966). These non-encapsulated nerve terminals, as well as rat VPRs, have been suggested to be mainly restricted to the mediastinal and interlobar surfaces of the lung lobes (Larsell 1922; Pintelon et al. 2007). According to physiological studies, an extensive sensory innervation was not to be expected in the visceral pleura (Moore 1992; Jeffery 1995; Burgers et al. 1999; review Brims et al. 2010). The discrepancy between physiological and morphological data may at least partly be explained by the fact that in most of the reported physiological experiments (Capps 1911; Larsell 1922) only the costal part of the pleural surface was stimulated, which indeed harbours a very low density of VPRs (Pintelon et al. 2007).

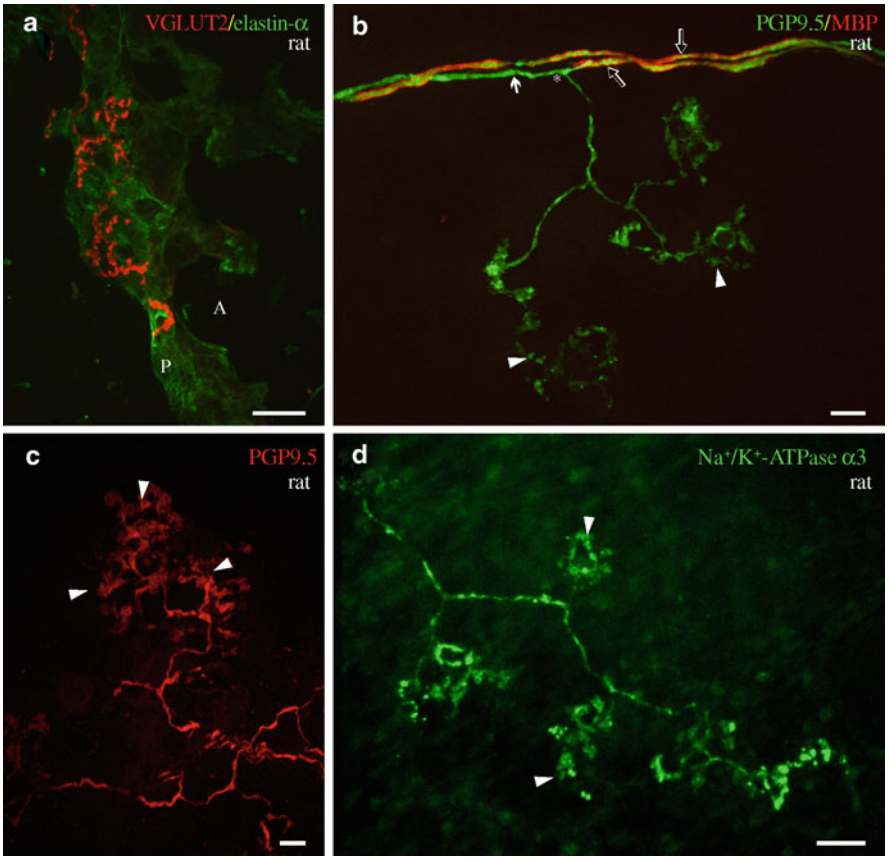

Fig. 5.16 Immunocytochemical staining of spinal sensory visceral pleura receptors in rats. (**a**) Cryostat section of an interlobar lung surface in rat lung showing extensively arborising laminar VGLUT2-ir nerve terminals of visceral pleura receptors (VPRs) protruding between the elastin α-ir elastic fibres in the visceral pleura (*P*). *A*: lung alveoli. (**b**) Whole-mount preparation of the visceral pleura double stained for PGP9.5 and MBP. The PGP9.5-ir nerve fibres in the small nerve bundle are surrounded by an MBP-ir myelin sheath (*open arrows*) that ends just before the dichotomous branching point (*asterisk*), where one of the branches gives rise to a VPR (*arrowheads*) and the other branch (*arrow*), now unmyelinated, continues its trajectory. (**c**) PGP9.5-immunostained VPR (*arrowheads*) in a whole-mount preparation. (**d**) VPR immunostained for Na^+/K^+-ATPase α3, accentuating the laminar appearance of the VPR-terminals (*arrowheads*). *Scale bars* = 10 μm

Immunocytochemical staining for MBP revealed myelin sheaths surrounding a substantial population of nerve fibres travelling the nerve bundles of the visceral pleura. Myelinated nerve fibres were seen to lose their myelin sheaths in the immediate neighbourhood of the first dichotomous branching point that gives rise to a collateral PGP9.5-ir fibre terminating as a VPR (Fig. 5.16b). Subsequently,

the remaining unmyelinated nerve branch often further traversed the visceral pleura for considerable distances, thereby regularly splitting off additional VPRs. This implies that many of the VPRs are located at a considerable distance from the myelinisation endpoint. Myelinated nerve fibres giving rise to VPRs had diameters ranging between 1.4 and 3.5 μm (Pintelon et al. 2007). Left or right unilateral infranodosal vagotomy revealed no reduction in the number of VPRs in the rat and, as such, confirms their non-vagal origin. This is in accordance with earlier assumptions that the receptor-like terminals in the visceral pleura may derive mainly from DRGs of the upper thoracic spinal nerves and reach the lungs via the sympathetic trunks (Larsell 1922; Larsell and Coffey 1928).

To explore the neurochemical coding of VPRs, the set of antibodies that is known to selectively characterise sensory (mechano)receptors in lungs (*see* higher) was applied.

5.3.1 Functional Morphological Characteristics of VPRs

Multiple immunocytochemical staining showed that the sodium/potassium transporter Na^+/K^+-ATPase α3 subunit is expressed in the surface membrane of both the laminar terminals of VPRs and in the nerve fibres from which they arise (Fig. 5.16d).

Antibodies against VGLUT1 or VGLUT2 revealed that VPR terminals are glutamatergic (Fig. 5.16a). While VGLUT1 IR was strong in nerve fibres and rather weak in VPR terminals, VGLUT2 could be mainly detected in the laminar nerve terminals and was weak in nerve fibres giving rise to VPRs, suggesting local accumulation of glutamatergic secretory vesicles and limited axonal transport (Pintelon et al. 2007). The assumption has been made that glutamate, released from peripheral sensory nerve endings, may play an important role in modulating the excitability of sensory nerve endings (Banks et al. 2002; Bewick et al. 2005).

A subpopulation of the PGP9.5-ir VPRs appeared to express the $P2X_3$ ATP receptor. $P2X_3$ receptor IR was predominantly displayed in the laminar terminals and was nearly undetectable in the branching nerve fibres. The predominant expression of $P2X_3$ receptors on the surface of VPR endings is likely due to the myelinated nature of the approaching nerve fibre (Pintelon et al. 2007).

IR for the calcium-binding protein CB, though often weak, appeared to be perfectly co-localised with the PGP9.5 IR and could be demonstrated both in the nerve fibres and in the VPR terminals.

Immunocytochemistry with antibodies against CGRP revealed non-myelinated thin varicose nerve fibres in the nerve bundles traversing the visceral pleura. These CGRP-ir nerve fibres often appeared to give rise to collateral branches that, however, seemed to be unrelated to the VPRs. Additionally, none of the observed VPRs was seen to express CGRP (Pintelon et al. 2007).

Studying the presence of potential motor components in the innervation pattern revealed the absence of cholinergic motor fibres in nerve bundles traversing the visceral pleura, and that VPRs do not exhibit VAChT IR. TH IR, on the other hand, was seen in a few adrenergic nerve fibres in the pleural nerve bundles and exceptionally also in the terminals of just a few rat VPRs. The presence of a typical marker for postganglionic sympathetic motor neurons in sensory endings may seem contradictory, but catecholamines and catecholamine-synthesising enzymes have been reported in subpopulations of mammalian (including rats) cranial and spinal sensory neurons (Katz et al. 1983, 1987; Kummer et al. 1990).

5.3.2 Functional Implications

Since VPRs express different sensory neuron-specific substances that have been used to selectively identify mechanoreceptor-like terminals in airways and lungs, i.e., Na^+/K^+-ATPase α3 (Dobretsov et al. 2003), VGLUT2 (Raab and Neuhuber 2003), calcium-binding proteins (Duc et al. 1994; Dütsch et al. 1998) and $P2X_3$-receptors (Wang and Neuhuber 2003), the neurochemical characterisation of VPRs provides strong evidence for a mechanosensory function.

In earlier anatomical studies, Larsell and Coffey (1928) have suggested that nerve endings in the visceral pleura function as "stretch receptor". Since all VPRs appear to originate from myelinated nerve fibres, VPRs show typical features of peripheral low-threshold mechanosensors. The preferential location of VPRs at the mediastinal and nearby interlobar lung surface suggests a potential role in sensing mechanical stress at the interface with other important organs located in the thorax.

In several species, it has been shown that pulmonary application of bradykinin is able to induce cardio-respiratory responses that at least partly persist after vagotomy. This suggests that spinal pulmonary afferents, such as the ones giving rise to VPRs, may also be involved in transmitting chemical and/or mechanical information from the lungs to the CNS. Multimodal mechano- and chemosensitive afferent units have been reported in the mediastinal parietal pleura (Wedekind 1997) and the costal parietal pleura (Jammes et al. 2005). The reported characteristics suggest that VPRs may be involved in the sensory transduction of mechanical and/or chemical (nociceptive) stimuli, related to normal lung function or as a consequence of pleural disease. With respect to the potential clinical relevance, VPRs may be regarded as candidates for mediating at least certain aspects of the hitherto unknown mechanisms involved in pain sensation and/or (reflex) dyspnoea regularly described to result from visceral pleura tumours (England et al. 1989; Rusch 1990), pulmonary embolism and pleuritis (Urban et al. 1983; Reissig and Kroegel 2003). Finally, it should be taken into account that the information carried by sensory terminals in the visceral pleura may give rise to, not necessarily conscious, sensations.

Chapter 6
In Situ Functional Imaging of Sensory Receptors in Lung Models

To unravel the complex maze of electrophysiologically and neurochemically identified pulmonary mechanoreceptors, combined morphological and physiological studies will be essential to achieve a better understanding of the sensory interactions between the periphery (lung and airways) and the central nervous system. Reliable data may be obtained using lung models that combine the visualisation of morphologically characterised pulmonary receptors, and the possibility to study their physiological properties directly. We therefore attempted to develop *ex vivo* lung models in combination with vital staining, as *in situ* models that potentially allow functional studies of the three morphologically identified pulmonary receptors.

Direct *in situ* physiological studies of pulmonary receptors are challenging because they are diffusely spread in the lung (NEBs and SMARs) and mainly in the mediastinal visceral pleura (VPR). Moreover, a precondition for performing physiological experiments is the ability to visualise the structures of interest in live tissue.

Because airway whole mounts have been proven to be useful tools to study airway innervation (Weichselbaum et al. 1996; Avadhanam et al. 1997; Larson et al. 2003) and the architecture of the airway smooth muscle layer (Smiley-Jewell et al. 2002), an *ex vivo* model of live airway whole mounts was optimised a few years ago (De Proost et al. 2007a). Intrapulmonary airways were stabilised with agarose to provide internal support and protection of the airways, and were subjected to micro-dissection under ice-cold physiological perfusion, leaving the airway tree intact for as many levels of branching as possible (Fig. 6.1a). Microscopic inspection of the preparation after fixation and immunostaining revealed an intact innervation and smooth muscle layer, and proved the presence of intact vagal receptors within the smooth muscle (SMARs) and epithelial layers (NEBs). Control staining has confirmed that the method used for airway whole-mount preparation offers excellent tools for studying SMARs *in situ*.

In order to study live VPR, whole mounts of the visceral pleura (Fig. 6.2a) were prepared by removing alveolar tissue from unfixed lungs, under continuous perfusion with ice-cold physiological solution. After fixation of these whole-mount preparations, immunostaining proved the presence of nerve tracts and

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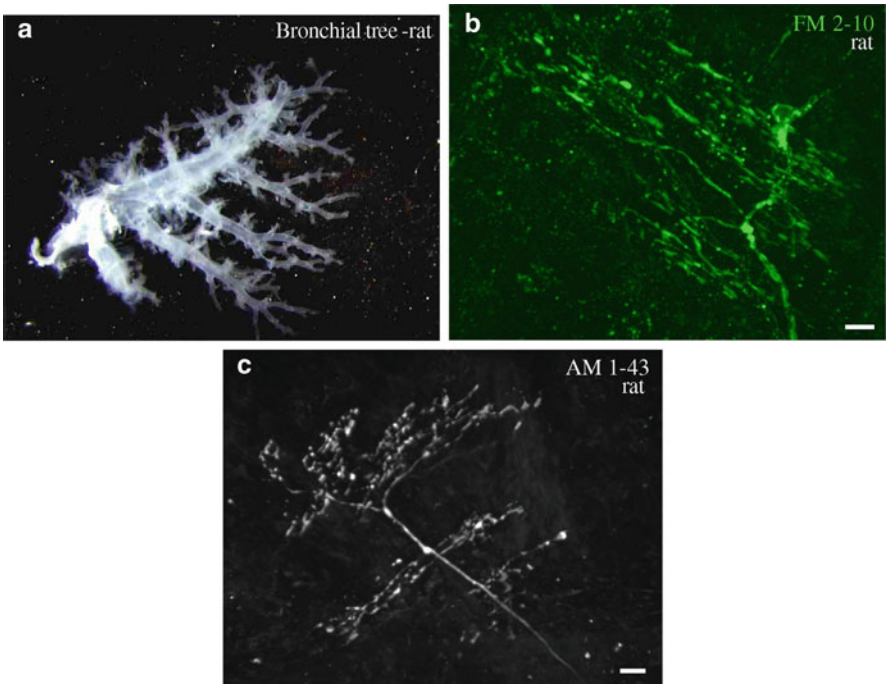

Fig. 6.1 (**a**) Stereomicroscopic image of a fresh airway whole-mount of the right caudal lung lobe of a rat. (**b**, **c**) Confocal images of SMARs after incubation of the airway whole mounts with the vital styryl pyridinium dyes FM 2-10 (**b**) and AM 1-43 (**c**). *Scale bars* = 10 μm

intact VPR (Fig. 6.2c–f). Fresh whole mounts of the visceral pleura therefore turned out to be promising tools for the functional study of VPR (Pintelon et al. 2007).

Although their presence was demonstrated in intrapulmonary micro-dissected airways (De Proost et al. 2007a), pulmonary NEBs are still largely inaccessible for direct measurements and manipulation in this model, because of their distribution in the almost unapproachable lung epithelium. Given the profuse innervation of pulmonary NEBs in rodents and their complex environment, an *in situ* model is needed that allows further insight into the working mechanism and roles of the complex pulmonary NEBs under physiological conditions. Fresh precision-cut lung slices (for review, see Liberati et al. 2010; Sanderson 2011) have been reported useful for physiological studies on NEBs for some time (Fu et al. 1999, 2002, 2007). Lung slices have the advantage that NEBs are still present in their natural environment, including not only neighbouring cells and tissues, but also the selective nerve endings (Adriaensen et al. 2003, 2006; Pintelon et al. 2005; De Proost et al. 2008; Liberati et al. 2010). Considering the extensive innervation of NEBs and the potential modulatory effects of the innervation on NEB function

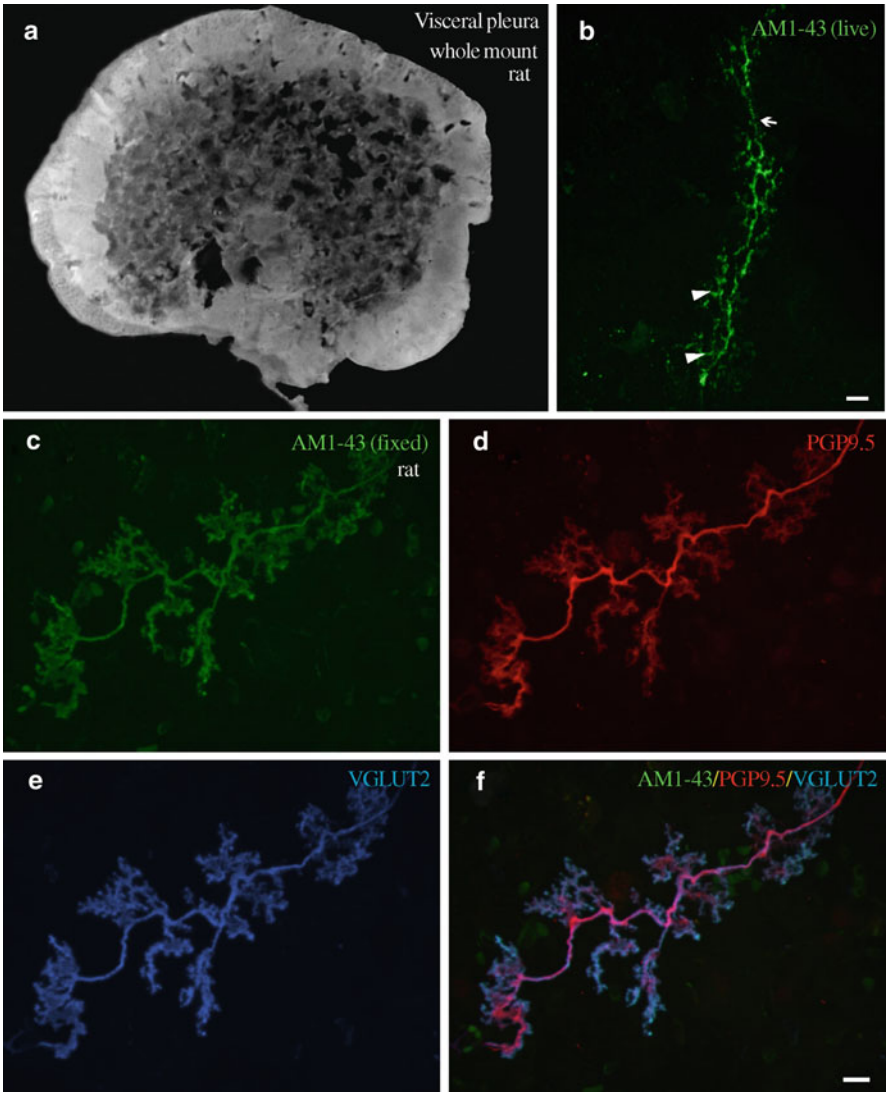

Fig. 6.2 (**a**) Stereomicroscopic image of a fresh whole-mount preparation of a visceral pleura of a rat, after removal of most of the alveolar tissue. (**b**) Vital staining of a visceral pleura whole mount with AM1-43, showing an approaching nerve fibre (*arrow*) and the laminar terminals of a VPR (*arrowheads*). (**c–f**) VPR in a whole mount that was fixed after vital staining with AM1-43 and subsequently immunostained for PGP9.5 (**d**) and VGLUT2 (**e**). (**f**) Merged image of (**d**) and (**e**). *Scale bars* = 10 μm

(Lauweryns and Van Lommel 1986; Adriaensen et al. 2006), an *ex vivo* lung slice setting was designed and optimised a few years ago that allows live cell imaging (Pintelon et al. 2005; De Proost et al. 2008, 2009). Vibratome sectioning of

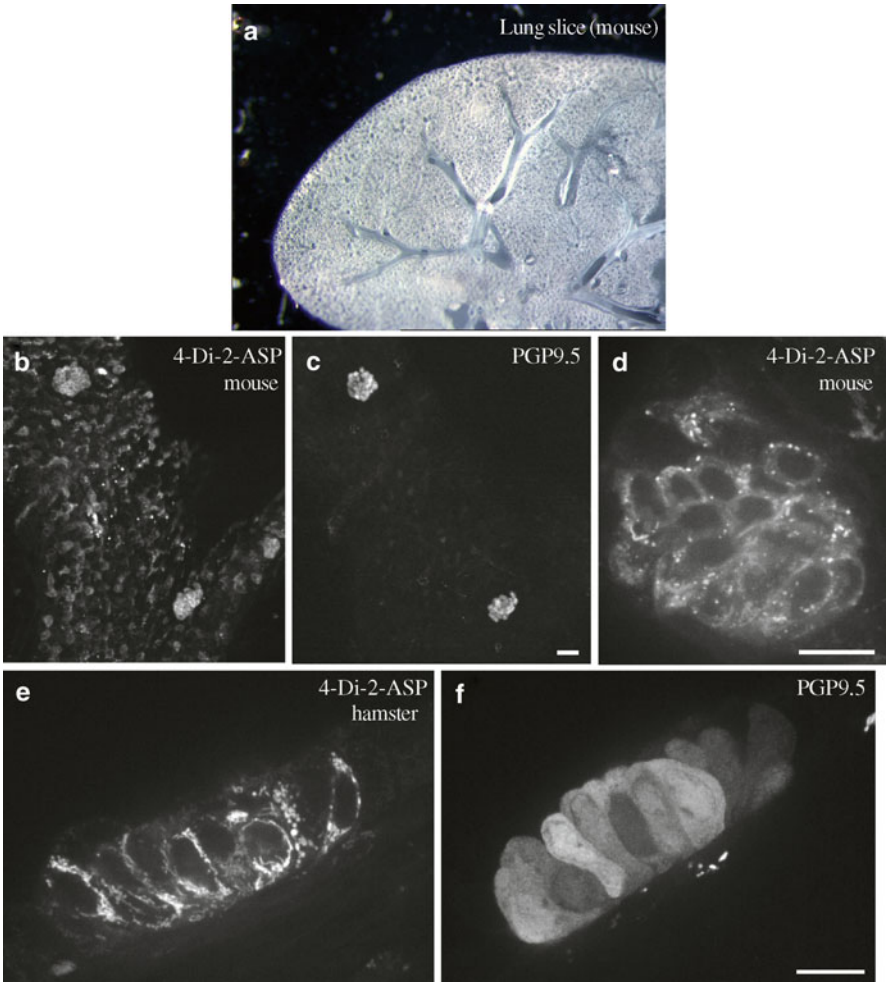

Fig. 6.3 (**a**) Stereomicroscopic image of a vibratome slice of living lung tissue. (**b**–**f**) Specific staining of pulmonary NEBs in fresh lung slices with the styryl pyridinium dye 4-Di-2-ASP. (**b**, **c**) Overview of a mouse lung slice showing two 4-Di-2-ASP fluorescent cell groups (*arrows*) that can be identified as NEBs by their PGP9.5 IR. (**d**) High magnification of pulmonary NEB cells in a mouse lung slice, showing a granular 4-Di-2-ASP pattern, represents mitochondrial staining. (**e**, **f**) 4-Di-2-ASP live staining, and subsequent fixation and PGP9.5 immunostaining of a pulmonary NEB in a hamster lung slice. *Scale bars* = 10 μm

agarose-filled rat and mouse lung lobes yielded lung slices that enclosed branching airways, blood vessels and alveolar areas (Fig. 6.3a). In accordance with other groups that used vibratome lung slices, we observed beating cilia (Hayashi et al. 2005; Delmotte and Sanderson 2006) and bronchoconstriction/relaxation

(Bergner and Sanderson 2002; Perez and Sanderson 2005; Kummer et al. 2006), indicating that the airway epithelial cells and muscle cells did not suffer irreversible damage from sectioning, and that agarose filling had no negative effects on normal airway physiology. When agarose-filled lung slices were fixed and subjected to diverse immunocytochemical procedures, NEBs and their extensive innervation could easily be detected (Fig. 6.3), offering additional possibilities for elucidating the function(s) of NEBs in integrated approaches.

A prerequisite to unequivocally recognise pulmonary receptors in *ex vivo* preparations is the reliable and specific labelling of the live structures. For pulmonary NEBs, neutral red staining, reported to stain rabbit NEBs (Fu et al. 1999, 2002), proved unsuccessful in rat and mouse lung slices. Because of the capacity of styryl pyridinium dyes to selectively label nerve terminals and neuronal cell bodies, as also demonstrated in extrapulmonary airways (Mazzone and Canning 2003; Mazzone 2004, 2005; Mazzone and McGovern 2008), these dyes have recently been used to selectively and reproducibly visualise lung receptors in whole-mount preparations and lung slices of different animal species (Pintelon et al. 2005, 2007; De Proost et al. 2007a).

6.1 Selective Visualisation of Sensory Lung Receptors with Styryl Pyridinium Dyes

Lipophilic styryl pyridinium "Fluorescent Marker (FM)" dyes are vital dyes that are virtually non-fluorescent in aqueous medium, but become intensely fluorescent after being incorporated into the outer leaflet of the cells' surface membrane. In most cells, the fluorescent dye disappears again from the outer membrane after removal of the dyes from the medium. However, long-term fluorescent labelling of specific cell types, such as neurons and secretory cells, has been shown to be possible, and two different entry pathways have been suggested. On the one hand, FM dyes are most commonly believed to enter neurons and selective groups of secretory cells during recycling of synaptic vesicles, whereby fluorescent membranes are internalised and fluorescence remains after wash-out of the dyes (Cochilla et al. 1999; Ryan 2001; Fukuda et al. 2003; Brumback et al. 2004). Efficient labelling often requires stimulation of the cells. Alternatively, direct entry into the cytoplasm through mechanically gated channels has been proposed as the mechanism of accumulation of the fluorescent dyes in several types of sensory cells (Meyers et al. 2003).

Styryl pyridinium dyes have proven to be great tools to selectively and reproducibly visualise SMAR-endings in whole-mount models of extra- and intrapulmonary airways of rats and mice (Fig. 6.1b, c) (De Proost et al. 2007a). Incubation of *ex vivo* airway whole mounts with styryl pyridinium dyes [AM1-43, FM2-10, FM4-64 and 4-(4-diethylaminostyryl)-*N*-methylpyridinium iodide (4-Di-2-ASP)] has been shown to visualise the laminar nerve terminals of SMARs in these live

whole-mount airway preparations. Interestingly, the myelinated nerve fibres that give rise to the SMARs also appeared to incorporate the dyes. In this way, the *in situ* model allows direct access to both the sensory receptor terminals and the myelinated vagal afferent nerve fibres that give rise to the receptors (De Proost et al. 2007a).

Live VPRs can be studied by incubation of whole-mount preparations of the visceral pleura with a fixable form of FM 1–43 (AM 1–43). This method resulted in specific and reproducible visualisation of VPRs (Fig. 6.2b) and allowed to track the nerve fibres from which they arise over considerable distances, opening up new perspectives for further physiological studies of VPRs (Pintelon et al. 2007).

Although no conclusive evidence is available at the moment, selective accumulation of the styryl dyes in SMARs and VPRs in *ex vivo* lung models might involve non-selective ion channels, because these lung receptors are believed to represent mechanosensors.

Besides accumulating in nerve terminals, it is clear that the probe 4-Di-2-ASP accumulates in pulmonary NEBs, as was shown in *ex vivo* vibratome lung slices (Pintelon et al. 2005) and in airway whole mounts of different animal species (De Proost et al. 2007a). In live lung slices, 4-Di-2-ASP was able to clearly differentiate intact living NEBs from other tissue elements (Figs. 6.3, 6.4 and 6.5) in an easy and reproducible way. Moreover, 4-Di-2-ASP staining of NEBs in agarose-filled lung slices offers good access to pulmonary NEBs.

Ex vivo airway whole-mount preparations, whole mounts of the visceral pleura and lung slices can subsequently be fixed and processed for immunostaining, making it possible to corroborate the reliable and specific labelling of pulmonary receptors by styryl pyridinium dyes (Fig. 6.2b–f) (Pintelon et al. 2005, 2007; De Proost et al. 2007a). Additionally, functional morphological data can be obtained by immunohistochemistry after using the *in situ* lung models in physiological/pharmacological experiments.

For SMARs and VPRs, the proposed *in situ* lung models should allow direct access to the sensory receptor terminals and the myelinated vagal afferent nerves that give rise to the receptors. The highly selective and reproducible visualisation of intact VPRs and SMARs in *in situ* lung models certainly offers realistic opportunities to directly manipulate the morphologically identified end-organs of these pulmonary receptors and to simultaneously monitor activity in the myelinated nerve fibres that give rise to them, using electrophysiological recording techniques. We are confident that such an approach will enable further selective investigation of the physiological characteristics of the different subgroups of morphologically identified pulmonary receptors.

For pulmonary NEBs, the *in situ* lung slice model, in which NEBs are selectively stained with 4-Di-2-ASP, was optimised for use in a confocal live cell imaging set-up to visualise potential reactions of NEBs (De Proost et al. 2008, 2009).

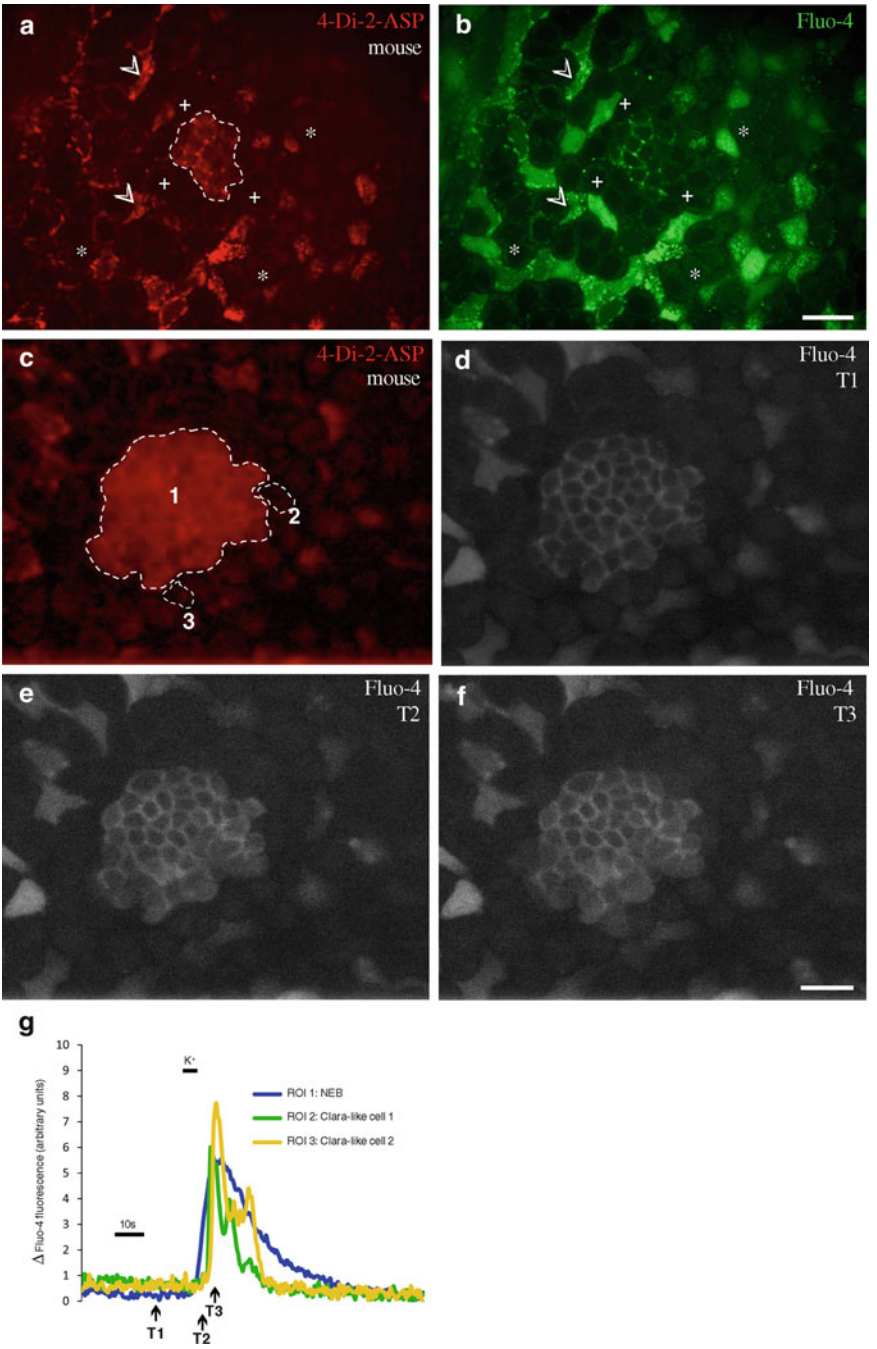

Fig. 6.4 Mouse airway epithelium in fresh lung slices stained with 4-Di-2-ASP and with the intracellular free calcium indicator Fluo-4. (**a**, **b**) Labelling with 4-Di-2-ASP allows for identification of the *encircled* NEB, revealing that basal Fluo-4 fluorescence in NEB cells is limited. Ciliated cells (*open arrowheads*) show bright 4-Di-2-ASP and Fluo-4 fluorescence,

6.2 Evaluation of the *Ex Vivo* Lung Slice Model to Explore the NEB Microenvironment Using Functional Fluorescent Indicators and Confocal Live Cell Imaging

To allow the unequivocal identification of NEBs in this live cell imaging set-up, lung slices are incubated with the styryl pyridinium dye 4-Di-2-ASP (Pintelon et al. 2005; De Proost et al. 2008, 2009). 4-Di-2-ASP-stained pulmonary NEBs appear as fluorescent clusters of small, rounded epithelial cells. Furthermore, 4-Di-2-ASP-incubated lung slices displayed a mosaic of polygonal fluorescent ciliated cells (Figs. 6.4 and 6.5), intermingled with almost non-fluorescent rounded cells, most of them being Clara cells (Figs. 6.4 and 6.5). NEB cells were found to be typically surrounded by CLCs that could be identified as a continuous layer of rounded non-fluorescent cells that are considerably larger than the 4-Di-2-ASP fluorescent NEB cells (Figs. 6.4 and 6.5). Based on the *ex vivo* 4-Di-2-ASP staining characteristics, light microscopic features and location, a straightforward and reliable identification of NEB cells, ciliated cells, non-ciliated bronchiolar Clara cells and CLCs can be achieved in live lung vibratome slices (Figs. 6.4 and 6.5). This observation offers the possibility to simultaneously visualise the responses of all these cell types to applied stimuli (De Proost et al. 2008, 2009), when functional fluorescent probes (e.g., Ca^{2+} indicators, membrane potential indicators, mitochondrial membrane potential probes) are used. Since activation of excitable cells is most of the time accompanied by a rise in intracellular Ca^{2+} concentration, Ca^{2+} indicators, such as Fluo-4 are often used in live cell imaging experiments.

6.2.1 Functional Fluorescent Indicators

6.2.1.1

Ca^{2+}-Imaging Experiments Using Fluo-4

Loading of fresh lung vibratome slices with Fluo-4 also showed a typical "basal" labelling pattern, enabling the discrimination of bright-fluorescent ciliated cells

Fig. 6.4 (continued) while Clara cells (*asterisks*) and Clara-like cells (*crosses*) are virtually non-fluorescent. (**c–g**) Representative recording of Fluo-4 fluorescence changes measured in NEB cells and surrounding Clara-like cells after a short (5s) challenge with 50 mM extracellular potassium $[K^+]_O$. (**c**) 4-Di-2-ASP stained NEB (region of interest (ROI)1) surrounded by Clara-like cells (ROI2 and ROI3 in the graph of **g**) imaged before the recording. (**d–f**) Time-lapse images of Fluo-4 fluorescence in the NEB and surrounding epithelial cells indicated in the graph, respectively as T1-T3. (**e**) NEB cells respond with a clear rise in Fluo-4 fluorescence right after the high $[K^+]_O$ challenge. (**f**) A few seconds later Clara-like cells respond with a rise in Fluo-4 fluorescence, due to their secondary activation by ATP released from the NEB cells. *Scale bars* = 10 μm

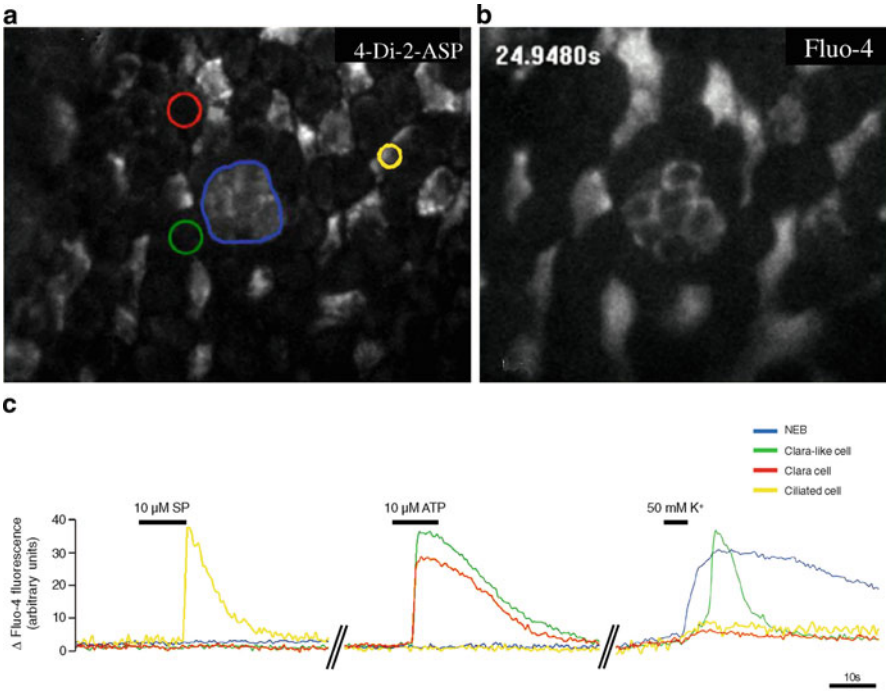

Fig. 6.5 Recording of Fluo-4 fluorescence changes in NEB cells (*blue* ROI), the surrounding Clara-like cells (*green* ROI), Clara cells (*red* ROI) and ciliated cells (*brown* ROI). (**a**) Labelling with 4-Di-2-ASP allows for the selective identification of these different epithelial cell types. (**b**) Image at the start of the experiment of the NEB microenvironment and the surrounding epithelium after loading with the calcium-indicator Fluo-4. (**c**) Consecutive application of 10 µM Substance P (SP), 10 µM ATP and high $[K^+]_O$ shows the selective stimulation of ciliated cells (by SP), Clara and Clara-like cells (by ATP) and NEB cells (by high $[K^+]_O$). Note the delayed Ca^{2+} rise in Clara-like cells in the right panel of the graph. *Scale bars* = 10 µm

and virtually non-fluorescent Clara and CLCs (De Proost et al. 2008, 2009). Upon activation of Fluo-4 loaded cells, a rise in intracellular Ca^{2+} concentration is reflected by a rise in Fluo-4 intensity. By selecting specific "regions of interest" (ROIs), changes in fluorescence intensity can then be plotted against time (Figs. 6.4c–g and 6.5).

Because Fluo-4 labelling on its own does not allow the clear identification of NEBs, performing selective Ca^{2+}-imaging experiments of pulmonary NEBs needs the combination with 4-Di-2-ASP live staining. Fluo-4 has fluorescence spectra compatible with the simultaneous visualisation of 4-Di-2-ASP.

6.2.1.2

4-Di-2-ASP as a Fluorescent Indicator for Changes in Mitochondrial Membrane Potential

4-Di-2-ASP belongs to the class of voltage-sensitive fluorescent probes that are taken up in mitochondria (Loew et al. 1985) and allow us to evaluate real-time changes in mitochondrial membrane potential ($\Delta\psi_m$) (Mewes and Rafael 1981; Rafael and Nicholls 1984; Loew et al. 1985). As a proof of principle, loading of lung slices with JC-1, an established $\Delta\psi_m$ indicator, was shown to result in similar observations compared to loading with 4-Di-2-ASP, after application of the mitochondrial uncoupler FCCP, thereby confirming that 4-Di-2-ASP detects changes in $\Delta\psi_m$ in the current lung slice model (De Proost et al. 2008). The observed rise in cytoplasmic 4-Di-2-ASP fluorescence in NEB cells is believed to be due to release of the dye from the depolarised mitochondria, where it was quenched.

6.2.2 Control Stimuli for Activation of the Different Epithelial Cell Types in the *Ex Vivo* Lung Slice Model

Stimulation of Fluo-4- and 4-Di-2-ASP-loaded lung slices with a physiological solution containing an elevated extracellular potassium concentration ($[K^+]_o$; 50 mM, 5s) evoked a reversible and reproducible rise in Fluo-4 fluorescence in NEB cells approximately 1.5 s after application (Figs. 6.4c–g and 6.5). This short-term elevation of $[K^+]_o$ causes an influx of extracellular Ca^{2+}, without signs of a negative effect on the physiological properties of the cells. Forced depolarisation with high $[K^+]_o$ therefore proved to be an appropriate and reliable positive control stimulus to confirm the loading of NEB cells with Fluo-4. Time-lapse imaging of 4-Di-2-ASP fluorescence revealed that high $[K^+]_o$ results in a delayed (5.5 s) depolarisation of the $\Delta\psi_m$ in NEBs. Analogous to observations that have been reported in excitable cells in many other tissues (Biscoe and Duchen 1990; Duchen 1992, 2000; Xiong et al. 2004), this mitochondrial membrane depolarisation appears to be dependent on the cytoplasmic Ca^{2+} rise that is buffered by uptake in mitochondria (De Proost et al. 2008).

Interestingly, after application of high $[K^+]_o$ to 4-Di-2-ASP and Fluo-4-loaded lung slices, the CLCs that encircle NEB cells also displayed a Ca^{2+} response (Figs. 6.4c–g and 6.5c). Onset of the $[Ca^{2+}]_i$ rise in individual CLCs appeared to be variable, and was typically delayed compared to the response in NEB cells. It has recently been shown that application of high $[K^+]_o$ to the lung slice evokes quantal ATP secretion from NEB cells, which subsequently activates the surrounding Clara-like cells via $P2Y_2$ receptors (De Proost et al. 2009). The possibility for visualisation of this paracrine interaction in the confocal lung slice model implies that both real-time NEB activation and the consequent transmitter exocytosis can be evaluated.

Application of ATP (1–50 μM) to lung slices as a potentially physiological stimulus results in activation of not only CLCs, but also Clara cells and to a lesser extent ciliated cells (Fig. 6.5) [refs. in vivo (Knowles et al. 1991) and *in vitro* (Van Scott et al. 1995)]. In mice, NEB cells never showed a rise in Fluo-4 fluorescence intensity after stimulation with ATP (Fig. 6.5). Application of ATP is therefore a good control stimulus to evaluate Fluo-4 loading of the airway epithelium surrounding NEBs.

Stimulation of mouse lung slices with substance P (10 μM) appears to result in selective Ca^{2+} rise in ciliated cells (own unpublished observations; Fig. 6.5)

The use of functional fluorescent indicators in a confocal live cell imaging set-up proved perfectly capable of detecting real-time changes in physiological parameters of the various airway epithelial cell types in lung slices. The opportunity to simultaneously study multiple NEB cells and their interactions with surrounding cells and tissues are important advantages of this new approach.

Chapter 7
Concluding Remarks and Future Prospects

Based on the obtained morphological and neurochemical evidence, at the moment three essentially different types of mechanoreceptors are described in lungs, sharing a nearly identical neurochemical coding and arising from myelinated nerve fibres. Although these three types of sensory nerve terminals in lungs reveal a somewhat different morphology, location and origin, it may be hypothesised that they share complementary mechanosensory roles in supporting normal lung function.

Given the multiplicity of physiologically characterised airway receptors, and the presently limited number of morphologically identified airway receptors, it seems logical to assume that a single morphologically identified airway receptor may likely combine multiple sensory activities. Because it appears to be extremely difficult to correlate electrophysiologically identified receptor activity to morphologically identified receptor end-organs, it may be more efficient to try to link morphology to physiology, i.e., identifying the functional properties for any given receptor structure. The recently developed *in situ* lung models, in which morphologically and neurochemically identified receptors can be specifically visualised, will definitely facilitate direct physiological studies of pulmonary NEBs, SMARs and VPRs in airways and lungs.

For SMARs and VPRs it is clear that both the visualised receptor terminals and the myelinated fibres giving rise to the "sensors" are reachable in whole-mount preparations, which offer great opportunities for future *in situ* physiological studies of the function of these structures.

For NEBs, it is obvious that the optimised *in situ* live cell imaging model based on precision-cut vibratome slices of live mouse lungs (Pintelon et al. 2005; De Proost et al. 2008, 2009), allows visualisation of pulmonary NEBs and simultaneous monitoring of physiological events, such as changes in the intracellular Ca^{2+} concentration or in mitochondrial membrane potential. Confocal multipoint analysis can be used to address the complexity of the NEB environment in lung slices, since it allows us to discriminate between recordings from NEB cells and nearby other cell types in the airway epithelium, based on morphological characteristics and the specific fluorescent patterns obtained with 4-Di-2-ASP staining and Fluo-4 loading. The unique advantage of this model is the visualization of

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potential activation of all cells in a NEB, and the simultaneous collection of information from many other surrounding cells and tissues in the lungs. In this way, the use of our *ex vivo* lung slice imaging model, in combination with electrophysiological "sniffer patching", exposed a potentially important paracrine purinergic signalling pathway between pulmonary neuroendocrine cells and CLCs, provided the first evidence for cell-cell communication in the NEB microenvironment, and characterised the molecular determinants of this paracrine pathway. Whereas it is generally accepted that the bioactive substances present in NEB cells are potential modulators of vascular and airway tone, and neurotransmitters connecting NEBs with the CNS via afferent nerve terminals, a direct functional interaction between NEB cells and CLCs has never been considered. The current observation certainly implies that CLCs may be more closely implicated in some of the NEB functions than just by forming a "protective shield".

Several recent reviews suggest that in the NEB microenvironment, CLCs would be the actual bronchial airway stem cells (Rawlins and Hogan 2006; Snyder et al. 2009; Kratz et al. 2010; Roomans 2010; Sullivan et al. 2010). This hypothesis is largely supported indirectly by the observation that in naphthalene-injured lungs PNECs on their own are unable to repopulate the airway epithelium. Whether these CLCs behave as dedicated stem cells in the steady state healthy lungs is, however, not yet confirmed. Their potential role as tumour-initiating cells of origin of SCLC and other neuroendocrine-like lung tumours seems important to consider, since under certain circumstances CLCs have been reported to express features of neuroendocrine cells (Reynolds et al. 2000a; Linnoila 2006), suggestive of a neuroendocrine differentiation capability. Since airway stem cells maintain protumourigenic characteristics, including high proliferation capacity and multipotent differentiation, a considerable amount of evidence exists for a link between the development of SCLCs and the NEB microenvironment (Sutherland et al. 2011). Thorough functional *in situ* studies of the NEB microenvironment will provide additional information on this important stem cell niche and will likely be essential to understand the pathogenesis of neuroendocrine-like lung cancers with a so far poor prognosis. Moreover, the current live cell imaging set-up offers additional possibilities to investigate the stem cell-like properties of Clara cells and CLCs, extending the study of the functional characteristics of these cell types in (patho) physiological conditions.

Indisputably, pulmonary NEBs are constructed as integrated receptor complexes, consisting of NEB cells, surrounding Clara-like cells and extensive nerve fibre populations. Investigating NEBs in their versatile natural environment has emphasised that NEBs form a unique, but tightly sealed, complex entity in the airway epithelium. Our preliminary observations that NEBs are not easily manipulated by a plethora of external stimuli, makes the NEB microenvironment uniquely placed to respond to very specific and selective airway stimuli. The possibility of NEBs not primarily being the generally believed oxygen sensors involved in the healthy physiological regulation of breathing, implicates that the exact role of NEBs in healthy lungs remains an intriguing question, open for

speculation and discussion. In the near future, the *ex vivo* lung slice model for live cell imaging of NEBs may turn out to be critical for determining which of the established "local" stimuli for the different electrophysiologically identified airway receptors are translated by NEB cells, and could possibly answer the question what may be the meaning and possible input of the neuroendocrine cell groups in the NEB microenvironment.

Currently, basic molecular information on the NEB microenvironment is still largely lacking, mainly because NEBs occur sparsely and widely spread in the airway epithelium. Recently, technical advances in state-of-the-art laser capture microdissection allow the collection of individual cells or cell groups from the NEB microenvironment, and the use of these samples for RNA isolation and subsequent gene expression analyses (Cutz et al. 2009a). Getting broader molecular signatures for NEB cells in different animal species, using e.g., whole genome micro-array analysis and PCR arrays, should provide information on the expression of ion channels, molecular receptors and signal transduction pathways that are involved in the healthy function of the NEB microenvironment, enabling more targeted future functional studies.

In the NEB microenvironment, many different motor and sensory nerve fibre populations are in place that may transduce and conduct sensory information to the CNS, and/or modulate the activity (transmitter release) of NEB cells. ATP released from NEB cells will undoubtedly have the potential to activate $P2X_{2/3}$ receptors expressed on the intraepithelial nerve terminals of a vagal nerve fibre population that selectively contacts pulmonary NEBs, representing a pathway to relay information towards the CNS. In this respect, it is important to notice that the secreted ATP is apparently able to additionally activate $P2Y_2$ receptors on the immediately surrounding CLCs, illustrating that one transmitter (i.e., ATP) can probably exert distinct effect by binding to different molecular receptor types. Localised endogenous ectonucleotidases may function as guards degrading all ATP before it can "escape" the NEB microenvironment and disturb purinergic processes in neighbouring epithelial cells that are well known to also express P2 receptors.

Although a direct link between the NEB-related vagal sensory populations and measurable activities in pulmonary vagal afferents is presently lacking, we strongly believe that the large number of vagal afferent fibres that specifically contact NEBs, indeed participate, and will eventually be recognised as such, in the plethora of known sensory airway receptors. There is now convincing evidence for the idea that the different populations of myelinated vagal afferents that are part of the very complex intraepithelial innervation of pulmonary NEBs should no longer be regarded as a separate group of vagal airway receptors with "unknown properties", but more likely will turn out to represent subpopulations of the extensive group of known electrophysiologically characterised myelinated airway receptors. Although the location and appearance of intramuscular SMARs leave little doubt that they are the structural counterparts of at least part of the electrophysiologically identified SARs, the observation that a majority of the

vagal afferents in lungs seem to contact NEB cells (Adriaensen et al. 2006) undermines the straightforward correlation between so-called SAR activity and SMARs. Morphologically and neurochemically, both SMARs and the NEB-connected vagal sensory nerve terminals seem to have everything to perform a mechanosensory function.

Together with SMARs and the vagal connections of NEBs, VPRs with a sympathetic origin have now been characterised. It is hypothesised that all three morphologically identified myelinated pulmonary receptors share complementary mechanosensory roles in supporting normal lung function. To fully understand the physiology of these newly identified pulmonary receptors, live lung models that combine visualisation and the possibilities for selective manipulation and direct physiological studies create great opportunities for future functional investigation of the structurally identified sensory receptors, and for finally unravelling the complex maze of pulmonary receptors.

References

Adcock JJ (2002) Airway nerves: in vitro electrophysiology. Curr Opin Pharmacol 2:280–282

Adriaensen D, Scheuermann DW (1993) Neuroendocrine cells and nerves of the lung. Anat Rec 236:70–85

Adriaensen D, Timmermans J-P (2004) Purinergic signalling in the lung: important in asthma and COPD? Curr Opin Pharmacol 4:207–214

Adriaensen D, Timmermans JP (2011) Breath-taking complexity of vagal C-fibre nociceptors: implications for inflammatory pulmonary disease, dyspnoea and cough. J Physiol 589:3–4

Adriaensen D, Timmermans J-P, Brouns I, Berthoud HR, Neuhuber WL, Scheuermann DW (1998) Pulmonary intraepithelial vagal nodose afferent nerve terminals are confined to neuroepithelial bodies: an anterograde tracing and confocal microscopy study in adult rats. Cell Tissue Res 293:395–405

Adriaensen D, Brouns I, Van Genechten J, Timmermans J-P (2003) Functional morphology of pulmonary neuroepithelial bodies: extremely complex airway receptors. Anat Rec 270A:25–40

Adriaensen D, Brouns I, Pintelon I, De Proost I, Timmermans J-P (2006) Evidence for a role of neuroepithelial bodies as complex airway sensors: comparison with smooth muscle-associated airway receptors. J Appl Physiol 101:960–970

Adrian ED (1933) Afferent impulses in the vagus and their effect on respiration. J Physiol Lond 79:332–358

Agostoni E, Chinnock JE, De Daly MB, Murray JG (1957) Functional and histological studies of the vagus nerve and its branches to the heart, lungs and abdominal viscera in the cat. J Physiol 135:182–205

Aguayo SM (1993) Pulmonary neuroendocrine cells in tobacco-related lung disorders. Anat Rec 236:122–127

Amara SG, Jonas V, Rosenfeld MG (1982) Alternative RNA processing in calcitonin gene expression generates mRNAs encoding different polypeptide products. Nature 298:240–244

Avadhanam KP, Plopper CG, Pinkerton KE (1997) Mapping the distribution of neuroepithelial bodies of the rat lung. A whole-mount immunohistochemical approach. Am J Pathol 150:851–859

Bak PM, Panos RJ (1997) Protease antigen recovery decreases the specificity of bromodeoxyuridine detection in formalin-fixed tissue. J Histochem Cytochem 45:1165–1170

Baluk P, Gabella G (1991) Afferent nerve endings in the tracheal muscle of guinea-pigs and rats. Anat Embryol 183:81–87

Baluk P, Nadel JA, McDonald DM (1992) Substance P-immunoreactive sensory axons in the rat respiratory tract: a quantitative study of their distribution and role in neurogenic inflammation. J Comp Neurol 319:586–598

Banks RW, Bewick GS, Reid B, Richardson C (2002) Evidence for activity-dependent modulation of sensory-terminal excitability in spindles by glutamate release from synaptic-like vesicles. Adv Exp Med Biol 508:13–18

Barnes PJ (2001) Neurogenic inflammation in the airways. Respir Physiol 125:145–154

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DOI 10.1007/978-3-642-22772-1

Barnes NC, Piper PJ, Costello JF (1984) Comparative effects of inhaled leukotriene C4, leukotrine D4, and histamine in normal human subjects. Thorax 39:500–504

Bartlett D Jr, Jeffery P, Sant'Ambrogio G, Wise JC (1976) Location of stretch receptors in the trachea and bronchi of the dog. J Physiol 258:409–420

Bayliss DA, Barret QB (2008) Emerging roles for two-pore-domain potassium channels and their potential therapeutic impact. Trends Pharmacol Sci 29:566–575

Belvisi MG (2003) Sensory nerves and airway inflammation: role of A delta and C-fibres. Pulm Pharmacol Ther 16:1–7

Bergner A, Sanderson MJ (2002) ATP stimulates Ca^{2+} oscillations and contraction in airway smooth muscle cells of mouse lung slices. Am J Physiol Lung Cell Mol Physiol 283:L1271–L1279

Bergren DR, Peterson DF (1993) Identification of vagal sensory receptors in the rat lung: are there subtypes of slowly adapting receptors? J Physiol 464:681–698

Berkley HJ (1894) The intrinsic pulmonary nerves in mammalia. John Hopkins Hosp Res 4: 240–247

Berthoud H-R, Patterson LM, Neumann F, Neuhuber WL (1997) Distribution and structure of vagal afferent intraganglionic laminar endings (IGLEs) in the rat gastrointestinal tract. Anat Embryol 195:183–191

Bewick GS, Reid B, Richardson C, Banks RW (2005) Autogenic modulation of mechanoreceptor excitability by glutamate release from synaptic-like vesicles: evidence from the rat muscle spindle primary sensory ending. J Physiol 562:381–394

Biscoe TJ, Duchen MR (1990) Cellular basis of transduction in carotid chemoreceptors. Am J Physiol 258:L271–L278

Bishop AE (2004) Pulmonary epithelial stem cells. Cell Prolif 37:89–96

Bitensky L, Chambers DJ, Chayen J, Cross BA, Guz A, Jain SK, Johnstone JJ (1975) Proceedings: evidence concerning the site of receptors mediating the Hering-Breuer inflation reflex. J Physiol 249:30P–31P

Bollé T, Lauweryns JM, Van Lommel A (2000) Postnatal maturation of neuroepithelial bodies and carotid body innervation: a quantitative investigation in the rabbit. J Neurocytol 29:241–248

Bonham AC, Chen CY, Mutoh T, Joad JP (2001) Lung C-fiber CNS reflexes: role in the respiratory consequences of extended environment tobacco smoke exposure in young guinea-pigs. Environ Health Perspect 109:573–578

Bousbaa H, Fleury-Feith J (1991) Effects of a long-standing challenge on pulmonary neuroendocrine cells of actively sensitized guinea pigs. Am Rev Resp Dis 144:714–717

Bousbaa H, Poron F, Fleury-Feith J (1994) Changes in chromogranin A-immunoreactive guinea-pig pulmonary neuroendocrine cells after sensitization and challenge with ovalbumin. Cell Tissue Res 275:195–199

Brierley SM (2010) Molecular basis of mechanosensitivity. Auton Neurosci Basic Clin 153:58–68

Brims FJ, Davies HE, Lee YC (2010) Respiratory chest pain: diagnosis and treatment. Med Clin North Am 94:217–232

Brouns I, Adriaensen D, Burnstock G, Timmermans J-P (2000) Intraepithelial vagal sensory nerve terminals in rat pulmonary neuroepithelial bodies express $P2X_3$ receptors. Am J Respir Cell Mol Biol 23:52–61

Brouns I, Van Genechten J, Scheuermann DW, Timmermans J-P, Adriaensen D (2002a) Neuroepithelial bodies: a morphologic substrate for the link between neuronal nitric oxide and sensitivity to airway hypoxia? J Comp Neurol 449:343–354

Brouns I, Van Nassauw L, Van Genechten J, Majewski M, Scheuermann DW, Timmermans J-P, Adriaensen D (2002b) Triple immunofluorescence staining method with antibodies raised in the same species to study the complex innervation pattern of intrapulmonary chemoreceptors. J Histochem Cytochem 50:575–582

Brouns I, Van Genechten J, Burnstock G, Timmermans J-P, Adriaensen D (2003a) Ontogenesis of P2X_3 receptor-expressing nerve fibres in the rat lung, with special reference to neuroepithelial bodies. Biomed Res 14:80–86

Brouns I, Van Genechten J, Hayashi H, Gajda M, Gomi T, Burnstock G, Timmermans J-P, Adriaensen D (2003b) Dual sensory innervation of pulmonary neuroepithelial bodies. Am J Respir Cell Mol Biol 28:275–285

Brouns I, Pintelon I, Van Genechten J, De Proost I, Timmermans J-P, Adriaensen D (2004) Vesicular glutamate transporter 2 is expressed in different nerve fibre populations that selectively contact pulmonary neuroepithelial bodies. Histochem Cell Biol 121:1–12

Brouns I, De Proost I, Pintelon I, Timmermans J-P, Adriaensen D (2006a) Sensory receptors in the airways: neurochemical coding of smooth muscle-associated airway receptors and pulmonary neuroepithelial body innervation. Auton Neurosci 126–127:307–319

Brouns I, Pintelon I, De Proost I, Alewaters R, Timmermans J-P, Adriaensen D (2006b) Neurochemical characterisation of sensory receptors in airway smooth muscle: comparison with pulmonary neuroepithelial bodies. Histochem Cell Biol 125:351–367

Brouns I, Pintelon I, De Proost I, Timmermans J-P, Adriaensen D (2009a) Diverse and complex airway receptors in rodent lungs. In: Zaccone G, Cutz E, Adriaensen D, Nurse CA, Mauceri A (eds) Airway chemoreceptors in the vertebrates. Structure, evolution and function. Science publishers, Enfield, New Hampshire, pp 235–268

Brouns I, Oztay F, Pintelon I, De Proost I, Lembrechts R, Timmermans J-P, Adriaensen D (2009b) Neurochemical pattern of the complex innervation of neuroepithelial bodies in mouse lungs. Histochem Cell Biol 131:55–74

Brumback AC, Lieber JL, Angleson JK, Betz WJ (2004) Using FM1-43 to study neuropeptide granule dynamics and exocytosis. Methods 33:287–294

Buckley K, Kelly RB (1985) Identification of a transmembrane glycoprotein specific for secretory vesicles of neural and endocrine cells. J Cell Biol 100:1284–1294

Burgers JA, Meerbeeck JP, Postmus PE (1999) De Pleura. In: Hoogsteden HC, Dekhuijzen PNR, Joos GF, Postmus PE (eds) Leerboek Longziekten. Elsevier/Bunge, Maarssen, pp 247–255

Burki NK, Lee LY (2010) Mechanisms of dyspnea. Chest 138:1196–1201

Burnet H, Hilaire G (1999) Pulmonary stretch receptor discharge and vagal regulation of respiration differ between two mouse strains. J Physiol 519:581–590

Burnstock (2009) Purinergic mechanosensory transduction and visceral pain. Mol Pain 5: 69

Cadieux A, Springall DR, Mulderry PK, Rodrigo J, Ghatei MA, Terenghi G, Bloom SR, Polak JM (1986) Occurrence, distribution and ontogeny of CGRP immunoreactivity in the rat lower respiratory tract: effect of capsaicin treatment and surgical denervations. Neuroscience 19:605–627

Campanucci VA, Nurse CA (2007) Autonomic innervation of the carotid body: role in efferent inhibition. Respir Phsyiol Neurobiol 157:83–92

Canning BJ (2006) Reflex regulation of airway smooth muscle tone. J Appl Physiol 101:971–985

Canning BJ (2011) Functional implications of the multiple afferent pathways regulating cough. Pulm Pharmacol Ther 24:295–299

Canning BJ, Mazzone SB (2005) Reflexes iniated by activation of the vagal afferent nerves innervating the airways and lungs. In: Undem BJ, Weinreich D (eds) "Advances in vagal afferent neurobiology", frontiers in neuroscience series. CRC, Boca Raton, FL, pp 403–430

Canning BJ, Spina D (2009) Sensory nerves and airway irritability. Handb Exp Pharmacol (194):139–183

Canning BJ, Reynolds SM, Anukwu LU, Kajekar R, Myers AC (2002) Endogenous neurokinins facilitate synaptic transmission in guinea pig airway parasympathetic ganglia. Am J Physiol Regul Integr Comp Physiol 283:R320–R330

Canning BJ, Mazzone SB, Meeker SN, Mori N, Reynolds SM, Undem BJ (2004) Identification of the tracheal and laryngeal afferent neurons mediating cough in anaesthetized guinea-pigs. J Physiol 557(2):543–558

Canning BJ, Mori N, Mazzone SB (2006) Vagal afferent nerves regulating the cough reflex. Respir Physiol Neurobiol 152:223–242

Capps JA (1911) An experimental study of the pain sense in the pleural membranes. Arch Intern Med 8:717–733

Carabba VH, Sorokin SP, Hoyt RFJ (1985) Development of neuroepithelial bodies in intact and cultured lungs of fetal rats. Am J Anat 173:1–27

Carr MJ, Undem BJ (2003) Bronchopulmonary afferent nerves. Respirology 8:291–301

Castro CM, Yang Y, Zhang Z, Linnoila RI (2000) Attenuation of pulmonary neuroendocrine differentiation in mice lacking Clara cell secretory protein. Lab Invest 80:1533–1540

Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD, Julius D (1997) The capsaicin receptor: a heat-activated ion channel in the pain pathway. Nature 389:816–824

Catterall WA, Striessnig J, Snutch TP, Perez-Reyes E (2003) International union of pharmacology. XL. Compendium of voltage-gated ion channels: calcium channels. Pharmacol Rev 55:579–581

Chen MF, Kimizuka G, Wang NS (1987) Human fetal lung changes associated with maternal smoking during pregnancy. Pediatr Pulmonol 3:51–58

Cho T, Chan W, Cutz E (1989) Distribution and frequency of neuro-epithelial bodies in post-natal rabbit lung: quantitative study with monoclonal antibody against serotonin. Cell Tissue Res 255:353–362

Chuaychoo B, Lee M-G, Kollarik M, Undem BJ (2005) Effect of 5-hydroxytryptamine on vagal C-fiber subtypes in guinea pig lungs. Pulm Pharmacol Ther 18:269–276

Clara M (1937) Zur Histobiologie des Bronchialepithels. Z Microsk Anat Forsch 41:321–347

Cochilla AJ, Angleson JK, Betz WJ (1999) Monitoring secretory membrane with FM1-43 fluorescence. Annu Rev Neurosci 22:1–10

Coleridge HM, Coleridge JC (1978) II. Effect of CO_2 on afferent vagal endings in canine lung. Respir Physiol 34:135–151

Coleridge HM, Coleridge JC (1984) Afferent vagal C-fibre innervation of the lungs and airways and its functional significance. Rev Physiol Biochem Pharmacol 99:1–110

Coleridge HM, Coleridge JC (1994) Pulmonary reflexes: neural mechanisms of pulmonary defense. Annu Rev Physiol 56:69–91

Coleridge HM, Coleridge JC (1997) Afferent nerves in the airways. In: Barnes PJ (ed) Autonomic control of the respiratory system. pp 39–58

Cook RD, King AS (1969) A neurite-receptor complex in the avian lung: electron microscopical observations. Experientia 25:1162–1164

Coulson FR, Fryer AD (2003) Muscarinic acetylcholine receptors and airway diseases. Pharmacol Ther 98:59–69

Cuttitta F, Carney DN, Mulshine J, Moody TW, Fedorko J, Fischler A, Minna JD (1985) Bombesin-like peptides can function as autocrine growth factors in human small-cell lung cancer. Nature 316:823–826

Cutz E (1997) Studies on neuroepithelial bodies under experimental and disease conditions. In: Cutz E (ed) Cellular and molecular biology of airway chemoreceptors. Landes Bioscience, Austin, pp 109–129

Cutz E, Jackson A (1999) Neuroepithelial bodies as airway oxygen sensors. Respir Physiol 115:201–214

Cutz E, Chan W, Wong V, Conen PE (1974) Endocrine cells in rat fetal lungs. Ultrastructural and histochemical study. Lab Invest 30:458–464

Cutz E, Chan W, Sonstegard KS (1978) Identification of neuro-epithelial bodies in rabbit fetal lungs by scanning electron microscopy: a correlative light, transmission and scanning electron microscopic study. Anat Rec 192:459–466

Cutz E, Chan W, Track NS (1981) Bombesin, calcitonin and leu-enkephalin immunoreactivity in endocrine cells of human lung. Experientia 37:765–767

Cutz E, Gillan JE, Track NS (1984) Pulmonary endocrine cells in the developing human lung and during neonatal adaptation. In: Becker KL, Gazdar AF (eds) The endocrine lung in health and disease. WB Saunders, Philadelphia, pp 210–327

Cutz E, Gillan JE, Bryan AC (1985) Neuroendocrine cells in the developing human lung: morphologic and functional considerations. Pediatr Pulmonol 1:S21–S29

Cutz E, Fu XW, Yeger H, Peers C, Kemp PJ (2003) Oxygen sensing in pulmonary neuroepithelial bodies and related tumor cell models. In: Lahiri S, Semenza GL, Prabhakar NR (eds) Lung oxygen sensing. Marcel Dekker, New York, pp 567–602

Cutz E, Fu XW, Yeger H (2004) Methods to study neuroepithelial bodies as airway oxygen sensors. Methods Enzymol 381:26–40

Cutz E, Yeger H, Pan J, Ito T (2008) Pulmonary neuroendocrine cell system in health and disease. Curr Respir Med Rev 4:174–186

Cutz E, Pan J, Yeger H (2009a) The role of NOX2 and 'Novel oxidases' in airway chemoreceptor O_2 sensing. Adv Exp Med Biol 648:427–438

Cutz E, Fu XW, Yeger H, Pan J, Nurse CA (2009b) Oxygen sensing in mammalian pulmonary neuroepithelial bodies. In: Zaccone G, Cutz E, Adriaensen D, Nurse CA, Mauceri A (eds) Airway chemoreceptors in the vertebrates. Structure, evolution and function. Science Publishers, Enfield, New Hampshire, pp 269–290

Dakhama A, Kanehiro A, Mäkelä MJ, Loader JE, Larsen GL, Gelfand EW (2002) Regulation of airway hyperresponsiveness by calcitonin gene-related peptide in allergen sensitised and challenged mice. Am J Respir Crit Care Med 165:1137–1144

Dakhama A, Larsen GL, Gelfand EW (2004) Calcitonin gene-related peptide: role in airway homeostasis. Curr Opin Pharmacol 4:215–220

Daly MD (1986) Interactions between respiration and circulation. In: Cherniack NS, Widdicombe J (eds) Handbook of physiology, section 3: the respiratory system. Vol II: control of breathing. American Physiological society, Bethesda, MD, pp 529–594

Day IN, Thompson RJ (2010) UCHL1 (PGP 9.5): neuronal biomarker and ubiquitin system protein. Prog Neurobiol 90:327–362

De Proost I, Pintelon I, Brouns I, Timmermans J-P, Adriaensen D (2007a) Selective visualisation of sensory receptors in the smooth muscle layer of ex vivo airway whole mounts by styryl pyridinium dyes. Cell Tissue Res 329:421–431

De Proost I, Brouns I, Pintelon I, Timmermans J-P, Adriaensen D (2007b) Pulmonary expression of voltage-gated calcium channels: special reference to sensory airway receptors. Histochem Cell Biol 128:301–316

De Proost I, Pintelon I, Brouns I, Kroese ABA, Riccardi D, Kemp PJ, Timmermans J-P, Adriaensen D (2008) Functional live cell imaging of the pulmonary neuroepithelial body microenvironment. Am J Respir Cell Mol Biol 39:180–189

De Proost I, Pintelon I, Wilkinson WJ, Goethals S, Brouns I, Van Nassauw L, Riccardi D, Timmermans J-P, Kemp PJ, Adriaensen D (2009) Purinergic signaling in the pulmonary neuroepithelial body microenvironment unraveled by live cell imaging. FASEB J 23:1153–1160

Delmotte P, Sanderson MJ (2006) Ciliary beat frequency is maintained at a maximal rate in the small airways of mouse lung slices. Am J Respir Cell Mol Biol 35:110–117

Dey RD, Hoffpauir JM (1986) Ultrastructural colocalization of the bioactive mediators 5-hydroxytryptamine and bombesin in endocrine cells of human fetal airways. Cell Tissue Res 246:119–124

Dey RD, Altemus JB, Zervos I, Hoffpauir J (1990) Origin and colocalization of CGRP- and SP-reactive nerves in cat airway epithelium. J Appl Physiol 68:770–778

Dinh QT, Groneberg D, Peiser C, Mingomataj E, Joachima RA, Witt C, Arck PC, Klappa FH, Fischer A (2004) Substance P expression in TRPV1 and trkA-positive dorsal root ganglion neurons innervating the mouse lung. Respir Physiol Neurobiol 144:15–24

Dinh QT, Groneberg DA, Peiser C, Joachim RA, Frossard N, Arck PC, Klapp BF, Fisher A (2005) Expression of substance P and nitric oxide synthase in vagal sensory neurons innervating the mouse airways. Regul Pept 126:189–194

Dobretsov M, Stimers JR (2005) Neuronal function and alpha3 isoform of the Na/K-ATPase. Front Biosci 10:2373–2396

Dobretsov M, Hastings SL, Sims TJ, Stimers JR, Romanovsky D (2003) Stretch receptor-associated expression of alpha 3 isoform of the Na+, K+-ATPase in rat peripheral nervous system. Neuroscience 116:1069–1080

Domnik NJ, Cutz E (2011) Pulmonary neuroepithelial bodies as airway sensors: putative role in the generation of dyspnea. Curr Opin Pharmacol 11:211–217

Duc C, Barakat-Walter I, Droz B (1994) Innervation of putative rapidly adapting mechanoreceptors by calbindin- and calretinin-immunoreactive primary sensory neurons in the rat. Eur J Neurosci 6:264–271

Duchen MR (1992) Ca^{2+}-dependent changes in the mitochondrial energetics in single dissociated mouse sensory neurons. Biochem J 283:41–50

Duchen MR (2000) Mitochondria and calcium: from cell signalling to cell death. J Physiol 529 (1):57–68

Duffin J (2004) Functional organization of respiratory neurones: a brief review of current questions and speculations. Exp Physiol 89:517–529

Dütsch M, Eichhorn U, Wörl J, Wank M, Berthoud H-R, Neuhuber WL (1998) Vagal and spinal afferent innervation of the rat esophagus: a combined retrograde tracing and immunocytochemical study with special emphasis on calcium- binding proteins. J Comp Neurol 398:289–307

Dwinnell FL (1966) Studies on the nerve endings in the visceral pleura. Am J Anat 118:217–226

e Silva MJ, Lewis DL (1995) L- and N-type Ca2+ channels in adult rat carotid body chemoreceptor type I cells. J Physiol 489:689–699

Elftman AG (1943) The afferent and parasympathetic innervation of the lungs and trachea of the dog. Am J Anat 72:1–27

England DM, Hochholzer L, McCarthy MJ (1989) Localized benign and malignant fibrous tumors of the pleura. Am J Surg Pathol 13:640–658

Ertel EA, Campbell KP, Harpold MM, Hofmann F, Mori Y, Perez-Reyes E, Schwartz A, Snutch TP, Tanabe T, Birnbaumer L, Tsien RW, Catterall WA (2000) Nomenclature of voltage-gated calcium channels. Neuron 25:533–535

Feyrter F (1938) In: Barth JA (ed) Uber diffuse endokrine epitheliale Organe. Leipzig, pp 1–62

Feyrter F (1953) Über die peripheren endokrinen (parakrinen) Drüsen des Menschen. Wilhelm Maudrich, Wien

Fischer A, Kummer W, Couraud JY, Adler D, Branscheid D, Heym C (1992) Immunohistochemical localization of receptors for vasoactive intestinal peptide and substance P in human trachea. Lab Invest 67:387–393

Fox AJ (1996) Modulation of cough and airway sensory fibres. Pulm Pharmacol 9:335–342

Fox EA, Phillips RJ, Baronowsky EA, Byerly MS, Jones S, Powley TL (2001) Neurotrophin-4 deficient mice have a loss of vagal intraganglionic mechanoreceptors from the small intestine and a disruption of short-term satiety. J Neurosci 21:8602–8615

Fröhlich F (1949) Die "Helle Zelle" der Bronchialschleimhaut und ihre Beziehungen zum Problem der Chemoreceptoren. Frankf Z Pathol 60:517–559

Fu XW, Spindel ER (2009) Recruitment of GABA(A) receptors in chemoreceptor pulmonary neuroepithelial bodies by prenatal nicotine exposure in monkey lung. Adv Exp Med Biol 648:439–445

Fu XW, Nurse CA, Wang YT, Cutz E (1999) Selective modulation of membrane currents by hypoxia in intact airway chemoreceptors from neonatal rabbit. J Physiol 514:139–150

Fu XW, Wang D, Nurse CA, Dinauer MC, Cutz E (2000) NADPH oxidase is an O_2 sensor in airway chemoreceptors: evidence from K^+ current modulation in wild-type and oxidase-deficient mice. Proc Natl Acad Sci USA 97:4374–4379

Fu XW, Wang D, Pan J, Farragher SM, Wong V, Cutz E (2001) Neuroepithelial bodies in mammalian lung express functional serotonin type 3 receptor. Am J Physiol Lung Cell Mol Physiol 281:L931–L940

Fu XW, Nurse CA, Wong V, Cutz E (2002) Hypoxia-induced secretion of serotonin from intact pulmonary neuroepithelial bodies in neonatal rabbit. J Physiol 539:503–510

Fu XW, Nurse CA, Farragher SM, Cutz E (2003) Expression of functional nicotinic acetylcholine receptors in neuroepithelial bodies of neonatal hamster lung. Am J Physiol Lung Cell Mol Physiol 285:1203–1212

Fu XW, Nurse C, Cutz E (2004) Expression of functional purinergic receptors in pulmonary neuroepithelial bodies and their role in hypoxia chemotransmission. Biol Chem 385:275–284

Fu XW, Nurse C, Cutz E (2007) Characterization of slowly inactivating KV current in rabbit pulmonary neuroepithelial bodies: effects of hypoxia and nicotine. Am J Physiol Lung Cell Mol Physiol 293:L892–L902

Fujimura M, Sakamoto S, Kamio Y, Matsuda T (1992) Effects of metacholine induced bronchoconstriction and procaterol induced bronchodilatation on cough receptor sensitivity to inhaled capsaicin and tartratic acid. Thorax 47:441–445

Fujita T, Kanno T, Kobayashi S (1988) The paraneuron. Springer, Tokyo

Fukuda J, Ishimine H, Masaki Y (2003) Long-term staining of live Merkel cells with FM dyes. Cell Tissue Res 311:325–332

Gallego R, Garcia-Caballero T, Roson E, Beiras A (1990) Neuroendocrine cells of the human lung express substance-P-like immunoreactivity. Acta Anat (Basel) 139:278–282

Ganong WF (1999) Regulation of respiration. In: Barnes A, Ransom J, Roche J (eds) Review of medical physiology. Appleton and Lange, Stamford, pp 640–649

Giangreco A, Groot KR, Janes SM (2007) Lung cancer and lung stem cells: strange bedfellows? Am J Respir Crit Care Med 175:547–553

Gomez-Pascual A, Martin-Lacave I, Moreno AM, Fernandez A, Galera H (1990) Neuroendocrine (NE) cells in rat neonatal lungs. A histochemical and immuncytochemical study. Anat Histol Embryol 19:158–163

Gosney JR (1993) Pulmonary neuroendocrine cells in species at high altitude. Anat Rec 236:105–107

Gosney JR, Sissons M (1985) Widespread distribution of bronchopulmonary endocrine cells immunoreactive for calcitonin in the lung of the normal adult rat. Thorax 40:194–198

Groth M, Helbig T, Grau V, Kummer W, Haberberger RV (2006) Spinal afferent neurons projecting to the rat lung and pleura express acid sensitive channels. Respir Res 7:96

Hage E (1976) Endocrine-like cells of the pulmonary epithelium. In: Coupland RE, Fujita T (eds) Chromaffin, enterochromaffin and related cells. Elsevier, Amsterdam

Haller CJ (1994) A scanning and transmission electron microscopic study of the development of the surface structure of neuroepithelial bodies in the mouse lung. Micron 25:527–538

Hartness ME, Lewis A, Searle GJ, O'Kelly I, Peers C, Kemp PJ (2001) Combined antisense and pharmacological approaches implicate hTASK as an airway O_2 sensing K^+ channel. J Biol Chem 276:26499–26508

Haxhiu MA, Kc P, Moore CT, Acquah SS, Wilson CG, Zaida SI, Massari VJ, Ferguson DG (2005) Brain stem excitatory and inhibitory signaling pathways regulating bronchoconstrictive responses. J Appl Physiol 98:1961–1982

Hayashi T, Kawakami M, Sasaki S, Katsumata T, Mori H, Yoshida H, Nakahari T (2005) ATP regulation of ciliary beat frequency in rat tracheal and distal airway epithelium. Exp Physiol 90:535–544

Helle KB (2010) Regulatory peptides from chromogranin A and secretogranin II: putative modulators of cells and tissues involved in inflammatory conditions. Regul Pept 165:45–51

Helliwell RJA, McLatchie LM, Clarke M, Winter J, Bevan S, McIntyre P (1998) Capsaicin sensitivity is associated with the expression of the vanniloid (capsaicin) receptor (VR1) mRNA in adult rat sensory ganglia. Neurosci Lett 250:177–180

Helset E, Kjaeve J, Bjertnaes L, Lundberg JM (1995) Acute alveolar hypoxia increases endothelin-1 release but decreases release of calcitonin gene-related peptide in isolated perfused rat lungs. Scand J Clin Lab Invest 55:369–376

Hering E (1868) Die Selbesteuerung der Athmung durch den Nervus vagus. Sber Akad Wiss Wein 57:672–677

Hille B (1986) Ionic channels: molecular pores of excitable membranes. Harvey Lect 82:47–69

Ho C-Y, Lee LY (1998) Ozone enhances excitabilities of pulmonary C fibers to chemical and mechanical stimuli in anesthetized rats. J Appl Physiol 85:1509–1515

Ho C-Y, Gu Q, Hong J-L, Lee L-Y (2000) Prostaglandin E_2 enhances chemical and mechanical sensitivities of pulmonary C fibers in the rat. Am J Respir Crit Care Med 162:528–533

Ho C-Y, Gu Q, Lin YS, Lee L-Y (2001) Sensitivity of vagal afferent endings to chemical irritants in the rat lung. Respir Physiol 127:113–124

Holzer P (1991) Capsaicin: cellular targets, mechanisms of action, and selectivity for thin sensory neurons. Pharmacol Rev 43:143–201

Hong KU, Reynolds SD, Giangreco A, Hurley CM, Stripp BR (2001) Clara cell secretory protein-expressing cells of the airway neuroepithelial body microenvironment include a label-retaining subset and are critical for epithelial renewal after progenitor cell depletion. Am J Respir Cell Mol Biol 24:671–681

Honig MG (1993) DiI Labelling. Neurosci Prot 93-050-16-01-20

Honjin R (1956) On the nerve supply of the lung of the mouse, with special reference to the structure of the peripheral vegetative nervous system. J Comp Neurol 105:587–625

Housley GD, Bringmann A, Reichenbach A (2009) Purinergic signaling in special senses. Trends Neurosci 32:128–141

Hung K-S (1980) Innervation of rabbit fetal lungs. Am J Anat 159:78–83

Hung K-S (1982) Development of neuroepithelial bodies in pre- and postnatal mouse lungs: scanning electron microscopic study. Anat Rec 203:285–291

Hung K-S (1984) Histology, ultrastructure, and development of the pulmonary endocrine cell. In: Becker KL, Gazdar AF (eds) The endocrine lung in health and disease. WB Saunders, Philadelphia, pp 162–192

Hung K-S, Loosli CG (1974) Bronchiolar neuro-epithelial bodies in the neonatal mouse lungs. Am J Anat 140:191–200

Hung K-S, Hertweck MS, Hardy JD, Loosli CG (1973) Ultrastructure of nerves and associated cells in bronchiolar epithelium of the mouse lung. J Ultrastr Res 43:426–437

Hunter DD, Undem BJ (1999) Identification and substance P content of vagal afferent neurons innervating the epithelium of the guinea-pif trachea. Am J Respir Crit Care Med 159:1943–1948

Hutchings HA, Morris S, Eccles R, Jawad MS (1984) Voluntary suppression of cough induced by inhalation of capsaicin in healthy volunteers. Respir Med 87:379–382

Ito T, Nozawa A, Usuda Y, Kitamura H, Kanisawa M (1995) Hamster pulmonary endocrine cells with neural cell adhesion molecule (NCAM) immunostaining. Histochem Cell Biol 104:357–362

Ito T, Udaka N, Inayama Y, Kitamura H, Kanisawa M (1998) Hamster pulmonary endocrine cells with positive immunostaining for calbindin-D-28K. Histochem Cell Biol 109:67–73

Ito T, Udaka N, Kawano N, Nogawa H, Kitamura H (1999) Ontogeny of pulmonary neuroendocrine cells which express the alpha-subunit of guanine nucleotide-binding protein Go. Histochem Cell Biol 111:289–295

Jammes Y, Fornaris E, Mei N, Barrat E (1982) Afferent and efferent components of the bronchial vagal branches in the cat. J Auton Nerv Syst 5:165–176

Jammes Y, Trousse D, Delpierre S (2005) Identification and properties of parietal pleural afferents in rabbits. J Physiol 567:641–650
Jeffery PK (1995) Structure: microscopic structure of normal lung. In: Corrin B, Geddes DM, Gibson GJ (eds) Respiratory medicine. WB Saunders, London, pp 3–72
Jeffery PK, Reid L (1975) New observations of rat airway epithelium: a quantitative and electron microscopic study. J Anat 120:195–320
Joad JP, Kott KS, Bonham AC (1997) Nitric oxide contributes to substance P-induced increases in lung rapidly adapting receptor activity in guinea-pigs. J Physiol 503:635–643
Joos GF, Pauwels RA, Van Der Straeten ME (1987) Effect of inhaled substance P and neurokinin A on the airways of normal and asthmatic subjects. Thorax 42:779–783
Jordan D (2001) Central nervous pathways and control of the airways. Respir Physiol 125:67–81
Kajekar R, Proud D, Myers A, Meeker SN, Undem BJ (1999) Characterization of vagal afferent subtypes stimulated by bradykinin in guinea-pig trachea. J Pharmacol Exp Ther 289:682–687
Kannari K, Sato O, Maeda T, Iwanaga T, Fujita T (1991) A possible mechanism of mechanoreception in Ruffini endings in the peridontal ligament of hamster incisors. J Comp Neurol 313:368–376
Kappagoda CT, Skepper JN, McNaughton L, Siew EEL, Navaratnam V (1990) Morphology of presumptive rapidly adapting receptors in the rat bronchus. J Anat 168:265–276
Karlsson J-A (1996) The role of capsaicin sensitive C-fibre afferent nerves in the cough reflex. Pulm Pharmacol 9:315–321
Karlsson J-A, Sant'Ambrogio G, Widdicombe J (1988) Afferent neural pathways in cough and reflex bronchoconstriction. J Appl Physiol 65:1007–1023
Kasacka I (2008) Quantitative characteristics of calcitonin-producing cells in the thyroid and lungs of uremic rats. Folia Histochem Cytobiol 46:525–530
Kasprzak A, Zabel M, Biczysko W (2007) Selected markers (chromogranin A, neuron-specific enolase, synaptophysin, protein gene product 9.5) in diagnosis and prognosis of neuroendocrine pulmonary tumours. Pol J Pathol 58:23–33
Katz DM, Markey KA, Goldstein M, Black IB (1983) Expression of catecholaminergic characteristics by primary sensory neurons in the normal adult rat in vivo. Proc Natl Acad Sci USA 80:3526–3530
Katz DM, Adler JE, Black IB (1987) Catecholaminergic sensory neurons: autonomic targets and mechanisms of transmitter regulation. FASEB J 46:24–29
Kaufman MP, Cassidy SS (1987) Reflex effects of the lung inflation and other stimuli on the heart and circulation. In: Scharf SM, Cassidy SS (eds) Lung biology in health and disease: heart-lung interactions in health and disease, vol 42. Marcel Dekker, New York, pp 339–363
Keith IM, Pelto-Huikko M, Schalling M, Hokfelt T (1991) Calcitonin gene-related peptide and its mRNA in pulmonary neuroendocrine cells and ganglia. Histochemistry 96:311–315
Keller CJ, Loeser A (1929) Der zentripetale Lungenvagus. Z Biol 89:373–395
Kemp PJ (2006) Detecting acute changes in oxygen: will the real sensor please stand up? Exp Physiol 91:829–834
Kemp PJ, Peers C (2009) Enzyme-linked acute oxygen sensing in airway and arterial chemoreceptors–invited article. Adv Exp Med Biol 648:39–48
Kemp PJ, Lewis A, Hartness M, Searle GJ, Miller P, O'Kelly I, Peers C (2002) Airway chemotransduction: from oxygen sensor to cellular effector. Am J Respir Crit Care Med 166:S17–S24
Kemp PJ, Searle GJ, Hartness ME, Lewis A, Miller P, Williams S, Wootton P, Adriaensen D, Peers C (2003) Acute oxygen sensing in cellular models: relevance to the physiology of pulmonary neuroepithelial and carotid bodies. Anat Rec 270:41–50
Kemp PJ, Telezhkin V, Wilkinson WJ, Mears R, Hanmer SB, Gadeberg HC, Muller CT, Riccardi D, Brazier SP (2009) Enzyme-linked oxygen sensing by potassium channels. Ann N Y Acad Sci 1177:112–118
Kiefer JC (2011) Primer and interviews: the dynamic stem cell niche. Dev Dyn 240:737–743

Kim D (2003) Fatty acid-sensitive two-pore domain K^+ channels. Trends Pharmacol Sci 24:648–654
Knowles MR, Clarke LL, Boucher RC (1991) Activation by extracellular nucleotides of chloride secretion in the airway epithelia of patients with cystic fibrosis. N Engl J Med 325:533–538
Knowlton GC, Larrabee MG (1946) A unitary analysis of pulmonary volume receptors. Am J Phsyiol 147:100–114
Kollarik M, Undem BJ (2006) Sensory transduction in cough-associated nerves. Respir Physiol Neurobiol 152:243–254
Kollarik M, Dinh QT, Fischer A, Undem BJ (2003) Capsaicin-sensitive and -insensitive vagal bronchopulmonary C-fibres in the mouse. J Physiol 551:869–879
Kollarik M, Ru F, Brozmanova M (2010) Vagal afferent nerves with the properties of nociceptors. Auton Neurosci Basic Clin 153:12–20
Kou YR, Lee LY (1990) Stimulation of rapidly adapting receptors in canine lungs by a single breath of cigarette smoke. J Appl Physiol 68:1203–1210
Kou YR, Lee LY (1991) Mechanisms of cigarette smoke-induced stimulation of rapidly adapting receptors in canine lungs. Respir Physiol 83:61–75
Kratz JR, Yagui-Beltran A, Jablons DM (2010) Cancer stem cells in lung tumorigenesis. Ann Thorac Surg 89:S2090–S2095
Kubin L, Alheid GF, Zuperku EJ, McCrimmon DR (2006) Central pathways of pulmonary and lower airway vagal afferents. J Appl Physiol 101:618–627
Kummer W, Gibbins IL, Stefan P, Kapoor V (1990) Catecholamines and catecholamine-synthesizing enzymes in guinea-pig sensory ganglia. Cell Tissue Res 261:595–606
Kummer W, Fischer A, Mayer B (1992a) Substance P and nitric oxide: participation in airway innervation. Regul Peptides S1:S92
Kummer W, Fischer A, Kurkowski R, Heym C (1992b) The sensory and sympathetic innervation of guinea pig lung and trachea as studied by retrograde neuronal tracing and double-labelling immunohistochemistry. Neuroscience 49:715–737
Kummer W, Wiegand S, Akinci S, Wessler I, Schinkel AH, Wess J, Koepsell H, Haberberger RV, Lips KV (2006) Role of acetylcholine and polyspecific cation transporters in serotonin-induced bronchoconstriction in the mouse. Respir Res 7:65
Kummer W, Lips KS, Pfeil U (2008) The epithelial cholinergic system of the airways. Histochem Cell Biol 130:219–234
Kuo HP, Rohde JA, Tokuyama K, Barnes PJ, Rogers DF (1990) Capsaicin and sensory neuropeptide stimulation of goblet cell secretion in guinea-pig trachea. J Physiol 431:629–641
Kuramoto H, Kuwano R (1994) Immunohistochemical demonstration of calbindin-containing nerve endings in the rat esophagus. Cell Tissue Res 278:57–64
Kwong K, Lee L-Y (2002) PGE_2 sensitizes cultured pulmonary vagal sensory neurons to chemical and electrical stimuli. J Appl Physiol 93:1419–1428
Kwong K, Kollarik M, Nassenstein C, Ru F, Undem BJ (2008) $P2X_2$ receptors differentiate placodal versus neural crest C-fiber phenotypes innervating guinea pig lungs and esophagus. Am J Physiol Lung Cell Mol Physiol 295:L858–L865
Lachamp P, Crest M, Kessler JP (2006) Vesicular glutamate transporters type 1 and 2 expression in axon terminals of the rat nucleus of the solitary tract. Neuroscience 137:73–81
Larsell O (1921) Nerve termination in the lung of the rabbit. J Comp Neurol 33:105–132
Larsell O (1922) The ganglia, plexuses, and nerve-terminations of the mammalian lung and pleura pulmonalis. J Comp Neurol 35:97–132
Larsell O, Coffey JR (1928) The effect on respiration of stimulating the nerve terminations in the visceral pleura. Anat Rec 28:20
Larsell O, Dow RS (1933) The innervation of the human lung. Am J Anat 52:125–146
Larson SD, Schelegle ES, Hyde DM, Plopper CG (2003) The three-dimensional distribution of nerves along the entire intrapulmonary airway tree of the adult rat and the anatomical relationship between nerves and neuroepithelial bodies. Am J Respir Cell Mol Biol 28:592–599

Lauweryns JM, Cokelaere M (1973a) Hypoxia-sensitive neuro-epithelial bodies. Intrapulmonary secretory neuroreceptors modulated by the CNS. Z Zellforsch 143:521–540
Lauweryns JM, Cokelaere M (1973b) Intrapulmonary neuro-epithelial bodies: hypoxia-sensitive neuro(chemo-)receptors. Experientia 29:1384–1386
Lauweryns JM, Peuskens JC (1972) Neuro-epithelial bodies (neuroreceptor or secretory organs?) in human infant bronchial and bronchiolar epithelium. Anat Rec 172:471–481
Lauweryns JM, Van Lommel A (1982) Morphometric analysis of hypoxia-induced synaptic activity in intrapulmonary neuroepithelial bodies. Cell Tissue Res 226:201–214
Lauweryns JM, Van Lommel A (1986) Effect of various vagotomy procedures on the reaction to hypoxia of rabbit neuroepithelial bodies: modulation by intrapulmonary axon reflexes. Exp Lung Res 11:319–339
Lauweryns JM, Van Lommel A (1987) Ultrastructure of nerve endings and synaptic junctions in rabbit intrapulmonary neuroepithelial bodies: a single and serial section analysis. J Anat 151:65–83
Lauweryns JM, Van Ranst L (1987) Calcitonin gene related peptide immunoreactivity in rat lung: light and electron microscopic study. Thorax 42:183–189
Lauweryns JM, Van Ranst L (1988a) Protein gene product 9.5 expression in the lungs of humans and other mammals. Immunocytochemical detection in neuroepithelial bodies, neuroendocrine cells and nerves. Neurosci Lett 85:311–316
Lauweryns JM, Van Ranst L (1988b) Immunocytochemical localization of aromatic L-amino acid decarboxylase in human, rat, and mouse bronchopulmonary and gastrointestinal endocrine cells. J Histochem Cytochem 36:1181–1186
Lauweryns JM, de Bock V, Verhofstad AA, Steinbusch HW (1982) Immunohistochemical localization of serotonin in intrapulmonary neuro-epithelial bodies. Cell Tissue Res 226:215–223
Lauweryns JM, Van Lommel AT, Dom RJ (1985) Innervation of rabbit intrapulmonary neuroepithelial bodies. Quantitative and qualitative ultrastructural study after vagotomy. J Neurol Sci 67:81–92
Lauweryns JM, de Bock V, Decramer M (1987) Effects of unilateral vagal stimulation on intrapulmonary neuroepithelial bodies. J Appl Physiol 63:1781–1787
Lawrence AJ (1995) Neurotransmitter mechanisms of rat vagal afferent neurons. Clin Exp Pharmacol Physiol 22:869–873
Lee LY (2009) Respiratory sensations evoked by activation of bronchopulmonary C-fibers. Respir Physiol Neurobiol 167:26–35
Lee L-Y, Pisarri TE (2001) Afferent properties and reflex functions of bronchopulmonary C-fibers. Respir Physiol 125:47–65
Lee L-Y, Undem BJ (2005) Bronchopulmonary vagal afferent nerves. In: Undem BJ, Weinreich D (eds) "Advances in vagal afferent neurobiology", frontiers in neuroscience series. CRC, Boca Raton, FL, pp 279–313
Lee I, Gould VE, Moll R, Wiedenmann B, Franke WW (1987) Synatophysin expressed in the bronchopulmonary tract: neuroendocrine cells, neuroepithelial bodies, and neuroendocrine neoplasms. Differentiation 34:115–125
Lee BP, Sant'Ambrogio G, Sant'Ambrogio FB (1992) Afferent innervation and receptors in the canine extrathoracic trachea. Respir Physiol 90:55–65
Lee LY, Kwong K, Lin YS, Gu Q (2002) Hypersensitivity of bronchopulmonary C-fibres induced by airway mucosal inflammation: cellular mechanisms. Pulm Pharmacol Ther 15:199–204
Lee L-Y, Lin YS, Gu Q, Chung E, Ho CY (2003) Functional morphology and physiological properties of bronchopulmonary C-fiber afferents. Anat Rec 270 A:17–24
Lee LY, Burki N, Gerhardstein D, Yu Q, Kou YR, Xu J (2007) Airway irritation and cough evoked by inhaled cigarette smoke: role of neuronal nicotinic acetylcholine receptors. Pulm Pharmacol Ther 20:355–364. doi:10.1016/j.pupt.2006.10.007

Lee LY, Gu Q, Lin YS (2010) Effect of smoking on cough reflex sensitivity: basic and preclinical studies. Lung 188(Suppl 1):S23–S27

Lembrechts R, Pintelon I, Schnorbusch K, Timmermans J-P, Adriaensen D, Brouns I (2011) Expression of mechanogated Two-pore-domain potassium channels in mouse lungs: special reference to mechanosensory airway receptors. Histochem Cell Biol DOI: 10.1007/s00418-011-0837-8

Liberati TA, Randle MR, Toth LA (2010) In vitro lung slices: a powerful approach for assessment of lung pathophysiology. Expert Rev Mol Diagn 10:501–508

Liedtke W, Kim C (2005) Functionality of the TRPV subfamily of TRP ion channels: add mechano-TRP and osmo-TRP to the lexicon! Cell Mol Life Sci 62:2985–3001

Lieu T, Undem BJ (2011) Neuroplasticity in vagal afferent neurons involved in cough. Pulm Pharmacol Ther 24:276–279

Li K, Nagalla SR, Spindel ER (1994) A rhesus monkey model to characterize the role of gastrin-releasing peptide (GRP) in lung development. Evidence for stimulation of airway growth. J Clin Invest 94: 1605–1615

Linnoila RI (2006) Functional facets of the pulmonary neuroendocrine system. Lab Invest 86:425–444

Liu X, Driskell RR, Engelhardt JF (2006) Stem cells in the lung. Methods Enzymol 419:285–321

Loew LM, Cohen LB, Salzberg BM, Obaid AL, Bezanilla F (1985) Charge-shift probes of membrane potential. Characterization of aminostyrylpyridinium dyes on the squid giant axon. Biophys J 47:71–77

Looga R (1997) Reflex cardiovascular responses to lung inflation: a review. Respir Physiol 109:95–106

Lopez-Barneo J (1994) Oxygen-sensitive ion channels: how ubiquitous are they? Trends Neurosci 17:133–135

Lundberg JM, Saria A (1987) Polypeptide-containing neurons in airway smooth muscle. Annu Rev Physiol 40:557–572

Lundberg JM, Hokfelt T, Martling C-R, Saria A, Cuello C (1984) Substance P-immunoreactive sensory nerves in the lower respiratory tract of various mammals including man. Cell Tissue Res 235:251–261

Luts A, Uddman R, Absood A, Håkanson R, Sundler F (1991) Chemical coding of endocrine cells of the airways: presence of helodermin-like peptides. Cell Tissue Res 265:425–433

Mahvi D, Bank H, Harley R (1977) Morphology of a napthalene-induced bronchiolar lesion. Am J Pathol 86:558–572

Maingret F, Fosset M, Lesage F, Lazdunski M, Honoré E (1999) TRAAK is a mammalian neuronal mechano-gated K^+ channel. J Biol Chem 274:1381–1387

Marangos PJ, Schmechel D, Zis AP, Goodwin FK (1979) The existence and neurobiological significance of neuronal and glial forms of the glycolytic enzyme enolase. Biol Psychiatry 14:563–579

Martling C-R, Saria A, Fischer JA, Hökfelt T, Lundberg JM (1988) Calcitonin gene-related peptide and the lung: neuronal coexistence with substance P, release by capsaicin and vasodilatory effect. Regul Pept 20:125–139

Mazzone SB (2004) Sensory regulation of the cough reflex. Pulm Pharmacol Ther 17:361–368

Mazzone SB (2005) An overview of the sensory receptors regulating cough. Cough 1:2

Mazzone SB, Canning BJ (2003) Identification of the afferent nerves mediating cough in guinea pigs. FASEB J 17:A822

Mazzone SB, McGovern AE (2008) Immunohistochemical characterization of nodose cough receptor neurons projecting to the trachea of guinea pigs. Cough 4:9

Mazzone SB, Mori N, Canning BJ (2002) Bradykinin-induced cough in conscious guinea pigs. Am J Respir Crit Care Med 165:A773

Mazzone SB, Reynolds SM, Mori N, Koller DL, Farmer DG, Myers AC, Canning BJ (2009) Selective expression of a sodium pump isozyme by cough receptors and evidence for its essential role in regulating cough. J Neurosci 29:13662–13671
McBride JT, Springall DR, Winter RJ, Polak JM (1990) Quantitative immunocytochemistry shows calcitonin gene-related peptide-like immunoreactivity in lung neuroendocrine cells is increased by chronic hypoxia in the rat. Am J Respir Cell Mol Biol 3:587–593
McLaughlin AIG (1933) Nerves and nerve endings in the visceral pleura of the cat. J Physiol 80:101–104
Melville GN, Iravani J (1975) Factors affecting ciliary beat frequency in the intrapulmonary airways of rats. Can J Physiol Pharmacol 53:1122–1128
Meuwissen R, Berns A (2005) Mouse models for human lung cancer. Genes Dev 19:643–664
Mewes H-W, Rafael J (1981) The 2-(dimethylaminostyryl)-1-methylpyridinium cation as indicator of the mitochondrial membrane potential. FEBS Lett 131:7–10
Meyers JR, MacDonald RB, Duggan A, Standaert DG, Corwin JT, Corey DP (2003) Lighting up the senses: FM1-43 loading of sensory cells through nonselective ion channels. J Neurosci 23:4054–4065
Miserocchi G, Mortola J, Sant'Ambrogio G (1973) Localization of pulmonary stretch receptors in the airways of the dog. J Physiol Lond 235:775–782
Montuenga LM, Guembe L, Burrell MA, Bodegas ME, Calvo A, Sola JJ, Sesma P, Villaro CA (2003) The diffuse endocrine system: from embryogenesis to carcinogenesis. Prog Histochem Cytochem 38:155–272
Moore KL (1992) The thorax. In: Satterfield ST (ed) Clinically oriented anatomy. Williams and Wilkins, Baltimore, USA, pp 33–126
Morikawa Y, Donahoe PK, Hendren WH (1978a) Cholinergic nerve development in fetal lung. Dev Biol 65:541–546
Morikawa Y, Donahoe PK, Hendren WH (1978b) Cholinergic nerve development of fetal lung in vitro. J Pediatr Surg 13:653–661
Morton DR, Klassen KP, Curtis GM (1951) The clinical physiology of the human bronchi. II. The effect of vagus section upon pain of tracheobronchial origin. Surgery 30:800–809
Murray JF (2010) The structure and function of the lung. Int J Tuberc Lung Dis 14:391–396
Myers AC, Kajekar R, Undem BJ (2002) Allergic inflammation-induced neuropeptide production in rapidly adapting afferent nerves in guinea pig airways. Am J Physiol Lung Cell Mol Physiol 282:L775–L781
Nassenstein C, Taylor-Clark TE, Myers AC, Ru F, Nandigama R, Bettner W, Undem BJ (2010) Phenotypic distinctions between neural crest and placodal derived vagal C-fibres in mouse lungs. J Physiol 588:4769–4783
Navone F, Jahn R, Di Gioia G, Stukenbrok H, Greengard P, De Camilli P (1986) Protein p38: An integral membrane protein specific for small vesicles of neurons and neuroendocrine cells. J Cell Biol 103:2511–2527
O'Kelly I, Peers C, Kemp PJ (1998) O_2-sensitive K^+ channels in neuroepithelial body-derived small cell carcinoma cells of the human lung. Am J Physiol 275:L709–L716
O'Kelly I, Peers C, Kemp PJ (2001) NADPH oxidase does not account fully for O_2-sensing in model airway chemoreceptor cells. Biochem Biophys Res Commun 283:1131–1134
Ochi K, Wakisaka S, Youn SH, Hanada K, Maeda T (1997a) Calretinin-like immunoreactivity in the Ruffini endings, slowly adapting mechanoreceptors, of the periodontal ligament of the rat incisor. Brain Res 769:183–187
Ochi K, Wakisaka S, Youn SH, Hanada K, Maeda T (1997b) Immunohistochemical localization of calbindin D28k in the periodontal Ruffini endings of rat incisors. Neurosci Lett 228: 195–198
Oh EJ, Mazzone SB, Canning BJ, Weinreich D (2006) Reflex regulation of airway sympathetic nerves in guinea-pigs. J Physiol 573:549–564

O'Kelly I, Stephens RH, Peers C, Kemp PJ (1999) Potential identification of the O_2-sensitive K^+ current in a human neuroepithelial body-derived cell line. Am J Physiol 276:L96–L104

O'Kelly I, Lewis A, Peers C, Kemp PJ (2000) O_2 sensing by airway chemoreceptor-derived cells: protein kinase C activation reveals functional evidence for involvement of NADPH oxidase. J Biol Chem 275:7684–7692

Overholt JL, Prabhakar NR (1997) Ca^{2+} current in rabbit carotid body glomus cells is conducted by multiple types of high-voltage-activated Ca^{2+} channels. J Neurophysiol 78:2467–2474

Oztay F, Brouns I, Pintelon I, Raab M, Neuhuber WL, Timmermans J-P, Adriaensen D (2010) Neurotrophin-4 dependency of intraepithelial vagal sensory nerve terminals that selectively contact pulmonary NEBs in mice. Histol Histopathol 25:975–984

Pack RJ, Barker S, Howe A (1986) The effect of hypoxia on the number of amine-containing cells in the lung of the adult rat. Eur J Respir Dis 68:121–130

Paintal AS (1973) Vagal sensory receptors and their reflexes. Physiol Res 53:159–227

Paintal AS (1995) Some recent advances in studies on J receptors. Adv Exp Med Biol 381:15–25

Pan J, Yeger H, Cutz E (2004) Innervation of pulmonary neuroendocrine cells and neuroepithelial bodies in developing rabbit lung. J Histochem Cytochem 52:379–389

Pan J, Luk C, Kent G, Cutz E, Yeger H (2006a) Pulmonary neuroendocrine cells, airway innervation, and smooth muscle are altered in Cftr null mice. Am J Respir Cell Mol Biol 35:320–326

Pan J, Copland I, Post M, Yeger H, Cutz E (2006b) Mechanical stretch-induced serotonin release from pulmonary neuroendocrine cells: implications for lung development. Am J Physiol Lung Cell Mol Physiol 290:L185–L193

Pearsall AD, Hoyt RF, Sorokin SP (1985) Three-dimensional reconstruction of a small-granule paracrine cell cluster in an adult hamster bronchus. Anat Rec 212:132–142

Pearse AG (1977) The diffuse neuroendocrine system and the APUD concept: related "endocrine" peptides in the brain, intestine, pituitary, placenta, and anuran cutaneous glands. Med Biol 55:115–125

Peers C, Kemp PJ (2001) Acute oxygen sensing: diverse but convergent mechanisms in airway and arterial chemoreceptors. Respir Res 2:145–149

Perez JF, Sanderson MJ (2005) The frequency of calcium oscillations induced by 5-HT, Ach, and KCl determine the contraction of smooth muscle cells of intrapulmonary bronchioles. J Gen Physiol 125:535–553

Perez-Pinera P, García-Suarez O, Germanà A, Díaz-Esnal B, de Carlos F, Silos-Santiago I, del Valle ME, Cobo J, Vega JA (2008) Characterization of sensory deficits in TrkB knockout mice. Neurosci Lett 433:43–47

Phillips RJ, Powley TL (2000) Tension and stretch receptors in gastrointestinal smooth muscle: re-evaluating vagal mechanoreceptor physiology. Brain Res Rev 34:1–26

Pintelon I, Brouns I, Van Genechten J, Scheuermann DW, Timmermans J-P, Adriaensen D (2003) Pulmonary expression of the vesicular acetylcholine transporter with special reference to neuroepithelial bodies. Auton Neurosci 106:47

Pintelon I, De Proost I, Brouns I, Van Herck H, Van Genechten J, Van Meir F, Timmermans J-P, Adriaensen D (2005) Selective visualisation of neuroepithelial bodies in vibratome slices of living lung by 4-Di-2-ASP in various animal species. Cell Tissue Res 321:21–33

Pintelon I, Brouns I, De Proost I, Van Meir F, Timmermans J-P, Adriaensen D (2007) Sensory receptors in the visceral pleura. Neurochemical coding and live staining in whole mounts. Am J Respir Cell Mol Biol 36:541–551

Plato M, Kummer W, Haberberger RV (2006) Structural and neurochemical comparison of vagal and spinal afferent neurons projecting to the rat lung. Neurosci Lett 395:215–219

Plopper CG, Suverkropp C, Morin D, Nishio S, Buckpitt A (1992) Relationship of cytochrome P-450 activity to Clara cell cytotoxicity. I. Histopathologic comparison of the respiratory tract of mice, rats and hamsters after parenteral administration of naphthalene. J Pharmacol Exp Ther 261:353–363

Plummer HKI, Sheppard B, Schuller HM (2000) Interaction of tobacco-specific toxicants with nicotinic cholinergic regulation of pulmonary neuroendocrine cells: implications for pediatric lung disease. Exp Lung Res 26:121–135
Polak JM, Becker KL, Cutz E, Gail DB, Goniakowska-Witalinska L, Gosney JR, Lauweryns JM, Linnoila I, McDowell EM, Miller YE (1993) Lung endocrine cell markers, peptides, and amines. Anat Rec 236:169–171
Porzionato A, Macchi V, Parenti A, Matturri L, De CR (2008) Peripheral chemoreceptors: postnatal development and cytochemical findings in Sudden Infant Death Syndrome. Histol Histopathol 23:351–365
Powley TL, Phillips RJ (2011) Vagal intramuscular array afferents form complexes with interstitial cells of Cajal in gastrointestinal smooth muscle: analogues of muscle spindle organs? Neuroscience 186:188–200
Qing X, Svaren J, Keith IM (2001) mRNA expression of novel CGRP1 receptors and their activity-modifying proteins in hypoxic rat lung. Am J Physiol Lung Cell Mol Physiol 280:L547–L554
Raab M, Neuhuber WL (2003) Vesicular glutamate transporter 2 immunoreactivity in putative vagal mechanosensor terminals of mouse and rat esophagus: indication of a local effector function. Cell Tissue Res 312:141–148
Raab M, Neuhuber WL (2007) Glutamatergic functions of primary afferent neurons with special emphasis on vagal afferents. Int Rev Cytol 256:223–275
Raab M, Wörl J, Brehmer A, Neuhuber WL (2003) Reduction of NT-3 or TrkC results in fewer putative vagal mechanoreceptors in the mouse esophagus. Auton Neurosci 108:22–31
Racké K, Matthiesen S (2004) The airway cholinergic system: physiology and pharmacology. Pulm Pharmacol Ther 17:181–198
Rafael J, Nicholls DG (1984) Mitochondrial membrane potential monitored in situ within isolated guinea pig brown adipocytes by a styryl pyridinium fluorescent indicator. FEBS Lett 170:181–185
Rawlins EL, Hogan BL (2006) Epithelial stem cells of the lung: privileged few or opportunities for many? Development 133:2455–2465
Redecker P, Grube D, Jahn R (1990) Immunohistochemical localization of synaptophysin (p38) in the pineal gland of the Mongolian gerbil (Meriones unguiculatus). Anat Embryol 181:433–440
Redick ML, Hung K-S (1984) Quantitation of pulmonary neuroepithelial bodies in pre- and postnatal rabbits. Cell Tissue Res 238:583–587
Rehm H, Wiedenmann B, Betz H (1986) Molecular characerization of synaptophysin, a major calcium-binding protein of the synaptic vesicle membrane. EMBO J 5:535–541
Reissig A, Kroegel C (2003) Transthoracic ultrasound of lung and pleura in the diagnosis of pulmonary embolism: a novel non-invasive bedside approach. Respiration 70:441–452
Reynolds SD, Giangreco A, Power JH, Stripp BR (2000a) Neuroepithelial bodies of pulmonary airways serve as a reservoir of progenitor cells capable of epithelial regeneration. Am J Pathol 156:269–278
Reynolds SD, Hong KU, Giangreco A, Mango GW, Guron C, Morimoto Y, Stripp BR (2000b) Conditional clara cell ablation reveals a self-renewing progenitor function of pulmonary neuroendocrine cells. Am J Physiol Lung Cell Mol Physiol 278:L1256–L1263
Reynolds SD, Reynolds PR, Pryhuber GS, Finder JD, Stripp BR (2002) Secretoglobins SCGB3A1 and SCGB3A2 define secretory cell subsets in mouse and human airways. Am J Respir Crit Care Med 166:1498–1509
Riccio MM, Kummer W, Biglari B, Myers AC, Undem BJ (1996) Interganglionic segregation of distinct vagal afferent fibre phenotypes in guinea-pig airways. J Physiol Lond 496:521–530
Rodrigo J, Hernansez CJ, Vidal MA, Pedrosa JA (1975) Vegetative innervation of the esophagus. II. Intraganglionic laminar endings. Acta Anat 92:79–100
Roncalli M, Springall DR, Maggioni M, Moradoghli-Haftvani A, Winter RJD, Zhao L, Coggi G, Polak JM (1993) Early changes in the calcitonin gene-related peptide (CGRP) content of

pulmonary endocrine cells concomitant with vascular remodeling in the hypoxic rat. Am J Respir Cell Mol Biol 9:467–474
Rong W, Gourine AV, Cockayne DA, Xiang Z, Ford AP, Spyer KM, Burnstock G (2003) Pivotal role of nucleotide $P2X_2$ receptor subunit of the ATP-gated ion channel mediating ventilatory responses to hypoxia. J Neurosci 23:11315–11321
Roomans GM (2010) Tissue engineering and the use of stem/progenitor cells for airway epithelium repair. Eur Cell Mater 19:284–299
Rusch VW (1990) Diagnosis and treatment of pleural mesothelioma. Semin Surg Oncol 6:279–285
Ryan TA (2001) Presynaptic imaging techniques. Curr Opin Neurobiol 11:544–549
Salathe M, Lipson EJ, Ivonnet PI, Bookman RJ (1997) Muscarinic signaling in ciliated tracheal epithelial cells: dual effects on Ca^{2+} and ciliary beating. Am J Physiol 272:L301–L310
Sanderson MJ (2011) Exploring lung physiology in health and disease with lung slices. Pulm Pharmacol Ther 24:452–465
Sant'Ambrogio G (1982) Information arising from the tracheobronchial tree of mammals. Physiol Rev 62:531–569
Sant'Ambrogio G, Sant'Ambrogio FB (1997) Reflexes from the upper airway, lungs, chest wall, and limbs. In: Crystal RG, West J (eds) The lung. Lippincott-Raven, Philadelphia, pp 1805–1819
Sant'Ambrogio G, Widdicombe J (2001) Reflexes from airway rapidly adapting receptors. Respir Physiol 125:33–45
Sant'Ambrogio G, Sant'Ambrogio FB, Davies A (1984) Airway receptors in cough. Bull Europ Physiopath Resp 20:43–47
Sartelet H, Maouche K, Totobenazara JL, Petit J, Burlet H, Monteau M, Tournier JM, Birembaut P (2008) Expression of nicotinic receptors in normal and tumoral pulmonary neuroendocrine cells (PNEC). Pathol Res Pract 204:891–898
Schäfer MKH, Weihe E, Varoqui H, Eiden LE, Erickson JD (1994) Distribution of the vesicular acetylcholine transporter (VAChT) in the central and peripheral nervous systems of the rat. J Mol Neurosci 5:1–26
Schelegle ES (2003) Functional morphology and physiology of slowly adapting pulmonary stretch receptors. Anat Rec 270:11–16
Schelegle ES, Green JF (2001) An overview of the anatomy and physiology of slowly adapting pulmonary stretch receptors. Respir Physiol 125:17–31
Scheuermann DW (1987) Morphology and cytochemistry of the endocrine epithelial system in the lung. Int Rev Cytol 106:35–88
Scheuermann DW, Adriaensen D, Timmermans J-P, De Groodt-Lasseel MH (1992) Comparative histological overview of the chemical coding of the pulmonary neuroepithelial endocrine system in health and disease. Eur J Morphol 30:101–112
Schuil PJ, Rosmalen JG, Graamans K, Huizing EH (1995) Calcitonin gene-related peptide in vitro stimulation of ciliary beat in human upper respiratory cilia. Eur Arch Otorhinolaryngol 252:462–464
Schuller HM, Witschi HP, Nylen E, Joshi PA, Correa E, Becker KL (1990) Pathobiology of lung tumors induced in hamsters by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and the modulating effect of hyperoxia. Cancer Res 50:1960–1965
Schuller HM, Jull BA, Sheppard BJ, Plummer HK (2000) Interaction of tobacco-specific toxicants with the neuronal alpha(7) nicotinic acetylcholine receptor and its associated mitogenic signal transduction pathway: potential role in lung carcinogenesis and pediatric lung disorders. Eur J Pharmacol 393:265–277
Schuller HM, Plummer HK III, Jull BA (2003) Receptor-mediated effects of nicotine and its nitrosated derivative NNK on pulmonary neuroendocrine cells. Anat Rec 270A:51–58
Schwaller B (2009) The continuing disappearance of "pure" Ca^{2+} buffers. Cell Mol Life Sci 66:275–300

Schwaller B (2010) Cytosolic Ca^{2+} buffers. Cold Spring Harb Perspect Biol 2:a004051
Sekhon HS, Jia Y, Raab R, Kuryatov A, Pankow JF, Whitsett JA, Lindstrom J, Spindel ER (1999) Prenatal nicotine increases pulmonary a7 nicotinic receptor expression and alters fetal lung development in monkeys. J Clin Invest 103:637–647
Seldeslagh KA, Lauweryns JM (1997) NCAM expression in the pulmonary neural and diffuse neuroendocrine cell system. Microsc Res Tech 37:69–76
Seuwen K, Pouyssegur J (1990) Serotonin as a growth factor. Biochem Pharmacol 39 (6):985–990
Sheldon MI, Green JF (1982) Evidence for pulmonary CO_2 sensitivity: effects on ventilation. J Appl Physiol 52:1192–1197
Shimosegawa T, Said SI (1991a) Co-occurrence of immunoreactive calcitonin and calcitonin gene-related peptide in neuroendocrine cells of rat lungs. Cell Tissue Res 264:555–561
Shimosegawa T, Said SI (1991b) Pulmonary calcitonin gene-related peptide immunoreactivity: nerve-endocrine cell interrelationships. Am J Respir Cell Mol Biol 4:126–134
Smiley-Jewell SM, Tran MU, Weir AJ, Johnson ZA, Van Winkle LS, Plopper CG (2002) Three-dimensional mapping of smooth muscle in the distal conducting airways of mouse, rabbit, and monkey. J Appl Physiol 93:1506–1514
Snyder JC, Teisanu RM, Stripp BR (2009) Endogenous lung stem cells and contribution to disease. J Pathol 217:254–264
Sonstegard KS, Mailman RB, Cheek JM, Tomlin TE, DiAugustini RP (1982) Morphological and cytochemical characterization of neuroepithelial bodies in fetal rabbit lung. I. Studies of isolated neuroepithelial bodies. Exp Lung Res 3:349–377
Sorhaug S, Steinshamn S, Munkvold B, Waldum HL (2008) Release of neuroendocrine products in the pulmonary circulation during intermittent hypoxia in isolated rat lung. Respir Physiol Neurobiol 162:1–7
Sorokin SP, Hoyt RF (1989) Neuroepithelial bodies and solitary small-granule cells. In: Massaro D (ed) Lung cell biology. Marcel Dekker, New York, pp 191–344
Sorokin SP, Hoyt RF (1990) On the supposed function of neuroepithelial bodies in adult mammalian lungs. News Physiol Sci 5:89–95
Sorokin SP, Hoyt RF (1993) Proceedings of workshop on pulmonary neuroendocrine cells in health and disease. Anat Rec 236:1–256
Sorokin SP, Hoyt RF, Shaffer MJ (1997) Ontogeny of neuroepithelial bodies: correlations with mitogenesis and innervation. Microsc Res Tech 37:43–61
Soukhova G, Wang Y, Ahmed M, Walker JF, Yu J (2003) Bradykinin stimulates respiratory drive by activating pulmonary sympathetic afferents in the rabbit. J Appl Physiol 95:241–249
Speirs V, Cutz E (1993) An overview of culture and isolation methods suitable for in vitro studies on pulmonary neuroendocrine cells. Anat Rec 236:35–40
Speirs V, Wang YV, Yeger H, Cutz E (1992) Isolation and culture of neuroendocrine cells from fetal rabbit lung using immunomagnetic techniques. Am J Respir Cell Mol Biol 6:63–67
Spencer H, Leof D (1964) The innervation of human lung. J Anat 98:599–609
Springall DR, Polak JM (1993) Calcitonin gene-related peptide and pulmonary hypertension in experimental hypoxia. Anat Rec 236:96–104
Springall DR, Polak JM (1997) Quantitative microscopical methods for the identification and localisation of nerves and neuroendocrine cell markers in mammalian lung. Microsc Res Tech 37:92–100
Springall DR, Cadieux A, Oliveira H, Su H, Rayston D, Polak JM (1987) Retrograde tracing shows that CGRP-immunoreactive nerves of rat trachea and lung originate from vagal and dorsal root ganglia. J Auton Nerv Syst 20:155–166
Springer J, Amadesi S, Trevisani M, Harrison S, Dinh QT, McGregor GP, Fisher A, Geppetti P, Groneberg DA (2004) Effects of alpha calcitonin gene-related peptide in human bronchial smooth muscle and pulmonary artery. Regul Pept 118:127–134

Stahlman MT, Gray ME (1984) Ontogeny of neuroendocrine cells in human fetal lung. I. An electron microscopic study. Lab Invest 51:449–463
Stahlman MT, Gray ME (1997) Immunogold EM localization of neurochemicals in human pulmonary neuroendocrine cells. Microsc Res Tech 37:77–91
Stahlman MT, Jones M, Gray ME, Kasselberg AG, Vaughn WK (1987) Ontogeny of neuroendocrine cells in human fetal lung. III. An electron microscopic immunohistochemical study. Lab Invest 56:629–641
Stripp BR, Maxson K, Mera R, Singh G (1995) Plasticity of airway cell proliferation and gene expression after acute naphthalene injury. Am J Physiol 269:L791–L799
Sullivan JP, Minna JD, Shay JW (2010) Evidence for self-renewing lung cancer stem cells and their implications in tumor initiation, progression, and targeted therapy. Cancer Metastasis Rev 29:61–72
Sutherland KD, Proost N, Brouns I, Adriaensen D, Song J-Y, Berns A (2011) Cells of origins of small cell lung cancer: inactivation of Trp53 and Rb1 in distinct cell types of adult mouse lung. Cancer Cell 19:754–764
Tabassian AR, Nylen ES, Giron AE et al (1988) Evidence for cigarette smoke induced calcitonin secretion from lungs of man and hamster. Life Sci 42:2323–2329
Tabassian AR, Snider RH, Nylen ES, Cassidy MM, Becker KL (1993) Heterogeneity studies of hamster calcitonin following acute exposure to cigarette smoke: evidence for a monomeric secretion. Anat Rec 236:253–256
Takamori S (2006) VGLUTs: 'Exciting' times for glutamatergic research ? Neurosci Res 55:343–351
Takamori S, Rhee JS, Rosenmund C, Jahn R (2000) Identification of a vesicular glutamate transporter that defines a glutamatergic phenotype in neurons. Nature 407:189–194
Takemura M, Quarcoo D, Niimi A, Dinh QT, Geppetti P, Fischer A, Chung KF, Groneberg DA (2008) Is TRPV1 a useful target in respiratory diseases? Pulm Pharmacol Ther 21:833–839
Tamaoki J, Kanemura T, Kobayashi K, Sakai N, Takizawa T (1989) Effects of calcitonin gene-related peptide on airway epithelial functions in dogs. Peptides 10:1007–1011
Tatar M, Webber SE, Widdicombe J (1988) Lung C-fiber receptors activation and defensive reflexes in anaesthetized cats. J Physiol 402:411–420
Tatar M, Sant'Ambrogio G, Sant'Ambrogio FB (1994) Laryngeal and tracheobronchial cough in anesthetized dogs. J Appl Physiol 76:2672–2679
Taylor-Clark T, Undem BJ (2006) Transduction mechanisms in airway sensory nerves. J Appl Physiol 101:950–959
Terada M, Iwanaga T, Takahashi-Iwanaga H, Adachi I, Arakawa M, Fujita T (1992) Calcitonin gene-related peptide (CGRP)-immunoreactive nerves in the tracheal epithelium of rats: an immunohistochemical study by means of whole mount preparations. Arch Histol Cytol 55:219–233
Tjen-A-Looi S, Kraiczi H, Ekman R, Keith IM (1998) Sensory CGRP depletion by capsaicin exacerbates hypoxia-induced pulmonary hypertension in rats. Regul Pept 74:1–10
Tsukiji J, Sango K, Udaka N, Kageyama H, Ito T, Saito H, Horie H, Inoue S, Kitamura H, Hagiwara E, Ikeda H, Okubo T, Ishigatsubo Y (2004) Long-term induction of beta-CGRP mRNA in rat lungs by allergic inflammation. Life Sci 76:163–177
Tsutsumi Y, Osamura RY, Watanabe K, Yanaihara N (1983) Simultaneous immunohistochemical localization of gastrin releasing peptide (GRP) and calcitonin (CT) in human bronchial endocrine-type cells. Virchows Arch A Pathol Anat Histopathol 400:163–171
Uddman R, Luts A, Sundler F (1985) Occurrence and distribution of calcitonin gene-related peptide in the mammalian respiratory tract and middle ear. Cell Tissue Res 241:551–555
Undem BJ, Carr MJ (2010) Targeting primary afferent nerves for novel antitussive therapy. Chest 137:177–184
Undem BJ, Kollarik M (2005) The role of vagal afferent nerves in chronic obstructive pulmonary disease. Proc Am Thorac Soc 2:355–360

Undem BJ, Nassenstein C (2009) Airway nerves and dyspnea associated with inflammatory airway disease. Respir Physiol Neurobiol 167:36–44
Undem BJ, Carr MJ, Kollarik M (2002) Physiology and plasticity of putative cough fibres in the guinea pig. Pulm Pharmacol Ther 15:193–198
Undem BJ, Chuaychoo B, Lee M-G, Weinreich D, Myers AC, Kollarik M (2004) Subtypes of vagal afferent C-fibres in guinea-pig lungs. J Physiol 556(3):905–917
Urban C, Nirenberg A, Caparros B, Anac S, Cacavio A, Rosen G (1983) Chemical pleuritis as the cause of acute chest pain following high-dose methotrexate treatment. Cancer 51:34–37
Van Genechten J, Brouns I, Scheuermann DW, Timmermans J-P, Adriaensen D (2003) Reduced number of intrinsic pulmonary nitrergic neurons in Fawn-Hooded rats as compared to control rat strains. Anat Rec 272A:446–453
Van Genechten J, Brouns I, Burnstock G, Timmermans J-P, Adriaensen D (2004) Quantification of neuroepithelial bodies and their innervation in Fawn-Hooded and Wistar rat lungs. Am J Respir Cell Mol Biol 30:20–30
Van Lommel A, Lauweryns JM (1993a) Neuroepithelial bodies in the Fawn Hooded rat lung: morphological and neuroanatomical evidence for a sensory innervation. J Anat 183:553–566
Van Lommel AT, Lauweryns JM (1993b) Ultrastructure and innervation of neuroepithelial bodies in the lungs of newborn cats. Anat Rec 236:181–190
Van Lommel A, Lauweryns JM (1997) Postnatal development of the pulmonary neuroepithelial bodies in various animal species. J Auton Nerv Syst 65:17–24
Van Lommel A, Lauweryns JM, De Leyn P, Wouters P, Schreinemakers H, Lerut T (1995) Pulmonary neuroepithelial bodies in neonatal and adult dogs: histochemistry, ultrastructure, and effects of unilateral hilar lung denervation. Lung 173:13–23
Van Lommel A, Lauweryns JM, Berthoud H-R (1998) Pulmonary neuroepithelial bodies are innervated by vagal afferent nerves: an investigation with in vivo anterograde Dil tracing and confocal microscopy. Anat Embryol 197:325–330
Van Lommel A, Bolle T, Fannes W, Lauweryns JM (1999) The pulmonary neuroendocrine system: the past decade. Arch Histol Cytol 62:1–16
Van Lommel A, Bollé T, Hellings P (2009) Pulmonary neuroepithelial bodies as hypothetical immunomodulators: some new findings and a review of the literature. In: Zaccone G, Cutz E, Adriaensen D, Nurse CA, Mauceri A (eds) Airway chemoreceptors in the vertebrates. Structure, evolution and function. Science Publishers, Enfield, New Hampshire, pp 311–330
Van Ranst L (1989) Immunocytochemisch onderzoek van de intrapulmonale neuroepitheliale lichamen en hun bezenuwing bij verschillende zoogdieren. Doctoraatsproefschrift, Katholieke Universiteit Leuven
Van Scott MR, Chinet TC, Burnette AD, Paradiso AM (1995) Purinergic regulation of ion transport across nonciliated bronchiolar epithelial (Clara) cells. Am J Physiol 269:L30–L37
Verstegui C, Fernandez-Vivero J, Prada A, Rodriguez F, Romero A, GonzalezMoreno M, deCastro JM (1997) Presence and distribution of 5HT-, VIP-, NPY-, and SP-immunoreactive structures in adult mouse lung. Histol Histopathol 12:909–918
Vicaut E, Laemmel E, Stucker O (2000) Impact of serotonin on tumour growth. Ann Med 32:187–194
von Düring M, Andres KH, Iravani J (1974) The fine structure of the pulmonary stretch receptor in the rat. Z Anat Entwickl Gesch 143:215–222
von Euler C (1997) Neural organization and rhythm generation. In: Crystal RG, West J (eds) The lung. Lippincott-Raven, Philadelphia, pp 1711–1724
Walsh C, McLelland J (1978) The development of the epithelium and its innervation in the avian extra-pulmonary respiratory tract. J Anat 125:171–182
Wang Y-Y, Cutz E (1993) Localization of cholecystokinin-like peptide in neuroendocrine cells of mammalian lungs: a light and electron microscopic immunohistochemical study. Anat Rec 236:198–205

Wang ZJ, Neuhuber WL (2003) Intraganglionic laminar endings in the rat esophagus contain purinergic $P2X_2$ and $P2X_3$ receptor immunoreactivity. Anat Embryol 207:363–371
Wang YF, Yu J (2002) Na^+/K^+-ATPase as a marker for detecting pulmonary sensory receptors. Sheng Li Xue Bao 54:390–394
Wang Y-F, Yu J (2004) Structural survey of airway sensory receptors in rabbit using confocal microscopy. Acta Physiologica Sinica 56:119–129
Wang D, Yeger H, Cutz E (1996a) Expression of gastrin-releasing peptide receptor gene in developing lung. Am J Respir Cell Mol Biol 14:409–416
Wang D, Youngson CR, Wong V, Yeger H, Dinauer MC, Vega-Saenz ME, Rudy B, Cutz E (1996b) NADPH-oxidase and a hydrogen peroxide-sensitive K^+ channel may function as an oxygen sensor complex in airway chemoreceptors and small cell lung carcinoma cell lines. Proc Natl Acad Sci USA 93:13182–13187
Wasano K (1977) Neuroepithelial bodies in the lung of rat and mouse. Arch Histol Jpn 40:207–219
Wasano K, Yamamoto T (1978) Monoamine-containing granulated cells in the frog lung. Cell Tissue Res 193:201–209
Wasano K, Yamamoto T (1981) A scanning and transmission electron-microscopic study on neuroepithelial bodies in the neonatal mouse lung. Cell Tissue Res 216:481–490
Webber SE, Lim JC, Widdicombe JG (1991) The effects of calcitonin gene-related peptide on submucosal gland secretion and epithelial albumin transport in the ferret trachea in vitro. Br J Pharmacol 102:79–84
Wedekind C (1997) Receptive properties of primary afferent fibers from rabbit pleura, in vitro. Somatosens Mot Res 14:229–236
Weichselbaum M, Everett AW, Sparrow MP (1996) Mapping the innervation of the bronchial tree in fetal and postnatal pig lung using antibodies to PGP9.5 and SV2. Am J Respir Cell Mol Biol 15:703–710
Wharton J, Polak JM, Bloom SR, Ghatei MA, Solcia E, Brown MR, Pearse AG (1978) Bombesin-like immunoreactivity in the lung. Nature 273:769–770
Widdicombe J (1954) Receptors in the trachea and bronchi of the cat. J Physiol 123:71–104
Widdicombe JG (1981) Nervous receptors in the respiratory tract and lungs. In: Hornbein T (ed) Lung biology in health and disease. Marcel Dekker, New York, pp 429–472
Widdicombe J (1986) The neural reflexes in the airways. Eur J Respir Dis Suppl 144:1–3
Widdicombe JG (1998) Afferent receptors in the airways and cough. Respir Physiol 114:5–15
Widdicombe JG (2001) Airway receptors. Respir Physiol 125:3–15
Widdicombe J (2003a) Functional morphology and physiology of pulmonary rapidly adapting receptors (RARs). Anat Rec 270:2–10
Widdicombe JG (2003b) Overview of neural pathways in allergy and asthma. Pulm Pharmacol Ther 16:23–30
Widdicombe J (2006) Reflexes from the lungs and airways: historical perspective. J Appl Physiol 101:628–634
Widdicombe J (2009) Lung afferent activity: implications for respiratory sensation. Respir Physiol Neurobiol 167:2–8
Widdicombe J, Nadel JA (1963a) Reflex effects of lung inflation on thracheal volume. J Appl Physiol 18:681–686
Widdicombe JG, Nadel JA (1963b) Airway volume, airway resistance, and work and force of breathing: theory. J Appl Physiol 18:863–868
Wiedenmann B, Franke WW, Kuhn C, Moll R, Gould VE (1986) Synaptophysin: a marker for neuroendocrine cells and neoplasms. Proc Natl Acad Sci USA 83:3500–3504
Will JA, Keith IM, Buckner CK, Chacko J, Olson EBJ, Weir EK (1984) Serotonin and the pulmonary circulation. In: Becker KL, Gazdar AF (eds) The endocrine lung in health and disease. WB Saunders, Philadelphia, pp 137–154

Wong AP, Keating A, Waddell TK (2009) Airway regeneration: the role of the Clara cell secretory protein and the cells that express it. Cytotherapy 11:676–687

Wu S-X, Koshimizu Y, Feng Y-P, Okamoto K, Fujiyama F, Hioki H, Li Y-Q, Kaneko T, Mizuno N (2004) Vesicular glutamate transporter immunoreactivity in the central and peripheral endings of muscle-spindle afferents. Brain Res 1011:247–251

Xiong J, Camello PJ, Verkhratsky A, Toescu EC (2004) Mitochondrial polarisation status and $[Ca^{2+}]_i$ signalling in rat cerebellar granule neurones aged in vitro. Neurobiol Aging 25:349–359

Yabumoto Y, Watanabe M, Ito Y, Maemura K, Otsuki Y, Nakamura Y, Yanagawa Y, Obata K, Watanabe K (2008) Expression of GABAergic system in pulmonary neuroendocrine cells and airway epithelial cells in GAD67-GFP knock-in mice. Med Mol Morphol 41:20–27

Yamamoto Y, Atoji Y, Suzuki Y (1995) Nerve endings in bronchi in the dog that react with antibodies against neurofilament protein. J Anat 187:59–65

Yamamoto Y, Atoji Y, Kuramoto H, Suzuki Y (1998) Calretinin-immunoreactive laminar nerve endings in the laryngeal mucosa of the rat. Cell Tissue Res 292:613–617

Yamamoto Y, Atoji Y, Suzuki Y (1999) Calretinin immunoreactive nerve endings in the trachea and bronchi of the rat. J Vet Med Sci 61:267–269

Yeger H, Pan J, Fu XW, Bear C, Cutz E (2001) Expression of CFTR and Cl^- conductances in cells of pulmonary neuroepithelial bodies. Am J Physiol Lung Cell Mol Physiol 281:L713–L721

Yoo SH, Huh YH, Hur YS (2010) Inositol 1,4,5-Trisphosphate receptor in chromaffin secretory granules and its relation to chromogranins. Cell Mol Neurobiol 30:1155–1161

Youngson C, Nurse C, Yeger H, Cutz E (1993) Oxygen sensing in airway chemoreceptors. Nature 365:153–155

Youngson C, Nurse C, Yeger H, Curnutte JT, Vollmer C, Wong V, Cutz E (1997) Immunocytochemical localization on O_2-sensing protein (NADPH oxidase) in chemoreceptor cells. Microsc Res Tech 37:101–106

Yu J (2009) Airway receptors and their reflex function. In: Gonzalez C, Peers C, Nurse CA (eds) Arterial Chemoreceptors. pp 411–420

Yu J, Wang YF, Zhang JW (2003) Structure of slowly adapting pulmonary stretch receptors in the lung periphery. J Appl Physiol 95:385–393

Yu J, Zhang JW, Wang YF, Fan F, Yu A (2004) Neuroepithelial bodies not connected to pulmonary slowly adapting stretch receptors. Respir Physiol Neurobiol 144:1–14

Yu J, Lin XS, Zhang JWWJ (2006) Pulmonary nociceptors are potentially connected with neuroepithelial bodies. In: The arterial chemoreceptors. Springer, pp 301–306.

Zhang JW, Walker JF, Guardiola J, Yu J (2006) Pulmonary sensory and reflex responses in the mouse. J Appl Physiol 101:986–992

Zhao H, Sprunger KL, Simasko MS (2010) Expression of transient receptor potential channels and two-pore potassium channels in subtypes of vagal afferent neurons in rat. Am J Physiol Gastrointest Liver Physiol 298:212–221

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