This item is the archived peer-reviewed author-version of:

Sensory input to the central nervous system from the lungs and airways : a prominent role for purinergic signalling via P2X2/3 receptors

Reference:

Adriaensen Dirk, Brouns Inge, Timmermans Jean-Pierre.- Sensory input to the central nervous system from the lungs and airways : a prominent role for purinergic signalling via P2X2/3 receptors
Autonomic neuroscience: basic and clinical - ISSN 1566-0702 - (2015), p. 1-9
DOI: <http://dx.doi.org/doi:10.1016/j.autneu.2015.04.006>

**SENSORY INPUT TO THE CENTRAL NERVOUS SYSTEM FROM THE LUNGS
AND AIRWAYS: A PROMINENT ROLE FOR PURINERGIC SIGNALLING VIA
P2X2/3 RECEPTORS**

Dirk Adriaensen, Inge Brouns and Jean-Pierre Timmermans*

University of Antwerp, Department of Veterinary Sciences, Laboratory of Cell Biology and Histology, Groenenborgerlaan 171, BE-2020 Antwerpen, Belgium.

(* Author for correspondence)

Corresponding author:

Jean-Pierre Timmermans

University of Antwerp, Department of Veterinary Sciences

Laboratory of Cell Biology and Histology / Core Facility Biomedical Microscopic Imaging

Groenenborgerlaan 171, BE-2020 Antwerpen, Belgium

Tel: +32 (0)3 265 3327 / Fax: +32 (0)3 265 3301

E-mail: jean-pierre.timmermans@uantwerpen.be

Running title: Purinergic afferent signalling in lungs

Key words: P2X2/3 receptors - Sensory airway receptors - Neuroepithelial bodies - Smooth muscle-associated airway receptors - Visceral pleura receptors - C-fibre receptors

Abstract

Specific subpopulations of lung-related primary afferent neurons in dorsal root and vagal sensory ganglia have been reported to express P2X2 and P2X3 receptors both in the neuronal cell bodies and in their peripheral terminals. The afferent innervation of airways and lungs is organised as sensory receptor structures, of which at least seven types with a vagal origin and two with a spinal origin have been reported.

In view of the recently suggested therapeutic promise of ATP antagonism – specifically at P2X3 receptor expressing nociceptive fibres – in respiratory disorders, the present work focusses on four distinct populations of pulmonary sensory receptors that have so far been reported to express P2X2/3 receptors. Three of them originate from myelinated nerve fibres that display similar mechanosensor-like morphological and neurochemical characteristics. Two of the latter concern vagal nodose sensory fibres, either related to pulmonary neuroepithelial bodies (NEBs), or giving rise to smooth muscle-associated airway receptors (SMARs); the third gives rise to visceral pleura receptors (VPRs) and most likely arises from dorsal root ganglia. The fourth population concerns C-fibre receptors (CFRs) that also derive from neuronal cell bodies located in vagal nodose ganglia.

Although the majority of the airway- and lung-related sensory receptors that express P2X2/3 receptors apparently do not belong to accepted nociceptive populations, these data definitely point out that ATP may be an important player in the physiological transduction of different lung-related afferent signals from the periphery to the CNS. The observed variety within the populations of pulmonary sensory receptors that express P2X2/3 receptors argues for a critical and careful interpretation of the functional data.

Introduction

Primary afferent neurons (PANs) link peripheral receptive fields in organs, to nerve cell somata in dorsal root ganglia (DRGs) and cranial ganglia. They can be classified into several subtypes based on morphology, speed of conduction, molecular markers and receptor expression, and a wide range of functions from high-threshold (nociceptive; fast A δ -fibers, slow C-fibres), sensitive to noxious mechanical and/or chemical stimuli, to low-threshold (non-nociceptive; mostly the faster) proprioceptive, mechanosensitive and thermosensitive fibres (Ford and Undem, 2013). Traditionally, vagal afferent nerves (whose somata are located in vagal jugular and nodose ganglia) have been considered as the sensory pathways for collecting physiological information from visceral organs, including lungs and airways, while spinal afferents would be involved in detecting ‘noxious’ information. According to the present knowledge, however, several subsets of vagal afferents may also be implicated in processing potentially harmful stimuli (nociceptors) from the airways (Yu, 2009; Kollarik et al., 2010; Nassenstein et al., 2010; Adriaensen and Timmermans, 2011), and, conversely, spinal pulmonary populations with potential physiological roles have been reported as well (Pintelon et al., 2007; Brouns et al., 2012).

In the lungs and airways, two different morphologically well-characterized populations of spinal afferents (Brouns et al., 2003b; Pintelon et al., 2007; Brouns et al., 2012), and at least seven vagal sensory airway receptors have been identified electrophysiologically or morphologically/neurochemically during the past decade (Adriaensen et al., 2006; Canning et al., 2006; Widdicombe, 2009; Yu, 2009; Nassenstein et al., 2010; Brouns et al., 2012). The characterised vagal sensory airway receptors include slowly adapting receptors (SARs), rapidly adapting receptors (RARs), high-threshold A δ -receptors (HTARs), bronchial and pulmonary C-fibre receptors (CFRs), neuroepithelial bodies (NEBs) and cough receptors.

Also functionally, a plethora of respiratory sensations have been reported, such as dyspnoea (breathlessness/ air-hunger/ sense of effort/ tightness), pain/ache, irritation, urge-to-cough, sense of lung volume/airflow, and temperature sense. Undoubtedly, many of these sensations find their origin in the lower airways and lungs, but hard evidence that specifically links them to any of the reported sensory airway receptors is still lacking (Widdicombe, 2009). Both HTARs, connecting to thin myelinated fibres, and CFRs, linked to non-myelinated fibres, are considered as subgroups of vagal airway nociceptors (Yu et al., 2007; Yu, 2009).

Today, basic knowledge of the potential involvement of both spinal and vagal sensory lung and airway receptors in collecting vital information for the regulation of breathing and immune responses remains fragmentary, with respect to for instance the exact location, morphology, neurochemical characteristics, physiological (and pathological) stimuli and reflex mechanisms, and their match with respiratory sensations.

ATP is an abundant molecule with an enormous panel of potential functions, such as the energy source in every cell, an ubiquitous transmitter and paracrine signalling molecule, and an extracellular ‘reporter’ of movement, distension, distress, ischemia, damage and inflammation (Burnstock, 2012). Many aspects of the mechanisms of selective or non-selective discharge of ATP in all of these processes are still being delineated (Burnstock, 2012), but it is clear that the release mechanisms may operate more aggressively in a variety of pathological settings, including airway pathology, leading to higher concentrations of both background and stimulated extracellular ATP (Ford, 2012).

Today, ATP acting via the P2X receptor family of ligand-gated ion channels is recognized as an important regulator of PANs (Burnstock, 2000; for review see Ford & Undem, 2013). A considerable fraction of PANs that project to the lungs and airways can be excited by ATP via P2X receptors (Undem et al., 2004; for reviews see Canning et al., 2006, Taylor-Clark and Undem, 2006). P2X2 and P2X3 receptors may function as homomeric or heteromeric

(P2X2/P2X3) channels, and have been reported to be expressed in lung-related 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 sensory receptors in airways and lungs, which will be the main focus of this short review.

Previous studies using P2X2 and P2X2/P2X3 double knockout mice have revealed a role for the P2X2 receptor subunit in mediating multiple sensory effects of ATP, and demonstrated an important contribution of heteromeric P2X2/3 receptors to nociceptive and mechanosensory transduction in the urinary bladder (Cockayne et al., 2005). In lungs and several other ‘hollow visceral organs’, it has been proposed that P2X3 or P2X2/3 receptor-expressing PANs are involved in mechanosensory and nociceptive transduction (for review see Burnstock, 2009), and that ATP sensitization of especially C-fibres may result in chronic discomfort, which would potentially benefit from the development of selective P2X3 antagonists (Ford and Udem, 2013).

Considering the suggested therapeutic promise of ATP antagonism specifically at P2X3 receptors in respiratory disorders (Ford and Udem, 2013), the present review summarizes available data on the involvement of afferent purinergic signalling via P2X2/3 receptors within the diverse group of morphologically/neurochemically and/or (electro)physiologically characterized sensory receptors in the lungs and intrapulmonary airways.

Diverse populations of sensory receptors in lungs and intrapulmonary airways express P2X2/P2X3 ATP receptors.

Especially during the past decade, both physiological and neurochemical studies have provided ample evidence for involvement of purinergic signalling, most prominently via P2X2/3 ATP receptors, in the transduction of sensory information from the airways and lungs to the central nervous system (CNS). As detailed below, at least four well-characterized subgroups of sensory receptors should be considered, the majority of which have a vagal origin. Three out of four are myelinated populations (two vagal and one presumably originating from thoracic DRGs), while the fourth population consists of pulmonary C-fibres.

1. Afferents that selectively interact with pulmonary neuroepithelial bodies

Neuroepithelial bodies (NEBs) are densely innervated groups of pulmonary neuroendocrine cells (PNECs) that are widely distributed in the epithelium of intrapulmonary airways of humans, mammals and all other groups of air-breathing vertebrates (Sorokin and Hoyt, 1989; Adriaensen et al., 2003). NEBs store various bioactive substances (Scheuermann, 1987; Adriaensen et al. 2003) that can be secreted upon specific stimulation (Fu et al., 2004; Pan et al., 2006; De Proost et al., 2008; Lembrechts et al., 2012, 2013). NEBs are invariably ‘shielded’ by so called Clara-like cells, a population of presumed airway epithelial stem cells that reside exclusively around and over NEB cells and, together with the innervation and PNECs, compose the NEB microenvironment (De Proost et al., 2008). Although still largely neglected in discussions about the regulation of lung function, pulmonary NEBs have been put forward as prime candidates to serve as complex sensory end-organs in the airways. Their typical organization, location as an integrated component of the epithelial lining of the airways, and close association with different populations of sensory nerve terminals, make

them ideally suited to sense changes in the airway environment and transduce this information to the CNS (Adriaensen et al., 2006; Brouns et al., 2012).

Intriguingly, the exact roles of NEBs in healthy lungs are still under debate (Cutz et al., 2013). NEBs have been suggested to fulfil several functions in the regulation of physiological processes in the lungs during prenatal, perinatal and postnatal life (Adriaensen et al., 2003; Linnoila, 2006), but by far the most frequently proposed and extensively documented evidence implicates them in airway oxygen sensing –mainly in the perinatal period– in different species (Cutz and Jackson, 1999; Cutz et al., 2003). Recently, evidence has also accumulated linking the NEB microenvironment to both mechanosensing (Pan et al., 2006; Lembrechts et al., 2011, 2012) and multimodal chemosensing (Lembrechts et al., 2013).

It has been reported and extensively illustrated that subpopulations of the myelinated sensory nerve fibres selectively contact NEBs in rodent intrapulmonary airways, express P2X2 and P2X3 receptors (**Fig.1**) and form laminar terminal arborisations that surround NEB cells. The NEB cells proper completely lack P2X2/3 receptor immunostaining in mice (Brouns et al., 2000, 2009, 2012; Adriaensen and Timmermans, 2004; Burnstock et al., 2012), whereas rat NEB cells express P2X2 but not P2X3 receptors (Brouns et al. 2012). Furthermore, both P2X2 and P2X3 receptors were identified on hamster NEBs by Fu and colleagues, who showed that ATP induced serotonin release and a non-desensitising inward current typical of P2X2 receptors, while the P2X3 receptor agonist α,β -methylene ATP (α,β -meATP) produced a slowly desensitising inward current, suggesting the involvement of a heteromultimeric P2X2/3 receptor (Fu et al., 2004).

Fig.1. Triple immunostaining for P2X2 (*red*) and P2X3 receptors (*green*), and for the general neuroendocrine marker protein gene-product 9.5 (PGP9.5; *blue*) of a mouse lung cryosection. **a.** A P2X2-immunoreactive (ir) nerve fibre (*arrow*) approaches the bronchiolar epithelium (*E*) and branches (*arrowheads*) between the epithelial cells. **b.** P2X3 immunohistochemistry shows a similar staining pattern. **c.** Combination of the red, green and blue channel show that the P2X2/3-ir nerve terminals coincide with the presence of a pulmonary NEB. *L:* airway lumen (*adapted from Brouns et al. 2009*).

Recently, this P2X3 receptor-positive population of nerve terminals in the NEB microenvironment has been elegantly visualized in genetically engineered mice that – in the lungs – selectively express GFP in NEB cells (**Fig.2**) (Schnorbusch et al., 2013).

Fig.2. Single immunohistochemical staining for P2X3 receptors (*red*) in a cryosection of the lungs of a mouse in which GFP is under the control of glutamic acid decarboxylase 67 (GAD67) regulatory elements, resulting in selective GFP expression in NEB cells (Schnorbusch et al. 2013). The P2X3-ir nerve fibre (*arrow*) approaches the NEB and branches between the NEB cells (*green*). *L:* airway lumen.

After unilateral infranodosal vagotomy, the P2X2/3-positive nerve terminals disappear from the intrapulmonary airways in the ipsilateral but not the contralateral lung, implying that P2X2/3 receptors are expressed on vagal sensory nerve endings that supply NEBs (Brouns et al., 2000, 2006a, 2009). These myelinated (diameter 1-3.5 µm) vagal P2X2/3 receptor positive nerve fibres lose their myelin sheaths just before branching and protruding between

NEB cells in the airway epithelium (**Fig.3**) and do not express the TRPV1 receptor (Brouns et al., 2003b), pointing most likely to a capsaicin-insensitive population.

Fig.3. Double immunostaining for P2X3 receptors (*green*) and myelin basic protein (MBP; *red*) in mouse lung, showing that a vagal afferent P2X3-ir nerve fibre loses its myelin sheath (*open arrow*) just before branching between *NEB* cells. *L*: airway lumen.

A considerable number of transmission electron microscopic (TEM) investigations have reported abundant nerve terminals, with a predominantly sensory phenotype and likely originating from vagal myelinated fibres, that penetrate between PNECs in the NEB microenvironment and form morphologically well-characterised afferent-like synaptic contacts with the NEB cells in several species, including rodents (for review see Adriaensen and Scheuermann, 1993; Van Lommel and Lauweryns, 1993). Both the location and morphology of the terminals between NEB cells, and the fact that vagal myelinated nerve fibres are involved, strongly suggest that at least part of these nerve terminals represent the TEM counterparts of the P2X2/3-expressing laminar terminals in NEBs.

In a developmental study, vagal nodose sensory nerve endings expressing P2X3 receptors were shown to be the first to selectively innervate NEBs, from gestational day 16 onwards in rats, suggesting the importance of a functional purinergic signalling pathway from NEBs to the CNS being established before birth (Brouns et al., 2003a).

Quinacrine histochemistry indicated high amounts of vesicular ATP in NEB cells (Brouns et al., 2000; De Proost et al., 2009), and real-time ‘reporter patching’ – using HEK 293 cells that over-express P2X2 receptors – revealed quantal ATP release following Ca^{2+} -mediated

activation of NEB cells (De Proost et al., 2009). It was also shown that ATP, released from activated NEB cells, induces a paracrine secondary Ca^{2+} -mediated activation of the surrounding stem cell-like Clara-like cells via P2Y_2 receptors, a phenomenon that is now used to visualise stimulated secretion from NEBs (Lembrechts et al., 2012, 2013; Schnorbusch et al. 2013).

Mechanical stretch has been reported as a potentially important physiological stimulus for PNECs. In cyclically stretched cultures of isolated foetal rabbit PNECs, serotonin was suggested to be released from cytoplasmic pools via mechanosensitive channels (Pan et al., 2006).

In rodent lungs, a major part of the myelinated vagal airway afferents, most of which have been found to represent physiologically characterised SAR-like mechanoreceptors (Yu et al., 2007), selectively contact pulmonary NEBs. It has been illustrated that NEB cells can be directly activated by physiological osmomechanical stimuli, including opening of the mechanosensitive Ca^{2+} channel TRPC5, subsequent intracellular Ca^{2+} increase, and calcium-dependent exocytosis of ATP. Since ATP may activate the NEB-associated $\text{P2X}_2/3$ receptor-expressing myelinated vagal afferents (**Fig.4**), PNECs in the NEB microenvironment seem to be fully equipped to initiate mechanosensory purinergic signal transduction from the airway epithelium to the CNS (Lembrechts et al., 2012). Purinergic signalling mechanisms, mediated by ATP receptors and ATP release upon mechanical distortion, have been implicated in mechanosensory transduction in several other visceral organs (Burnstock, 2007).

Fig.4. Triple immunohistochemical staining for the transient receptor potential canonical-5 (TRPC5; *red*) channel, P2X3 receptors (*green*) and calcitonin gene-related peptide (CGRP; *blue*), a well-known marker for mouse NEBs. **a.** Red channel revealing a group of epithelial cells expressing TRPC5 at their apical surface (*open arrowheads*). **b.** P2X3-ir intraepithelial arborisation. **c.** Combination of the red, green and blue channel shows the presence of the Ca^{2+} -permeable mechanogated TRPC5 channel in the apical membrane of CGRP-labelled NEB cells that are in close contact with P2X3 receptor expressing afferent nerve terminals. *L*: airway lumen.

Besides the apparent mechanosensing properties of NEB cells, it is intriguing that the populations of myelinated vagal nodose afferents that contact NEBs have since long been presumed to have their own intrinsic mechanosensory transduction capacities (Widdicombe, 2001), and the recent localisation of the mechanogated $\text{K}_{2\text{P}}$ channel TRAAK on P2X2/3 expressing vagal sensory terminals in NEBs (**Fig.5**) (Lembrechts et al., 2011) supports the idea that the NEB microenvironment is a complex mechanosensory receptor unit within the airway epithelium, the excitability of which can be modulated in different ways. Further functional interpretations of the expression of TRAAK in different populations of vagal sensory airway receptor can be found in the next section (‘smooth muscle-associated airway receptors’) and in the conclusions.

Fig.5. Double immunostaining for P2X3 receptors (*green*) and TRAAK (*red*) of a mouse lung cryosection. **a.** A vagal sensory P2X3-ir nerve fibre (*arrow*) approaches the epithelium and forms an intraepithelial P2X3-ir arborisation (*arrowheads*), indicative for the presence of a *NEB*. **b.** The P2X3-ir nerve terminals are co-stained for TRAAK. **c.** Combination of the red and green channel. *L*: lumen of the airway.

While it has been reported that peripheral ATP application can indeed stimulate vagal myelinated mechanosensitive airway afferents (Canning et al., 2004; Kollarik and Undem, 2006), the question remains whether also the physiological mechanically induced activity in these fibres is mediated by ATP as a neurotransmitter. The latter would make the difference between NEB cells potentially initiating mechanosensory transduction in vagal airway afferents, or just modulating (Lembrechts et al., 2012).

Finally, pulmonary NEBs in rodents also appear to be contacted by one or more populations of C-fibre-like nerve terminals that may arise from both thoracic DRGs and vagal ganglia, express calcitonin gene-related peptide (CGRP) and substance P (SP) but not P2X3 receptors (**Fig.6**), and are capsaicin sensitive (Brouns et al., 2003b, 2009, 2012).

Fig.6. Double immunohistochemical staining for P2X3 receptors (*red*) and CGRP (*green*) of a mouse lung cryosection. **a.** Red channel showing an approaching P2X3-ir nerve fibre (*arrow*) protruding between epithelial cells (*arrowheads*). **b.** Green channel showing CGRP-ir C-fibres (*open arrows*) very close to the base of a CGRP-ir NEB. **c.** Combination of the red and green channel shows that the CGRP- and P2X3-ir nerve terminals concern clearly different nerve fibre populations. *L*: airway lumen.

2. Smooth muscle-associated airway receptors

Myelinated nerve fibres that branch and give rise to nerve terminals in airway smooth muscle bundles have been described in the older anatomical literature (Larsell, 1922; Larsell and Dow, 1933; Elftman, 1943; Honjin, 1956). Immunocytochemical studies in rats revealed

branching nerve endings in the wall of the airways, suggestive of being located in the smooth muscle layer, using antibodies against calretinin (Yamamoto et al., 1999) and Na^+/K^+ -ATPase $\alpha 3$ (Yu et al., 2004). More recently, a combination of neurochemical coding, vagal denervation experiments and specific visualization of smooth muscle has identified the nature and origin of branching nerve terminals with well-delineated laminar end-organs that are typically found in the smooth muscle layer of rodent intrapulmonary conducting airways. The consistent association of the terminals of these receptor-like structures with airway smooth muscle, and the lack of a direct functional correlation, prompted us to suggest the name ‘smooth muscle-associated airway receptors (SMARs)’ a few years ago (Brouns et al., 2006a,b, 2012; De Proost et al., 2007; Lembrechts et al., 2011).

Nerve fibres that give rise to SMARs have been shown to be myelinated (diameter 1-3.5 μm) and to have a vagal origin. In rodent lungs, SMARs have been characterised by a panel of markers that is characteristic of well-established mechanosensitive nerve terminals in other organs, and very similar to at least part of the myelinated vagal afferents that selectively contact NEBs. Of particular interest for the present overview is the expression of P2X2 and P2X3 ATP receptors in subpopulations of the SMARs (**Fig.7**) (Brouns et al., 2006a,b, 2012; Burnstock et al., 2012).

Fig.7. P2X3 receptor-expressing (*green*) smooth muscle-associated airway receptor (SMAR) located in a bronchiolar smooth muscle bundle, as visualised by alpha smooth muscle actin (αSMA) immunostaining.

Multiple immunocytochemical staining revealed that all P2X3 receptor-stained SMARs, just like the P2X3-positive nerve terminals in NEBs (*see above*), also expressed TRAAK (**Fig.8**) (Lembrechts et al., 2011).

Fig.8. Double immunostaining for P2X3 receptors (*green*) and TRAAK (*red*) of a bronchiole in a mouse lung cryosection. **a.** P2X3 receptor expression reveals laminar nerve endings of a SMAR in the smooth muscle (*SM*) region, underneath the airway epithelium (*E*). **b.** TRAAK IR is present in the same SMAR. **c.** Combination of the red and green channel shows that all P2X3-positive nerve terminals of SMARs co-express the mechanosensitive TRAAK channel. *L*: airway lumen.

The observation that SMARs and NEBs are regularly found in each other's immediate neighbourhood, and the very similar characteristics of their nerve terminals, including P2X2/3 receptor expression (**Fig.9**), suggest that great caution should be exerted when interpreting electrophysiological data based on 'local' stimuli, such as application of ATP.

Fig.9. Double immunostaining for P2X3 receptors (*green*) and for the neuroendocrine marker CGRP (*red*) in a mouse lung cryosection. Note P2X3 IR both in a SMAR (*open arrowheads*) and in intraepithelial vagal sensory nerve terminals (*arrowheads*) protruding between NEB cells at an airway branching point. This image illustrates that NEBs and SMARs are regularly found in each other's immediate neighbourhood. *L*: airway lumen.

Both SMARs and NEBs share morphological and neurochemical characteristics with SARs (Yu et al., 2004; Adriaensen et al., 2006), and it has been suggested that SAR activity in rodents may have been recorded from SMARs and NEBs (Adriaensen et al., 2006).

Furthermore, inhibitory stretch-activated TRAAK channels were found to be co-expressed with P2X2/3 receptors on SAR-like SMAR terminals, suggesting possible involvement of TRAAK in the inspiratory off-switch mechanism, while activation may be ATP-mediated (Lembrechts et al. 2011).

3. Visceral pleura receptors

Although the visceral pleura has historically been considered insensitive to painful stimuli, commonly reported symptoms of pulmonary embolism, pleuritis and pleural tumours, the majority of which are associated with the visceral pleura, include severe chest pain, dyspnoea and urge to cough (Branch et al., 1983; Gulati et al., 2014). Diagnosis and adequate relief of the often angina-like chest pain and other sensations associated with pleural disease is typically problematic, an important aspect of which undoubtedly is related to a lack of knowledge concerning the location, morphology, and especially the neurochemical characteristics of potential sensory receptors involved in afferent pathways from the visceral pleura.

The morphology of extensive nerve bundles and different types of nerve terminals in certain areas of the visceral pleura of various animal species has been described in a few older nerve staining studies (Larsell, 1922; McLaughlin, 1933; Dwinnell, 1966). However, these data evidently did not yield specific neurochemical information that allowed differentiation between potential functionally distinct populations of sensory and motor terminals.

More recently, however, the neurochemical coding of sensory receptor-like end-organs in the visceral pleura has been described in detail, based on multiple immunostaining of whole

mount preparations and cryosections of the visceral pleura of control and vagally denervated rats (Pintelon et al., 2007; Brouns et al., 2012). Visceral pleura innervation was shown to be characterised by nerve bundles that enter the hilus region and gradually split into slender bundles with a few nerve fibres. At regular intervals, separate nerve fibres give rise to typical laminar terminals that are predominantly located on mediastinal and interlobar lung surfaces. Because of their unique association with the elastic fibres of the visceral pleura, they were referred to as ‘visceral pleura receptors (VPRs)’ (Pintelon et al., 2007; Brouns et al., 2012).

The sensory nerve fibres that give rise to VPRs appeared to be myelinated (diameter 1.4-3.5 μm) and to have a non-vagal origin (Pintelon et al., 2007). This non-vagal origin of VPRs 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).

VPRs were shown to selectively express a panel of markers that was very similar to the two above-described populations of afferent terminals in NEBs and SMARs; of particular interest for the present short review is the observation that subsets of VPRs also appeared to express P2X₃ ATP receptors. It was concluded that VPRs display the neurochemical characteristics of mechanosensory and/or nociceptive terminals (Pintelon et al., 2007; Brouns et al., 2012; Burnstock et al., 2012).

Based on the same combination of techniques, a limited amount of data could be obtained for the mouse visceral pleura, revealing VPRs with morphological and neurochemical characteristics that are very similar to those described for rats (own unpublished observations; **(Fig.10, 11)**). The latter findings suggest that purinergic signalling via P2X_{2/3} receptors may also be a common feature of at least subpopulations of this type of sensory lung receptors and therefore of potential interest for future studies regarding pain management.

Fig.10. Single immunostaining for P2X3 receptors of a cryosection of mouse lungs reveals the typical laminar nerve terminals (*arrowheads*) of a visceral pleura receptor (VPR) that is situated between two lung lobes.

Fig. 11. Triple immunostaining for P2X2 receptors (*red*), MBP (*blue*) and elastin α (*green*) of a whole mount preparation of a mouse lung visceral pleura. **a.** Combination of the red and blue channel reveals a myelinated P2X2-expressing nerve fibre (*arrow*) that branches into P2X2-ir laminar nerve terminals (*arrowheads*). **b.** Combination of all three channels shows the localisation of the laminar nerve terminals between the elastic fibres of the visceral pleura.

4. Pulmonary C-fibre receptors

Already almost two decades ago, ATP was found to effectively activate pulmonary CFRs and cause reflex bronchoconstriction in dogs (Pelleg and Hurt, 1996; Katchanov et al., 1998).

In guinea pigs, jugular C-fibres project to extrapulmonary airways (larynx, trachea, bronchus) and lung parenchyma, while nodose C-fibres primarily innervate structures within the lungs. Only the nodose C-fibre population appears to respond with action potential discharge to α,β -methylene ATP, a P2X-selective receptor agonist (Undem et al., 2004). Similarly, ATP has been reported to activate both intrapulmonary SARs and RARs, but not extrapulmonary cough receptors (Canning et al., 2004, 2006; Undem et al., 2004; Kollarik and Undem, 2006). Guinea pig nodose C-fibre neurons respond to α,β -methylene ATP with a sustained discharge that is sensitive to selective P2X3 (P2X2/3) antagonists, and express mRNA for both P2X2 and P2X3 subunits. Jugular and DRG C-fibre neurons either reveal a transient, rapidly

inactivating current or show no detectable current using the same stimulus, and express P2X3 but little or no P2X2 mRNA (Kwong et al., 2008). It was further shown that only the activation of P2X2/3 heteromeric receptors produces sustained currents, whereas homomeric P2X3 receptor activation gives rise to a rapidly inactivating current (Dunn et al., 2001). Recently, a potent and highly selective P2X3-P2X2/3 antagonist (AF-353) was shown to have no effect on induced bronchoconstriction but did block the consequent nodose C-fibre activation without interference with their capsaicin-induced activation, supporting the hypothesis that released ATP acts as a necessary intermediate in bronchoconstriction-induced nodose C-fibre activation (Weigand et al., 2012). The data obtained so far, however, provide little insight into the source of the activating ATP.

Also in mice, ATP was shown to activate lung-related C-fibres more than a decade ago (Kollarik et al., 2003). More recently, the electrophysiological characteristics and activation patterns of lung-related C-fibres were studied in an elegant innervated isolated *ex vivo* mouse trachea/lung preparation. Gene expression analysis of individual neurons characterised by retrograde tracing from the airways, identified many phenotypes of capsaicin-sensitive lung-projecting C-fibre neurons that fit into two major groups coinciding with their embryonic origin, i.e., an ATP-sensitive (nodose-like; epibranchial placode-derived) and an ATP-insensitive (jugular-like; neural crest-derived) population. The nodose subtype is non-peptidergic, expresses P2X2 and P2X3 receptors, and extensive gene analysis data are available (Nassenstein et al., 2010).

Increased levels of reactive oxygen species (ROS) in lungs have been shown to sensitise vagal lung C-fibres in rats, which leads to exaggerated reflex responses and the development of airway hypersensitivity; TRPV1, TRPA1 and P2X receptors all appear to be involved in this process (Ruan et al., 2005, 2014; Lin et al., 2013). Similarly, TRPV1, TRPA1 and P2X receptors, but not nicotinic acetylcholine receptors, appear to participate in the stimulation of

lung vagal CFRs by inhaled cigarette smoke, possibly via the action of ROS (Lin et al., 2010, Weng et al., 2013).

Functional evidence suggests that P2X3 receptor subunits are expressed predominantly and selectively in so-called C- and A δ -fibre primary afferent neurons (Ford, 2012; Ford and Undem, 2013). P2X receptor-mediated afferent activation has been implicated in inflammatory, visceral and neuropathic pain, as well as in airway hyperreactivity (Ford, 2012).

To our knowledge, however, no morphological evidence for the location of P2X2 and/or P2X3-expressing CFR terminals in the lungs and airways in any species has been published to date. In rodents, P2X2/3 receptors and CGRP are not co-expressed in C-fibres in intrapulmonary airways (Brouns et al., 2000, 2009). Taking into account that mouse lungs do not harbour intrapulmonary airways with cartilage reinforcements (bronchi), and that intraepithelial C-fibres are typically found at the bronchial level (own unpublished observations), we were able to locate separate populations of CGRP and P2X3 receptor-expressing intraepithelial C-fibre-like terminals in mouse bronchi, the first images of which are provided in the present publication (**Fig.12**).

Fig.12. Double immunostaining for P2X3 receptors (green; arrowheads) and CGRP (red; open arrowhead) reveals two separate C-fibre populations in mouse bronchial epithelium (E). L: airway lumen.

Concluding comments and reflections

As should be clear from the preceding description, purinergic signalling via P2X2/3 receptors has been characterised in at least four distinct well-defined populations of sensory receptors in lungs and intrapulmonary airways (NEBs, SMARs, VPRs, CFRs), implying that ATP may be an important player in the physiological transduction of a variety of lung-related afferent signals from the periphery to the CNS.

Although an exact match between the various groups of electrophysiologically characterised vagal airway receptors and the morphologically/neurochemically identified receptor end-organs in the lungs and airways is still far from complete, the reported P2X2/3 receptor-mediated purinergic activation and P2X2/3 expression of several of the sensory receptor subtypes may offer additional clues for classification.

Except for VPRs, which likely originate from thoracic DRGs, all P2X2 and/or P2X3 receptor-expressing intrapulmonary nerve terminals identified so far appear to be derived from neuronal cell bodies located in the vagal nodose (epibranchial placode-derived) ganglion.

Both the P2X2/3 receptor-positive population of nerve terminals that invades pulmonary NEBs in the airway epithelium, the fibres that give rise to SMARs in airway smooth muscle bundles, and VPRs in the elastic network of the visceral pleura, originate from myelinated nerve fibres that display similar mechanosensor-like morphological and neurochemical characteristics.

Within the different populations of P2X2/3 receptor-expressing intrapulmonary sensory nerve terminals, NEBs are so far the only receptor end-organs that have revealed a complete physiological purinergic signalling system. The NEB microenvironment includes a local source of ATP in NEB cells (Brouns et al., 2000; De Proost et al., 2009), which has been shown to be released via Ca^{2+} -mediated exocytosis as a response to diverse physiological

stimuli (De Proost et al., 2009; Lembrechts et al., 2012, 2013), as well as a neurochemically well-characterised population of myelinated vagal afferents with intraepithelial P2X2/3 receptor-positive nerve terminals (Brouns et al., 2000, 2003a,b, 2009, 2012; Burnstock et al., 2012; Schnorbusch et al., 2013) that establish real synaptic contacts with NEB cells (Adriaensen and Scheuermann, 1993; Van Lommel & Lauweryns, 1993). This strongly suggests purinergic synaptic neurotransmission via stimulated and strictly regulated exocytosis of ATP. Additionally, considering the stem cell characteristics of Clara-like cells in the NEB microenvironment, local paracrine purinergic signalling via stimulated ATP release from NEB cells, may be of great importance for normal airway function, for airway regeneration after injury and/or for the pathogenesis of small cell lung carcinomas (De Proost et al., 2009).

No data are available on the potential sources of extracellular ATP involved in purinergic signalling pathways in SMARs, VPRs and CFRs, or on the detailed morphology of the microenvironment that would allow interaction with the expressed P2X2/3 receptors. In addition to a number of cell types that may secrete ATP as a transmitter via stimulated exocytosis, many cells and tissues have been reported to release ATP via different other mechanisms, such as (non-selective) membrane channels, as a consequence of physiological mechanical or related stimuli (Burnstock, 2012; Burnstock et al., 2012).

From a morphological point of view, both SMARs and NEBs share features with presumed low-threshold slowly adapting vagal airway mechanoreceptors, i.e. SARs (Yamamoto et al., 1999; Yu et al., 2004; Wang and Yu, 2004; Adriaensen et al., 2006), suggesting that most likely the same structures are concerned. Based on the origin of SAR activity arising from the terminals of myelinated vagal sensory nerve fibres residing in airways and lungs, and on their detailed neurochemical characteristics, it has been suggested that the observed SAR activity in rodents (Zhang et al., 2006) may have been recorded from at least subpopulations of

SMARs and NEBs (Adriaensen et al., 2006). Airway SARs have been reported to mediate the so-called Hering-Breuer reflex (Hering, 1868; for review see Widdicombe, 2006) in many species, including rodents (Zhang et al., 2006). While continuously activated during inspiration, the reflex at a certain lung volume threshold induces an off-switch of SAR activation, resulting in termination of inspiration and initiation of expiration (Zhang et al., 2006).

So far, no functional data or electrophysiological recordings are available for thoracic DRG neurons that send their myelinated projections to the visceral pleura. The location, morphology and neurochemical characteristics of VPRs definitely point to healthy mechanosensing, but also their potential involvement in the generation of pleura pathology-related pain and other sensations that may involve purinergic signalling, such as dyspnoea and urge to cough, is intriguing (Pintelon et al. 2007; Brouns et al., 2012; Burnstock et al., 2012). The effects of ATP on the activity of the VPR population of sensory lung receptors in health and disease are unknown, but their specific expression of P2X2/3 receptors may open interesting perspectives for future pain management.

Gene expression for P2X2 and P2X3 receptors has been extensively described in a considerable proportion of the vagal (mainly nodose) C-fibre neurons that project to the lungs and airways in different species (Undem et al., 2004; Kwong et al., 2008; Nassenstein et al., 2010; Weigand et al., 2012). The CFRs apparently are not only activated but also sensitised by application –at both nerve cell body and peripheral receptive field level – of ATP and ATP analogues that are indicative of involvement of P2X2/3 receptors, resulting in hyperreactivity (Lin et al., 2013; Ruan et al., 2014; Weng et al., 2013; Ford, 2012; Ford and Undem, 2013). At present, however, a major mismatch exists between the numerous publications reporting functional evidence for purinergic signalling via P2X2/3 receptor-expressing pulmonary C-fibre neurons, and the absence of published morphological evidence for the location of P2X2

and/or P2X3-expressing CFR terminals in the lungs and airways in any species. Somewhat surprisingly, none of the many images of rodent lungs published so far appears to show obvious P2X2 and/or P2X3 immunostaining in intrapulmonary CFRs (Brouns et al., 2000, 2003b, 2006b, 2009, 2012; Burnstock et al., 2012). However, we recently succeeded in visualizing the location of bronchial P2X3 receptor-immunoreactive intraepithelial CFR-like nerve terminals in the mouse lung (as illustrated in **Fig. 12** in the present short review).

In patho(physio)logical situations, the extracellular concentrations of ATP are typically considerably higher, partly due to enhanced stimulation and release from physiological sources or less efficient breakdown, but importantly also because cytoplasmic ATP may be massively released in a non-controllable way when cells or tissues are damaged due to e.g. inflammation or physical destruction (Burnstock, 2012; Burnstock et al., 2012), which are phenomena that certainly also play a role in lung pathology (Ford, 2012; Ford and Undem, 2013). Inflammation is a prominent player in many diseases of the airways and lungs, including asthma, COPD, cystic fibrosis, allergy, infection and physical injury, and the enhanced release of ATP from airway epithelia during inflammatory processes is believed to play an important role in the pathophysiology of chronic lung disease (for reviews see Adriaensen and Timmermans, 2004; Burnstock et al., 2012; Ford, 2012; Ford and Undem, 2013). Signalling via ATP and P2X3 receptors seems to be not just another participant in the extracellular ‘soup’ of transmitters but a key common aggravator in the receptive field that promotes sensitisation of PANs (Ford and Undem, 2013). P2X receptor-mediated afferent activation has been implicated in inflammatory, visceral and neuropathic pain, as well as in airway hyperreactivity (Ford, 2012).

Literature data (Canning et al., 2004; Kollarik and Undem, 2006; Brouns et al., 2012) and the present short overview have reported both functional and extensive morphological/neurochemical evidence for the presence of at least four clearly distinct

populations of P2X2/3 receptor-expressing PANs that give rise to a variety of receptor end-organs in the lungs and airways, i.e., CFRs and three myelinated populations with mechanosensor-like properties. On the other hand, several recent publications describe functional evidence for P2X3 receptor subunits that are predominantly and selectively expressed in so-called C- and A δ -fibre PANs, suggesting a high degree of specificity to the pain sensing system in the body and hence a limited likelihood of adverse effects of potential P2X3 antagonists (Ford, 2012; Ford and Udem, 2013).

In conclusion, the accumulating evidence for ATP activation and sensitisation – via P2X3 receptors – of PANs in visceral hollow organs, including lungs and airways, which appears to prime them to chronic discomfort, is definitely interesting, and the great potential benefit of P2X3 antagonists is intriguing. The reviewed substantial variety of lung-related sensory receptors that express P2X2/3 receptors – the majority of which do not belong to accepted nociceptive populations – however argues for a critical and careful interpretation of the functional data.

Acknowledgments

We thank Dr. K. Schnorbusch and Dr. R. Lembrechts for their share in the data and images of the presented work, D. De Rijck for help with imaging and illustrations, and D. Vindevogel for aid with the manuscript. This study was supported by a grant of the Hercules Foundation (AUAH-09-001 to D.A.).

References

- Adriaensen, D., Scheuermann, D.W., 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, J.-P., 2011. Breath-taking complexity of vagal C-fibre nociceptors: implications for inflammatory pulmonary disease, dyspnoea and cough. *J. Physiol.* 589, 3-4.
- 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.
- Branch, W.T.J., McNeil, B.J., 1983. Analysis of the differential diagnosis and assessment of pleuritic chest pain in young adults. *Am. J. Med.* 75, 671-679.
- Brouns, I., Adriaensen, D., Burnstock, G., Timmermans J.-P., 2000. Intraepithelial vagal sensory nerve terminals in rat pulmonary neuroepithelial bodies express P2X₃ receptors. *Am. J. Respir. Cell Mol. Biol.* 23, 52-61.
- Brouns, I., Van Genechten, J., Burnstock, G., Timmermans, J.-P., Adriaensen, D., 2003a. Ontogenesis of P2X₃ 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., 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. Basic Clin.* 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., Oztay, F., Pintelon, I., De Proost, I., Lembrechts, R., Timmermans, J.-P., Adriaensen, D., 2009. Neurochemical pattern of the complex innervation of neuroepithelial bodies in mouse lungs. *Histochem. Cell Biol.* 131, 55-74.

Brouns, I., Pintelon, I., Timmermans, J.-P., Adriaensen, D., 2012. Novel insights in the neurochemistry and function of pulmonary sensory receptors. *Adv. Anat. Embryol. Cell Biol.* 211, 1-115.

Burnstock, G., 2000. P2X receptors in sensory neurons. *Br. J. Anaesth.* 84, 476-488.

Burnstock, G., 2007. Physiology and pathophysiology of purinergic neurotransmission. *Physiol. Rev.* 87, 659-797.

Burnstock, G., 2009. Purinergic mechanosensory transduction and visceral pain. *Mol. Pain* 5, 69.

- Burnstock, G., 2012. Discovery of purinergic signaling, the initial resistance and current explosion of interest. *Br. J. Pharmacol.* 167, 238-255.
- Burnstock, G., Brouns, I., Adriaensen, D., Timmermans, J.-P., 2012. Purinergic signaling in the airways. *Pharmacol. Rev.* 64, 834-868.
- Canning, B.J., Mazzone, S.B., Meeker, S.N., Mori, N., Reynolds, S.M., Undem, B.J., 2004. Identification of the tracheal and laryngeal afferent neurones mediating cough in anaesthetized guinea-pigs. *J. Physiol.* 557, 543-558.
- Canning, B.J., Mori, N., Mazzone, S.B., 2006. Vagal afferent nerves regulating the cough reflex. *Respir. Physiol. Neurobiol.* 152, 223-242.
- Cockayne, D.A., Dunn, P.M., Zhong, Y., Rong, W., Hamilton, S.G., Knight, G.E., Ruan, H.Z., Ma, B., Yip, P., Nunn, P., McMahon, S.B., Burnstock, G., Ford, A.P., 2005. P2X2 knockout mice and P2X2/P2X3 double knockout mice reveal a role for the P2X2 receptor subunit in mediating multiple sensory effects of ATP. *J. Physiol.* 567, 621-639.
- Cutz, E., Jackson, A., 1999. Neuroepithelial bodies as airway oxygen sensors. *Respir. Physiol.* 115, 201-214.
- Cutz, E., Fu, X.W., Yeager, H., Peers, C., Kemp, P.J., 2003. Oxygen sensing in pulmonary neuroepithelial bodies and related tumor cell models. In: Lahiri, S., Semenza, G.L., Prabhakar, N.R. (Eds.), *Lung oxygen sensing*. Marcel Dekker, New York, pp. 567-602.
- Cutz, E., Pan, J., Yeager, H., Domnik, N.J., Fisher, J.T., 2013. Recent advances and controversies on the role of pulmonary neuroepithelial bodies as airway sensors. *Semin. Cell Dev. Biol.* 24, 40-50.

De Proost, I., Pintelon, I., Brouns, I., Timmermans, J.-P., Adriaensen, D., 2007. 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., Pintelon, I., Brouns, I., Kroese, A.B.A., Riccardi, D., Kemp, P.J., Timmermans, J.-P., Adriaensen, D., 2008. Functional live cell imaging of the pulmonary neuroepithelial body microenvironment. *Am. J. Respir. Cell Mol. Bio.* 39, 180-189.

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

Dunn, P.M., Zhong, Y., Burnstock, G., 2001. P2X receptors in peripheral neurons. *Prog. Neurobiol.* 65, 107-134.

Dwinnell, F.L., 1966. Studies on the nerve endings in the visceral pleura. *Am. J. Anat.* 118, 217-226.

Elftman, A.G., 1943. The afferent and parasympathetic innervation of the lungs and trachea of the dog. *Am. J. Anat.* 72, 1-27.

Ford, A.P., 2012. In pursuit of P2X3 antagonists: novel therapeutics for chronic pain and afferent sensitization. *Purinergic Signal.* 8, Suppl 1, 3-26.

Ford, A.P., Udem, B.J., 2013. The therapeutic promise of ATP antagonism at P2X3 receptors in respiratory and urological disorders. *Front. Cell. Neurosci.* 7, 267.

Fu, X.W., Nurse, C.A., Cutz, E., 2004. Expression of functional purinergic receptors in pulmonary neuroepithelial bodies and their role in hypoxia chemotransmission. *Biol. Chem.* 385, 275-284.

Gulati, A., Shah, R., Puttanniah, V., Hung, J.C., Malhotra, V., 2014. A retrospective review and treatment paradigm of interventional therapies for patients suffering from intractable thoracic chest wall pain in the oncologic population. *Pain Med.*, doi: 10.1111/pme.12558 [Epub ahead of print].

Hering, E., 1868. Die Selbststeuerung der Athmung durch den Nervus Vagus. *Sber. Akad. Wiss. Wien* 57, 672-677.

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.

Katchanov, G., Xu, J., Schulman, E.S., Pelleg, A., 1998. ATP causes neurogenic bronchoconstriction in the dog. *Drug Dev. Res.* 45, 342-349.

Kollarik, M., Dinh, Q.T., Fischer, A., Undem, B.J., 2003. Capsaicin-sensitive and -insensitive vagal bronchopulmonary C-fibres in the mouse. *J. Physiol.* 551, 869-879.

Kollarik, M., Undem, B.J., 2006. Sensory transduction in cough-associated nerves. *Respir. Physiol. Neurobiol.* 152, 243-254.

Kollarik, M., Ru, F., Brozmanova, M., 2010. Vagal afferent nerves with the properties of nociceptors. *Auton. Neurosci. Basic Clin.* 153, 12-20.

Kwong, K., Kollarik, M., Nassenstein, C., Ru, F., Undem, B.J., 2008. P2X2 receptors differentiate placodal vs. neural crest C-fiber phenotypes innervating guinea pig lungs and esophagus. *Am. J. Physiol. Lung Cell Mol. Physiol.* 295, L858-L865.

- 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, J.R., 1928. The effect on respiration of stimulating the nerve terminations in the visceral pleura. *Anat. Rec.* 28, 20.
- Larsell, O., Dow, R.S., 1933. The innervation of the human lung. *Am. J. Anat.* 52, 125-146.
- 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.* 136, 371-385.
- Lembrechts, R., Brouns, I., Schnorbusch, K., Pintelon, I., Timmermans, J.-P., Adriaensen, D., 2012. Neuroepithelial bodies as mechanotransducers in the intrapulmonary airway epithelium: involvement of TRPC5. *Am. J. Respir. Cell Mol. Biol.* 47, 315–323.
- Lembrechts, R., Brouns, I., Schnorbusch, K., Pintelon, I., Kemp, P.J., Timmermans, J.-P., Riccardi, D., Adriaensen, D., 2013. Functional expression of the multimodal extracellular calcium-sensing receptor in pulmonary neuroendocrine cells. *J. Cell Sci.* 126, 4490-4501.
- Lin, Y.J., Hsu, H.H., Ruan, T., Kou, Y.R., 2013. Mediator mechanisms involved in TRPV1, TRPA1 and P2X receptor-mediated sensory transduction of pulmonary ROS by vagal lung C-fibers in rats. *Respir. Physiol. Neurobiol.* 189, 1-9.
- Lin, Y.S., Hsu, C.C., Bien, M.Y., Hsu, H.C., Weng, H.T., Kou, Y.R., 2010. Activations of TRPA1 and P2X receptors are important in ROS-mediated stimulation of capsaicin-sensitive lung vagal afferents by cigarette smoke in rats. *J. Appl. Physiol.* 108, 1293-1303.
- Linnoila, R.I., 2006. Functional facets of the pulmonary neuroendocrine system. *Lab. Invest.* 86, 425-444.

- McLaughlin, A.I.G., 1933. Nerves and nerve endings in the visceral pleura of the cat. *J. Physiol.* 80, 101-104.
- Nassenstein, C., Taylor-Clark, T.E., Myers, A.C., Ru, F., Nandigama, R., Bettner, W., Undem, B.J., 2010. Phenotypic distinctions between neural crest and placodal derived vagal C-fibres in mouse lungs. *J. Physiol.* 588, 4769-4783.
- Pan, J., Copland, I., Post, M., Yeger, H., Cutz, E., 2006. Mechanical stretch-induced serotonin release from pulmonary neuroendocrine cells: implications for lung development. *Am. J. Physiol. Lung Cell Mol. Physiol.* 290, L185-L193.
- Pelleg, A., Hurt, C.M., 1996. Mechanism of action of ATP on canine pulmonary vagal C fibre nerve terminals. *J. Physiol.* 490, 265-275.
- 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.
- Ruan, T., Lin, Y.S., Lin, K.S., Kou, Y.R., 2005. Sensory transduction of pulmonary reactive oxygen species by capsaicin-sensitive vagal lung afferent fibres in rats. *J. Physiol.* 565, 563-578.
- Ruan, T., Lin, Y.S., Hsu, T.H., Lu, S.H., Jow, G.M., Kou, Y.R., 2014. Sensitization by pulmonary reactive oxygen species of rat vagal lung C-fibers: the roles of the TRPV1, TRPA1, and P2X receptors. *PLoS One* 9, e91763.
- Scheuermann, D.W., 1987. Morphology and cytochemistry of the endocrine epithelial system in the lung. *Int. Rev. Cytol.* 106, 35-88.

- Schnorbusch, K., Lembrechts, R., Pintelon, I., Timmermans, J.-P., Brouns, I., Adriaensen, D., 2013. GABAergic signaling in the pulmonary neuroepithelial body microenvironment: functional imaging in GAD67-GFP mice. *Histochem. Cell Biol.* 140,549-566.
- Sorokin, S.P., Hoyt, R.F., 1989. Neuroepithelial bodies and solitary small-granule cells. In: Massaro, D. (Ed.), *Lung cell Biology*. Marcel Dekker, New York, pp. 191-344.
- Taylor-Clark, T., Undem, B.J., 2006. Transduction mechanisms in airway sensory nerves. *J. Appl. Physiol.* 101, 950-959.
- Undem BJ, Chuaychoo B, Lee MG, Weinreich D, Myers AC and Kollarik M (2004) Subtypes of vagal afferent C-fibres in guinea-pig lungs. *J Physiol* 556:905-917.
- Van Iommel, A., Lauweryns, J.M., 1993. Neuroepithelial bodies in the Fawn Hooded rat lung: morphological and neuroanatomical evidence for a sensory innervation. *J. Anat.* 183, 553-566.
- Wang, Y.-F., Yu, J., 2004. Structural survey of airway sensory receptors in rabbit using confocal microscopy. *Acta Physiol. Sin.* 56, 119-129.
- Weigand, L.A., Ford, A.P., Undem, B.J., 2012. A role for ATP in bronchoconstriction-induced activation of guinea pig vagal intrapulmonary C-fibers. *J. Physiol.* 590, 4109-4120.
- Weng, W.H., Hsu, C.C., Chiang, L.L., Lin, Y.S., Su, C.L., 2013. Role of TRPV1 and P2X receptors in the activation of lung vagal C-fiber afferents by inhaled cigarette smoke in rats. *Mol. Med. Rep.* 7, 1300-1304.
- 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.G., 2001. Airway receptors. *Respir. Physiol.* 125, 3-15.
- 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.
- Yu, J., 2009. Airway receptors and their reflex function. *Adv. Exp. Med. Biol.* 648, 411-420.
- Yu, J., Zhang, J.W., Wang, Y.F., Fan, F., Yu, A., 2004. Neuroepithelial bodies not connected to pulmonary slowly adapting stretch receptors. *Respir. Physiol. Neurobiol.* 144, 1-14.
- Yu, J., Lin, S., Zhang, J. Otmishi, P., Guardiola, J.J., 2007. Airway nociceptors activated by pro-inflammatory cytokines. *Respir. Physiol. Neurobiol.* 156, 116-119.
- Zhang, J.W., Walker, J.F., Guardiola, J., Yu, J., 2006. Pulmonary sensory and reflex responses in the mouse. *J. Appl. Physiol.* 101, 986-992.