

## **Ontogenesis of P2X<sub>3</sub> receptor-expressing nerve fibres in the rat lung, with special reference to neuroepithelial bodies**

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### **Abstract**

Pulmonary neuroepithelial bodies (NEBs) are extensively innervated organoid groups of neuroendocrine cells present in the epithelium of intrapulmonary airways of all air-breathing vertebrates. In adult rat lungs, NEBs receive, among others, vagal nodose sensory nerve terminals that are immunoreactive for calbindin D28k (CB) and P2X<sub>3</sub> ATP receptors. In the present study, immunoreactivity (IR) for P2X<sub>3</sub> receptors, combined with immunostaining for CB (a marker for NEBs and their vagal sensory innervation), calcitonin gene-related peptide (CGRP; a marker for NEBs and their spinal sensory innervation) or myelin basic protein (MBP; a marker for myelin sheaths), was examined in the developing rat lung (gestational day (GD)15-PD40).

It was found that from GD15 onwards, P2X<sub>3</sub> receptor-IR nerve fibres were also IR for CB, but not for CGRP, providing strong evidence for a vagal sensory origin of the P2X<sub>3</sub>-IR nerve fibre population in the lung, even before NEBs could be demonstrated. From GD19 onwards, P2X<sub>3</sub> receptor-IR vagal sensory nerve fibres were seen to protrude between CB or CGRP-IR NEB cells, revealing many of the features of the nodose afferents in adult rat lungs. From PD10 onwards, the P2X<sub>3</sub>-IR nodose afferents contacting NEBs were revealed to be myelinated. The fact that vagal sensory nerve terminals in rat lungs express P2X<sub>3</sub> receptor IR from the first moment they make contact with pulmonary NEBs, a few days before birth, suggests that NEBs may carry out important functions during late-foetal life, and reflects the importance of fully functional vagal sensors in NEBs at the time of birth, in addition to the proposed chemoreceptor and/or mechanoreceptor functions during later life.

### **Introduction**

Neuroepithelial bodies (NEBs) [1] are normal components of the epithelium of intrapulmonary airways in man and all air-breathing vertebrates investigated so far [2-5]. NEBs consist of extensively innervated clusters of pulmonary neuroendocrine cells (PNECs), that are charged with amine, peptide and purine transmitters, and are derived from undifferentiated precursors in the endodermal pulmonary epithelium [for review see 3]. Since PNECs are the first cells to become recognisably differentiated in the airways, NEBs have for some time been suggested to play a role in organogenesis. Based on incomplete morphological data, NEBs are now considered to carry out different functions throughout prenatal, perinatal, early postnatal, and adult life [for reviews see 4,6]. The oxygen-sensing

properties [7-9], depolarisation due to hypoxic inhibition of O<sub>2</sub>-sensitive K<sup>+</sup> channels [10], and the consequent release of bioactive substances from NEB cells [11] have been described. The possibility that NEBs are also involved in purinergic mechanosensory transduction has also been raised [12].

Neuronal tracing, chemical or mechanical denervation and (immuno)cytochemistry, in combination with confocal microscopy, have recently provided evidence for a very complex innervation of pulmonary NEBs in rats [12-17]. NEBs in rat lungs are selectively contacted by two clearly distinct sensory nerve fibre populations, a vagal and a spinal one, by an intraepithelial nitrgergic nerve fibre population originating from neuronal cell bodies intrinsic to the lungs, and by several other so far not fully characterised populations. The vagal sensory component, has its

origin in the nodose ganglion, ramifies between the NEB cells, can be labelled by calbindin D28k (CB) and P2X<sub>3</sub> purinoreceptor immunoreactivity (IR), and is myelinated in postnatal lungs. The spinal sensory component, on the other hand, is calcitonin gene-related peptide (CGRP)/substance P(SP)-IR, contacts only the basal pole of NEBs, is sensitive to systemic capsaicin treatment, and reveals obvious C-fibre characteristics. Because of the close integration of NEBs and (sensory) nerve fibres, pulmonary NEBs have recently been added to the list of airway receptors [18].

Using growth-associated protein 43 (GAP-43) IR as a marker for developing, growing or remodelling nerve fibres, it has been proven that subepithelial GAP-43-IR nerve fibres in rat airways precede the ontogenetic formation of NEBs by several orders of airway branching [15]. From GD16 onwards GAP-43/CB-IR, most likely vagal sensory nerve endings contact pulmonary NEBs in rat lungs, and are as such the first nerve fibres that were seen to contact pulmonary NEBs selectively [15].

Vagal nodose sensory nerve terminals in 4-5 weeks old rat lungs, were shown to express the ATP-receptor P2X<sub>3</sub> [14], an observation that has led to the suggestion that ATP, secreted by NEB cells, may act as a neurotransmitter/neuromodulator in the vagal sensory transduction of NEBs [12,14]. It was proposed that the myelinated vagal sensory P2X<sub>3</sub> receptor-IR nerve fibres that selectively innervate NEBs are involved in pulmonary mechanosensory transduction [12,17].

The present study aimed at examining the distribution of P2X<sub>3</sub> receptor IR in rat lungs during development, with special reference to NEBs. Myelin basic protein (MBP) IR was used to label myelin sheaths. CB and CGRP IR were used as markers for NEB cells and for the vagal and spinal components of the innervation of rat pulmonary NEBs respectively.

## Material and Methods

### *Animals and tissue processing*

In this study, Wistar rats (Iffa Credo, Brussels, Belgium) were kept in acrylic cages with wood shavings in an acclimatised room (12/12H light/dark cycle; 22 ± 3°C) and were provided with water and rat pellets *ad libitum*. National and international principles of laboratory animal care were followed and the experiments were approved by the ethics committee for animal experiments of the University of Antwerp.

For developmental studies, 4 foetuses from two different mothers were obtained at gestational day (GD) 15, GD17, GD19 and GD21. The mothers (n=8; 2 for each gestational age) were killed by intraperitoneal injection of an overdose of sodium pentobarbital (Nembutal). Chests of the foetuses were opened prior to immersion-fixation of the whole animals in 4% phosphate-buffered paraformaldehyde (PF; 1 h, 4°C). The respiratory tract was removed *in toto* and reimmersed in the same solution (2 h, 4°C).

Rats (from two different litters in each age group) were sacrificed at postnatal day (PD) 0 (day of birth; n=4), PD2 (n=4), PD4 (n=4), PD10 (n=4), PD21 (n=4), PD28 (n=4) and PD40 (n=4). The animals were killed by intraperitoneal injection of an overdose of Nembutal. The chests were opened, followed by intratracheal instillation of 4% PF. The lungs were dissected, immersed in the same fixative, deaerated, and further fixed for 2h (4°C).

All lungs were rinsed in phosphate-buffered saline (PBS; 0.01 M, pH 7.4), stored overnight (ON) in sucrose (20 % in PBS; 4°C) and mounted in Tissue Tek (Sakura Finetek Europe, Zoeterwoude, The Netherlands) on a cryostat chuck by rapid freezing in a CO<sub>2</sub> chamber. Cryostat sections (20 µm-thickness) were thaw-mounted on poly-L-lysine coated microscope slides, air dried and stored at -80°C in a closed container.

### *Immunocytochemistry*

To acquire an enhanced staining of P2X<sub>3</sub> receptors on nerve fibre endings, and to allow for the consecutive application of two antisera raised in the same species, a biotinylated tyramide signal amplification (TSA) kit (PerkinElmer Life Sciences NEL700, Boston, MA, USA) was applied to detect P2X<sub>3</sub> receptors, as previously described [14]. In short, after blocking endogenous peroxidase activity, sections were rinsed in PBS, preincubated with the same solution as used for dilution of the primary antibodies, and incubated ON with a rabbit polyclonal antibody against P2X<sub>3</sub> receptors (gift from Roche Bioscience; diluted 0.625 µg/ml), followed by incubation with a biotinylated donkey anti-rabbit antibody (Jackson; diluted 1:500), and Extr-Avidin-horseradish peroxidase (Sigma; diluted 1:1500). Between subsequent steps the sections were washed in 0.05% Tween 20 in PBS. Sections were then incubated with biotinylated tyramide in 'amplification solution' (diluted 1:100; 8 min). Following TSA, visualisation was performed using FITC-conjugated streptavidin (Amersham; diluted 1:200).

For the simultaneous demonstration of P2X<sub>3</sub> receptor-IR nerve fibres, pulmonary NEBs, and their spinal or vagal sensory innervation, lung sections that were processed for P2X<sub>3</sub> receptor staining were additionally subjected to an indirect immunocytochemical staining using rabbit polyclonal antibodies against CGRP (Affiniti; diluted 1:200) or CB (SWant; diluted 1:2500), and a Cy3-conjugated goat anti-rabbit serum (GAR-Cy3; Jackson; 1:200). For double immunocytochemical staining of P2X<sub>3</sub> receptors and myelin sheaths, lung sections processed for P2X<sub>3</sub> receptor staining were subsequently incubated with a rabbit polyclonal antibody against MBP (DAKO; 1:1) and GAR-Cy3.

Positive and negative staining controls for all immunocytochemical procedures were performed as previously described [14].

All sections were mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA).

An epifluorescence microscope (Zeiss Axiophot) equipped with filters for the visualisation of FITC and

Cy3 was used to evaluate the results. To obtain detailed images of areas of interest, confocal laser scanning microscopes (Zeiss LSM 510 and PerkinElmer UltraVIEW LCI) and the attached image reconstruction facilities, respectively Imaris 2.7 software (Bitplane AG, Zurich, Switzerland) and Volocity (Improvision, Coventry, UK) were used.

## Results

From GD15 on, very strong P2X<sub>3</sub> receptor-IR nerve fibres, always co-localising with CB IR, were seen in cross-sections of the vagal nerves (Fig. 1). As early as GD15, P2X<sub>3</sub> receptor-IR nerve fibres could also be distinguished accompanying the pulmonary epithelial tubes, even in the most distal airway branches (Fig. 1). From GD19 on, abundant P2X<sub>3</sub> receptor-IR nerve fibres could be seen to approach the epithelial lining, ramify and protrude between the epithelial cells, giving rise to terminal arborisations (Figs. 2-8). These intraepithelial complexes appeared to be preferentially located at airway branching points (Figs. 3, 5). From PD2 onwards, P2X<sub>3</sub>-positive nerve fibres in nerve bundles in the lamina propria of intrapulmonary airways,

showed weaker P2X<sub>3</sub> receptor IR, while staining in nerve terminals was unchanged.

Only from PD2 onwards, MBP IR is observed intrapulmonary in myelin sheaths of solitary nerve fibres and of nerve fibres located in large bundles. From PD10 onwards, also some of the P2X<sub>3</sub> receptor-IR nerve fibres, that give rise to extensive intraepithelial terminals, appear to reveal MBP-IR myelin sheaths. Myelinated P2X<sub>3</sub> receptor-IR vagal sensory nerve fibres can be observed to lose their myelin sheaths in the immediate neighbourhood of the epithelium, after which they branch and give rise to nerve endings penetrating between epithelial cells (Fig. 6). From PD10 until postnatal week 5, the number of intrapulmonary P2X<sub>3</sub> receptor-IR nerve fibres that becomes myelinated increases.

On GD19, subsequent labelling of lung sections processed for P2X<sub>3</sub> receptor localisation, with antibodies against CB or CGRP (as markers for PNECs) showed that the P2X<sub>3</sub> receptor-IR intraepithelial nerve terminals in all cases co-occurred with the presence of an NEB (Figs. 7, 8). Single optical sections obtained by confocal microscopy revealed that the P2X<sub>3</sub>-IR nerve fibres protrude between and surround the NEB cells (Fig. 7d). From PD10 onwards, vagal sensory nerve fibres showed only a very weak or no P2X<sub>3</sub> receptor IR before entering the NEBs.

### **Figs. 1-8 Confocal microscopic images of P2X<sub>3</sub> receptor immunoreactivity (IR; FITC-fluorescence) and comparison with other markers during ontogenetic development of rat lungs**

**Fig. 1:** Gestational day (GD) 15. Low magnification overview of a cross-section of the oesophagus (O) and of a peripheral area in the lung. Green fluorescent P2X<sub>3</sub> receptor IR is present in nerve fibres of a cross-section of the vagal nerve (NX) and in nerve fibres (arrows) accompanying even the most distal branches of the primitive airway tubes. Single optical section. The red channel shows autofluorescent red blood cells.

**Figs. 2-5** P2X<sub>3</sub> receptor-stained (FITC-fluorescence) nerve fibres (arrows) approach the epithelium, protrude between the epithelial cells and form intraepithelial terminals (arrowheads) at different levels of rat intrapulmonary airways

**Fig. 2:** GD19; proximal airway. High magnification detail. Maximum intensity projection of 18 optical sections (1- $\mu$ m interval).

**Fig. 3:** GD21. Low-magnification overview of a branching point of proximal intrapulmonary airways. Single optical section.

**Fig. 4:** Postnatal day (PD) 0 (day of birth); peripheral lung area. In this high-magnification detail abundant P2X<sub>3</sub> receptor-IR nerve terminals are seen close to the luminal surface. (L) Lumen of the bronchus. Maximum intensity projection of 11 optical sections (1- $\mu$ m interval).

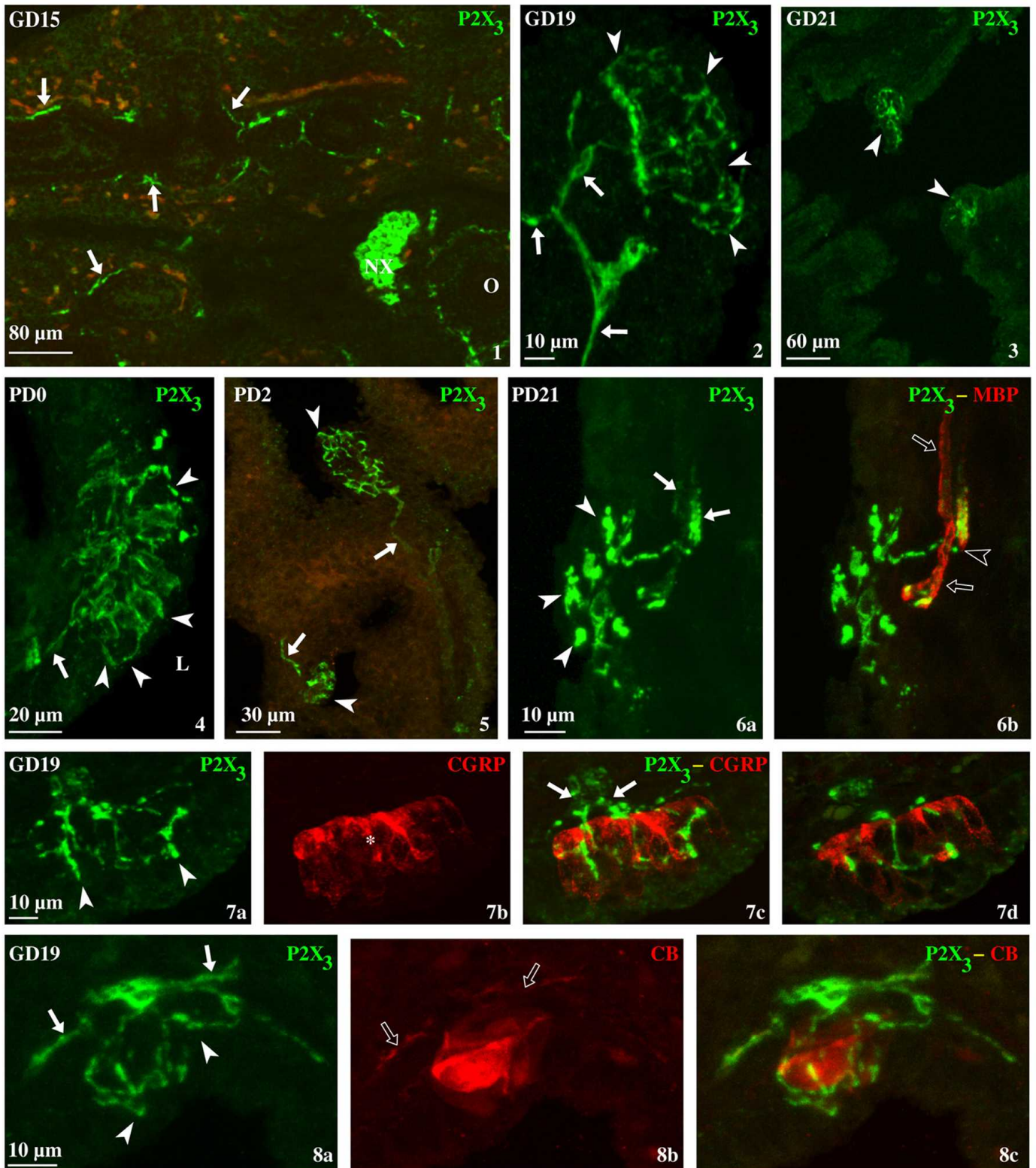
**Fig. 5:** PD2. Overview of two intraepithelial arborisations in the periphery of the lungs. The red channel shows non-specific background fluorescence as a reference for the lung tissue. Maximum intensity projection of 10 optical sections (2- $\mu$ m interval).

**Fig. 6a,b:** Confocal microscopic images of immunocytochemical double staining for P2X<sub>3</sub> receptors (green FITC fluorescence) and myelin basic protein (MBP; red Cy3 fluorescence) in a bronchus of a 21-day-old rat. Maximum intensity projections of 30 optical sections (0.8- $\mu$ m interval). **a.** FITC-labelled nerve fibres expressing P2X<sub>3</sub> receptors (arrows) approach the epithelium, branch, protrude between the epithelial cells and give rise to intraepithelial nerve terminals (arrowheads), many of which are seen close to the luminal surface. **b.** Combination of the red and green channels clearly demonstrates that MBP IR can be detected in myelin sheaths surrounding the P2X<sub>3</sub> receptor-IR vagal afferent nerve fibres (open arrows). MBP-IR myelin sheaths are lost in close proximity to the target (open arrowheads).

**Figs. 7, 8:** Co-labelling of pulmonary NEBs and P2X<sub>3</sub> receptor-IR nerve terminals in prenatal rat lungs

**Figs. 7a-d** GD19. Immunocytochemical double-staining for P2X<sub>3</sub> receptors (green FITC fluorescence) and CGRP (red Cy3 fluorescence). High magnification details of an NEB in a proximal tube of rat lung contacted by nerve fibres expressing P2X<sub>3</sub> receptors. **a-c.** Maximum intensity projections of 16 confocal optical sections (1- $\mu$ m interval). **a.** Green channel showing an intraepithelial complex of P2X<sub>3</sub> receptor-IR nerve terminals (arrowheads). **b.** Red channel showing a CGRP-IR NEB (asterisk) at the same location. **c.** Combination of both channels clearly demonstrating that the CGRP-IR NEB is contacted by extensively branching P2X<sub>3</sub>-IR nerve fibres. Note the absence of CGRP IR from the P2X<sub>3</sub>-positive fibres (arrows). **d.** Single confocal optical section revealing that P2X<sub>3</sub> receptor-IR nerve terminals protrude between the CGRP-positive neuroendocrine cells of the NEB.

**Fig. 8 a-c** GD19. Details of a CB-IR (red Cy-3 fluorescence) NEB contacted by CB- and P2X<sub>3</sub> receptor (green FITC-fluorescence)-IR nerve terminals. Maximum intensity projections of 12 confocal optical sections (1.5- $\mu$ m interval). **a.** Green channel showing an intraepithelial arborisation (arrowheads) of P2X<sub>3</sub> receptor-expressing nerve fibres (arrows). **b.** Red channel showing rather weakly-stained CB-IR nerve fibres (open arrows) in contact with a CB-IR NEB. **c.** Combination of both channels revealing that the nerve fibres in contact with the NEB express both P2X<sub>3</sub> receptors and CB, though the staining intensity varies.



From GD19 on, strongly CB-IR NEBs at all levels of the airways received thick P2X<sub>3</sub>-IR nerve endings, which showed a weak CB IR in more distal airways (Fig 8), but a strong CB IR in more proximal airways. All P2X<sub>3</sub>-IR intrapulmonary nerve fibres, however, co-localised CB, and vice versa.

Double staining for P2X<sub>3</sub> receptors and CGRP at GD 19 showed intensely-stained CGRP-IR NEBs, contacted by P2X<sub>3</sub>-IR nerve fibres (Fig. 7) at all levels of the intrapulmonary airways. P2X<sub>3</sub> receptor-IR nerve terminals did not show CGRP IR (Fig. 7). From GD 21 on, CGRP-IR NEBs, directly innervated by CGRP-IR nerve fibres could be discerned.

## Discussion

Purinergic P2X<sub>3</sub> receptors are expressed on terminal arborisations of vagal nodose afferent nerve fibres in contact with NEBs in the lungs of young and adult rats [14] and in the present study they have been shown to appear early during lung ontogenesis.

It is now generally agreed that pulmonary NEBs in rats receive at least two clearly distinct sensory nerve fibre populations, with different morphological, and therefore most likely functional, characteristics [12-14,17]. The vagal sensory component has its origin in the nodose ganglion, protrudes between the NEB cells, is marked by CB and P2X<sub>3</sub> receptor IR, does not express capsaicin receptors, and is apparently insensitive to systemic capsaicin treatment. Additionally, the vagal nodose sensory nerve fibre population innervating NEBs was recently shown to be myelinated in postnatal lungs [12]. The spinal sensory component of the innervation, on the other hand, is CGRP/SP-IR, contacts the basal pole of NEBs, expresses capsaicin receptors and was additionally proven to be sensitive to systemic capsaicin treatment.

CB IR has been proven to be a useful marker for rat pulmonary NEBs and for their vagal sensory innervation from GD16 onward [15]. Immunocytochemical double staining in the present study revealed that P2X<sub>3</sub> receptor and CB IR were co-localised in sections of the vagal nerve as early as on GD15. From GD19 onward, P2X<sub>3</sub> receptor-IR nerve fibres are seen to ramify between the neuroendocrine cells of NEBs, showing morphological characteristics of the vagal nodose nerve terminals described during postnatal life [13,14]. Since all P2X<sub>3</sub> receptor-IR nerve terminals contacting NEBs appeared to co-express CB, it may be concluded that the intraepithelial P2X<sub>3</sub>-IR nerve terminals selectively contacting NEBs, represent the vagal sensory component of the innervation of NEBs in rat lungs.

CGRP-IR NEBs can be detected in rat lungs from GD16 onwards, but are at that time restricted to the proximal 3 to 4 branching levels of the airways (out of 7-9 at that stage)[15]. From GD17 onward, scarce thin and varicose CGRP-IR nerve fibres could be seen following the bronchial tree, apparently unrelated to NEBs [15]. On GD19, NEBs in the most proximal airways can be observed to be contacted by CGRP-IR nerve fibres [3,15,19]. In the present study, the P2X<sub>3</sub> receptor-IR nerve fibre

population providing terminals between the CGRP-IR neuroendocrine cells of NEBs, was clearly CGRP negative from GD19 onwards. The P2X<sub>3</sub> receptor-IR nerve fibre population contacting pulmonary NEBs in rats, therefore, clearly forms a different nerve fibre population than the CGRP-IR spinal sensory one.

In 4-5 weeks old and adult rats, it has been observed that vagal sensory nerve fibres approaching NEBs do not always reveal a clear P2X<sub>3</sub> receptor expression [14]. In the present study, P2X<sub>3</sub> IR was weaker in intrapulmonary solitary nerve fibres and nerve bundles in the lamina propria, from PD2 onwards. From PD10 onwards, nerve fibres were not always clearly recognisable before entering the NEB. Intraepithelially, on the other hand, P2X<sub>3</sub> receptor IR was very strong and did not change during development. The myelination of vagal nodose sensory nerve fibres reaches the lungs in rats from PD2 onwards. Vagal nodose sensory fibres are seen to lose their myelin sheaths in the immediate neighbourhood of the target NEBs, then branch and give rise to the terminals that penetrate between NEB cells. It is likely that the presence of a myelin sheath hampers the detection of P2X<sub>3</sub> receptor IR along the nerve fibres, but not in the nerve terminals in contact with NEB cells in postnatal rat lungs.

High levels of GAP-43 are reported in growth cones of nerve processes during periods of embryonic [20] and postnatal [21] development. On GD16, GAP-43/CB-IR vagal nerve fibres have been described in rats to run along the pulmonary epithelial tubes, thereby preceding the formation of NEBs by several orders of airway branching [15]. The possibility of sensory nerve fibres being involved in the developmental formation of pulmonary NEBs [15] can not be rejected based on the suggestion that NEBs differentiate long before the sensory fibres reach the lungs [3]. Pulmonary NEBs in rats appear to be contacted by extensive vagal sensory GAP-43/CB-IR nerve fibres long before birth [15], the terminals of which were in the present study shown to express P2X<sub>3</sub> receptors as early as they are detectable with GAP-43 or CB IR.

It has been proposed that P2X receptors may have important functions in neuronal development and synaptogenesis [22,23], which is consistent with their ontogenetic expression on extensive populations of nerve fibres. When these highly-specialised functions are achieved, the receptors are down-regulated. The expression of P2X<sub>3</sub> receptors on the vagal sensory nerve terminals contacting pulmonary NEBs from GD17 until adulthood suggests that the expression of P2X<sub>3</sub> receptors is not just related to ontogenetic development, but points to an (additional) adult function.

The precise function of NEBs, widely distributed throughout the lung parenchyma and consisting of PNECs loaded with bioactive substances, is still a matter of debate. It has been proposed that pulmonary NEBs, acting as hypoxia-sensitive airway "chemoreceptors", may be important players in neonatal adaptation, particularly in the first breath or in the initiation of normal breathing after birth [24]. Since NEBs are richly supplied with vagal sensory nerve terminals which express most likely functional P2X<sub>3</sub> receptors already long before birth, and since NEBs are

strategically placed to perceive the first breath, NEBs could conceivably play a role in the resetting of the conventional (arterial) chemoreceptors [24]. Moreover, it has been established that the carotid body chemoreceptors are relatively immature at birth, and undergo a significant increase in their responsiveness to hypoxia during the first weeks of postnatal life, apparently mainly due to a higher level of afferent nerve activity [for review see 25]. In rats, the peak nerve activity to a strong hypoxic stimulus [26] and the number of nerve terminals per parent axon contacting carotid bodies [27] increase both about fourfold between 1 and 30 days of age, with most of the maturation occurring in the first week. The observation that at birth the innervation of pulmonary NEBs in rabbits is more mature than that of carotid body glomus cells, has led to the suggestion that NEBs may act as complementary chemoreceptors to the carotid body during the early postnatal period [28].

In conclusion, intraepithelial vagal nodose sensory nerve endings appear to be the first to selectively contact pulmonary NEBs in rats, expressing P2X<sub>3</sub> receptors from GD16 onwards. P2X<sub>3</sub> receptor IR is therefore a good marker for the vagal nodose component of the innervation of rat pulmonary NEBs throughout life. The appearance as well-differentiated clusters of PNECs, the high density of NEBs in foetal lungs, and their early connection to the central nervous system, among others, via vagal sensory nerve fibres long before birth, suggests an important role for NEBs during intrauterine life and/or in neonatal adaptation, next to the ability to accommodate local and central reflex actions in relation to both chemo- and mechanosensory stimuli, in postnatal lungs.

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