

Pulmonary Neuroendocrine Cell System in Health and Disease

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Abstract: Pulmonary neuroendocrine cell (PNEC) system consists of solitary cells and innervated clusters, neuroepithelial bodies (NEB), widely distributed throughout the airway mucosa of mammalian lungs. These cells are numerous in fetal/neonatal lungs and produce amine (serotonin, 5-HT) as well as a variety of neuropeptides (e.g. bombesin). The potential role and significance of these highly specialized lung cells in normal and diseased lung is only now beginning to be appreciated. The multifaceted role(s) of PNEC system include lung development, neonatal adaptation and during postnatal airway homeostasis as guardians of stem cell niche. Recent advances in cellular and molecular biology of PNEC system particularly their ontogeny and mechanisms of neuroendocrine differentiation in developing lung are reviewed.

The evidence for the role of NEB as hypoxia-sensitive airway chemoreceptors is presented including identification and characterization of O₂ sensor molecular complex that is activated by hypoxia leading to release of amine and peptide mediators acting locally or *via* NEB neural connections. Thus NEB are postulated to function as O₂ sensitive airway sensors involved in respiratory control, especially during adaptation to extrauterine life. Hyperplasia of PNEC/NEB cells, suggesting altered function, has been identified in a number of perinatal/pediatric lung disorders including bronchopulmonary dysplasia, central hypoventilation syndrome, Sudden Infant Death Syndrome and Neuroendocrine Hyperplasia of Infancy as well as Cystic Fibrosis and pediatric asthma. In the adults, PNEC/NEB may be involved in the pathogenesis of tobacco induced airway disease, pulmonary fibrosis and lung carcinogenesis.

Keywords: Lung development, neurogenic genes, oxygen sensing, airway chemoreceptors, pediatric and adult lung disease, lung carcinogenesis.

PULMONARY NEUROENDOCRINE CELL SYSTEM: MORPHOLOGY, MOLECULAR MARKERS, INNERVATION

The first description of Pulmonary Neuroendocrine cells (PNEC) dates back over 60 years when single and small clusters of cells with argyrophilic cytoplasm (stainable with silver impregnation method) were described within the airway epithelium of human and animal lungs [1, 2]. Initially these cells were considered to be a part of a diffuse endocrine system of cells distributed within both endocrine and non-endocrine tissues but their precise role was unknown. With the discovery of amine (serotonin, 5-HT) and peptide production by these cells and the expression of a whole range of neural and endocrine features, the term Pulmonary Neuroendocrine cells, emphasizing their mixed neural and endocrine cell phenotype was adapted [3]. Various aspects of PNEC system including recent advances in cellular and molecular biology of these cells have been subject of several recent reviews [4-7].

At light microscopy level single PNEC appear as flask – shaped cells with apical cytoplasm reaching the airway lumen (open-type), or as elongated cells, often without an apparent luminal contact, but instead forming lateral “dendritic-like” cytoplasmic processes extending along the airway basement membrane (closed-type). It is postulated that

in the fetal lung “open-type” PNEC receive stimuli (i.e. hypoxia) from the airway lumen, whereas in a closed-type PNEC the stimulus transduction leading to 5-HT release is mediated by mechanosensitive channels that are activated by mechanical stretch generated by lung fluid secretion and/or fetal breathing movements known to be important in lung development (see next section) [8].

While the solitary PNEC are distributed throughout the epithelium of trachea and bronchi up to the terminal bronchioles, Neuroepithelial bodies (NEB) form corpuscular structures or cell aggregates composed of 5-15 PNEC that are localized in the epithelium of intrapulmonary airways and appear concentrated at airway branch points [9]. Using immunocytochemistry, both single PNEC and NEB have been shown to express 5-HT as well as several neuropeptides (i.e. bombesin, calcitonin gene related peptide, CGRP) and a variety of neural and neuroendocrine molecular markers (Table 1). The availability of specific immunohistochemical markers for the identification of PNEC/NEB has facilitated studies on their distribution and frequency in the developing lung and postnatally. These studies revealed relative high numbers of PNEC/NEB in fetal/neonatal lungs and a decline in the adult lung [9]. These changes were interpreted as a dilutional effect of post-natal lung growth with apparent minimal variation from childhood to advanced age [10]. Other recent studies using whole mounts of airway epithelium examined by confocal microscopy and 3-D reconstruction reported high local concentration of solitary PNEC with non-homogeneous distribution in airways of adult lungs [11].

At the ultrastructural level, both single PNEC and NEB cells are characterized by the presence of numerous cyto-

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Table 1. Bioactive Secretory Products, Immunohistochemical and Molecular Markers of PNEC/NEB Cells in Mammalian Lungs*

Amines/Autocids: Serotonin (5-hydroxytryptophan,5-HT) ?Acetylcholine (Ach) Adenosine triphosphate (ATP) Amine metabolizing enzymes: Tryptophane hydroxylase (TH) Aromatic amino acid decarboxylase Vesicular acetylcholine transporter (VChat) Acetylcholine esterase	Neuroendocrine/Neurosecretion Markers: Neuron specific enolase (NSE) Protein gene product 9.5 (PGP9.5) Chromogranin A Neural adhesion molecules (NCAM/M) Leu 7/NHK Go alpha Synaptophysin Synaptic vesicle protein2 (SV2) Calbindin D28K
Regulatory Peptides: Bombesin/Gastrin releasing peptide (GRP) Calcitonin Calcitonin gene related peptide (CGRP) Substance P Cholecystokinin (CCK) Somatostatin Endothelin Peptide YY Helodermin Pituitary adenylyl cyclase activating protein	Functional Proteins: NADPH oxidase (gp 91phox/NOX2,p22phox,p47phox,p67phox,rac2) Transcription Factors: Mash 1(rodent); hASH1(human)

*Subject to species variation.

plasmic dense core vesicles, the storage site for amine and peptides. The apical membrane facing the airway lumen forms short microvilli. The cytoplasmic organelles include small mitochondria, rough endoplasmic reticulum, ribosomes, microtubules and bundles of microfilaments. A well developed Golgi complex shows a variety of associated vesicles related to the formation of dense core cytoplasmic granules. Electron microscopic studies on NEB innervation demonstrated two morphological types of nerve endings in contact with the granulated cells: afferent-like sensory nerve endings containing numerous small mitochondria and efferent-like nerve fibers with small agranular vesicles, microtubules and sparse mitochondria [12]. On serial section studies these two types of nerve fibers were found in continuity, an arrangement consistent with an axon reflex [13].

The nature and the origin of intraepithelial nerve endings contacting NEB cells have been studied thus far only in the lungs of rabbit and rat. Although there is species variation, the predominant neural connections innervating NEB are sensory vagal afferents with cell bodies residing in the nodose ganglion [14]. In the rat, two additional components have been demonstrated, CGRP immunoreactive nerves derived from the spinal ganglia and a nitrergic component originating from peribronchial ganglia [15]. The complex innervation together with the expression of several ionotropic neurotransmitter receptors likely reflect the multifunctional role of NEB in the lung [16].

THE ROLE OF PNEC DURING LUNG DEVELOPMENT AND MOLECULAR MECHANISMS OF NEUROENDOCRINE DIFFERENTIATION

Mammalian respiratory system develops as an out pouching of the foregut endoderm, forming a tube-like structure that grows progressively into adjacent splanchnopleuric mesenchyme. This lung primordium, or lung bud, undergoes an extensive series of tubular extensions and branching surrounded by an interactive mesenchyme. In the human, by 28 days after fertilization, the left and right bronchial buds (corresponding to left and right lungs) are established and by the

fifth week three bronchial stems on the right and two on the left determine the ultimate airway structure of the lung. The lung development is divided into five developmental stages, that in human lung correspond to: 1) embryonic period (3-7 weeks) to form the bronchopulmonary segments; 2) pseudoglandular period (7-16 weeks) to form up to 25 orders of branching up to the level of terminal bronchioles; 3) canalicular period (16-24 weeks) with rapidly advancing angiogenesis and differentiation of the mesenchyme surrounding the tubules lined by cuboidal epithelium and formation of several orders of respiratory bronchioles; 4) terminal sac period (24-36 weeks) with initiation of primitive alveolar ducts and then gradual maturation of a functional blood/gas barrier and extensive vascularization of these structures; and 5) alveolar period (36 weeks to term/adult) with full maturation of the alveoli and proportional reduction of lung parenchyma as lungs switch to air breathing. Alveoleogenesis continues into the third year postnatally concomitant with lung expansion. The following key observations led to the suggestion that PNEC may play a critical role in lung morphogenesis and/or affect cell growth and differentiation of other lung cells:

- 1) *PNEC are the first cell type to differentiate* within primitive airway epithelium [17]. In human fetal lung, the first PNEC (pre-NE cells), identified by the presence of sparse cytoplasmic DCV and positive immunoreactivity for 5-HT as well as neuron specific enolase (NSE), appear in proximal airways at about 8 weeks of gestation [18]. PNEC are distributed widely throughout the airways and their maturation and differentiation follows a centrifugal pattern similar to other airway cells that starts in the trachea and proximal airways and progresses distally [19]. Early on, PNEC appear as single cells and at about 12 weeks of gestation (in human), first NEB, often localized at airway bifurcations are observed at about 14 weeks [17, 18]. The development of the innervation of PNEC/NEB was recently investigated in rabbit lung [20]. The first contacts between intramucosal nerve fibers and PNEC/NEB cells were observed at E18, at

a time when PNEC become detectable in this species. However, some PNEC in these early fetal lungs lacked innervation, suggesting that PNEC differentiation may occur independently of the innervation. This finding is in agreement with an earlier observation showing differentiation of PNEC in explants of lung buds devoid of mesenchyma and neural elements [21]. The selective and close association between intramucosal nerve endings and PNEC/NEB cells also suggests that these cells *via* secretion of neurotrophins may guide their own innervation. The density and complexity of NEB innervation appeared completed by the time of birth, coinciding with their postulated function as airway sensors involved in neonatal respiratory adaptation [22]. This advanced maturation of NEB innervation contrasted with that of the carotid bodies, the principal arterial chemoreceptors, whose function at birth is relatively immature.

- 2) PNEC produce a variety of biologically active substances with growth factor and/or mitogen like *properties* (i.e. bombesin, CGRP, 5-HT) that affect lung morphogenesis *via* paracrine mechanisms [6]. The evidence in support for the PNEC role in branching morphogenesis as well as cell growth and differentiation in developing lung is derived from a number of experimental studies. Exogenously administered bombesin/GRP was shown to act as a potent growth and differentiation factor for fetal murine lung *in utero* and for human and rodent lung in organ culture [23]. Bombesin-like peptides (BLP) *in vitro* and *in vivo* promoted proliferation of both mesenchymal and epithelial cells in conducting airways and in primitive alveoli, increased branching morphogenesis, and induced differentiation of alveolar type II cells [24]. Furthermore, exposure to blocking monoclonal anti-bombesin antibody significantly reduced lung maturation in serum-free organ cultures [25]. These effects of bombesin/GRP-like peptides on lung tissues are mediated *via* expression of specific GRP-preferring receptors widely distributed on both epithelial and mesenchymal cells [26-28]. The mechanism of action that involves local, paracrine secretion of various amine and neuropeptide molecules with mitogenic/growth factor-like properties is supported by a study of fetal hamster lungs subjected to continuous 3H -thymidine or BrdU labeling [29]. In this experiment, epithelial cells immediately adjacent to NEB were consistently labeled, indicating active cell proliferation, whereas the amount of labeling decreased progressively with increasing distance from NEBs. The authors concluded that NEB's regulate local cell proliferation in developing airway that is activated in a proximal-to-distal wave.
- 3) PNEC derived bioactive *molecules (amine, peptides) are releasable by hypoxia and/or mechanical strain*, both recognized intrauterine "environmental" factors linked to lung morphogenesis [6, 30]. Initially, exposure to environmental hypoxia was identified as a potent stimulus that triggers 5-HT release from NEB cells both *in vivo* and *in vitro* [31, 32]. This hypoxia induced 5-HT release occurs at varying levels of hypoxia ranging from severe (pO₂ <20mm Hg) to mild

hypoxia (pO₂~90mm Hg) that is expected in the airway under physiological conditions [33]. The hypoxia induced release of both amine and neuropeptides is mediated by regulated secretory process involving Ca²⁺ dependent exocytosis of cytoplasmic DCV, the storage site for these biologically active substances. Since the intrauterine lung development occurs in a relatively hypoxic environment (fetal euoxia, pO₂ ~20-30 mmHg), hypoxia mediated release of bioactive substances from PNEC could modulate lung organogenesis [6]. Because physical forces (i.e. expansion by fetal lung fluid secretion; fetal breathing movements) are essential for lung growth and development, we have recently investigated the effects of mechanical strain on 5-HT release using cultures of PNEC isolated from early (E20) and late (E26) gestation rabbit fetal lungs [8]. Using PNEC cultures exposed to sinusoidal cyclic stretch(simulating intrauterine environment) we showed significant release of 5-HT that was inhibitable with gadolinium chloride, a specific blocker of mechanosensitive channels but was not affected by Ca²⁺ - free medium or nifedipine, specific blocker of voltage activated Ca²⁺ channels. These findings suggest that mechanical stretch-induced 5-HT release from PNEC is mediated by mechanosensitive channels and that it is independent of the exocytic pathway, the predominant mechanism for hypoxia stimulated 5-HT release. The stretch induced 5-HT release affects mostly the cytoplasmic pool of 5-HT that predominates during the early stages of lung development, when PNEC contain only few DCV [17, 18]. Since 5-HT is a recognized pleiotropic biogenic amine affecting cell growth [34], as well as modulation of amiloride -sensitive Na⁺ based reabsorptive system [35], it may play an important role in both lung morphogenesis and fluid resorption at the time of birth. Potential interactions between hypoxia and physical forces acting on PNEC may also be involved and need to be further investigated.

THE ONTOGENY OF PNEC AND MOLECULAR REGULATION OF THEIR DIFFERENTIATION

Although the precise origin of PNEC is at present unknown, current evidence suggests that they are derived from the foregut endoderm that give rise to pluripotent epithelial progenitors, similarly to analogous endocrine cells in the GI tract and pancreas [36]. To identify the putative progenitors of PNEC we have used an antibody against an early neuronal developmental marker, FORSE-1, that recognizes the fore-brain-surface-embryonic antigen expressed on progenitor cells in the developing CNS [37]. In early rabbit fetal lungs (E16), FORSE-1 antibody labeled all cells of the primitive airway epithelium and later became restricted to PNEC/NEBs that were 5-HT immunopositive. During later stages of lung development there was a gradual loss of FORSE-1 epitope with further PNEC differentiation. The identification of a few FORSE-1/5-HT expressing cells in postnatal lungs is consistent with the retention of progenitors in mature lungs. Colocalization of FORSE-1 with stage-specific embryonic antigen (SSEA), related to the Lewis-X antigen, in PNEC supports their embryonic phenotype of neuroendocrine programmed cells. The finding of FORSE-1/SSEA

epitope expression in primitive undifferentiated lung epithelium prior to PNEC differentiation supports the idea of pluripotent lung epithelial phenotype. Furthermore it appears that the PNEC lineage within distal lung arises separately from the other lung epithelial cells and that PNEC do not convert to other cell types [38]. In mouse studies the entire primitive lung epithelium at E13-15 (pseudoglandular period) shows expression of multiple epithelial cell markers including CGRP, CC10 and SPA suggesting a neuroendocrine/Clara-like progenitor state. In human fetal lungs the neuroendocrine marker PGP 9.5 (a ubiquitin C-terminal hydrolase) is universally expressed in primitive airway epithelium during the pseudoglandular phase and becomes restricted to PNEC and lung neural components during later stages of development [39]. At a molecular level it is now evident that neurogenic genes belonging to the family of basic loop-helix (bHLH) transcription factors such as *Achaete-scute-homolog-1* (*Mash1* in rodents and *hASH1* in humans) and *Hes-1* (hairy-enhancer of split) play related but opposite roles in governing PNEC lineage fate in the lung [7, 40] (Fig. 1). The activation of *Mash1* in mice and its human counterpart (*hASH1*) are essential for PNEC development. The disruption of *Mash1* gene in mice (*Mash1*^{-/-}) resulted in failure of PNEC differentiation in the lung [41, 42]. Interestingly, the lung morphogenesis did not appear to be affected by the loss of *Mash1* expression, although these mice die soon after birth due to respiratory failure. On the other hand, enhancement of PNEC differentiation was observed in *Hes1* knockout mice (*Hes1*^{-/-}), that showed precocious appearance of PNEC at E13 and a relative PNEC hyperplasia at E18 [42]. These studies indicate that *Hes1* acts as a neurogenic gene repressor and that *Mash1* and *Hes1* act in concert with Notch/Notch ligand signaling pathways that determine PNEC cell fate. The regulatory mechanisms involved in the differentiation and proliferation of PNEC are complex and include interactions between a number of intrinsic and extrinsic factors, depending whether they occur within immature epithelium of developing lung or post nately as part of epithelial renewal. The various aspects of PNEC differentiation and proliferation in normal developing lung and under experimental as well as in various pathological conditions have been reviewed in detail elsewhere [43].

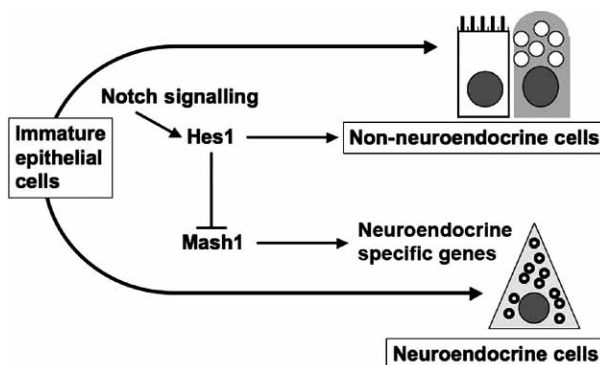


Fig. (1). Cell fate determination of fetal airway epithelium by the basic helix-loop-helix network. Neuroendocrine phenotype is dependent on *Mash1*. In non-neuroendocrine cells, *Hes1* represses *Mash1* expression and activation, and maintains the cells to be non-neuroendocrine. Moreover, Notch signaling activates *Hes1*.

PULMONARY STEM CELL NICHES

Stem cells are operationally defined as progenitors with the capacity for self-renewal and ability to generate progeny that include transit amplifying (TA) cells that eventually give rise to functionally differentiated phenotypes of the tissue or organ. In a recent review on lung epithelial stem cells, Rawlins and Hogan [44] distinguishes between dedicated stem cells (self-renewing population) and facultative stem cells (differentiated cells that revert to the dedicated stem cell state following injury), progenitors, and self-renewing differentiated cells (divide in response to injury and maintains homeostasis). In fact, there is evidence for different lung stem cell subtypes and thus the identification of pluripotent stem cells in the lung has been more difficult compared to other organs. Stem cells are thought to reside within specialized niches that protect them from insults and provide an optimal microenvironment for survival and expansion as needed. Cells meeting these criteria retain mitotic labeling for prolonged periods (i.e., non cycling compartment) [7, 45]. Label retaining cells (LRC; identified by BrdU labeling technique) have been identified in several distinct niches along the respiratory tract. LRC have been found within submucosal gland ducts and the cartilage-intercartilaginous junction [43, 46], in a physical association with NEB at airway bifurcations of bronchi and bronchioles [47, 48], and at bronchioalveolar junctions [49, 50]. Since the area of pulmonary stem cell biology is rather complex and continuously evolving, the discussion here will focus on PNEC/NEB. The evidence for the existence of PNEC/NEB related stem cell niche is derived from experiments using the naphthalene mouse model [47]. Intraperitoneal injections of naphthalene, an aromatic hydrocarbon that is converted by cytochrome P450 2F2 (encoded by *Cyp2f2*) into a toxic metabolite selectively ablates Clara cells that express high levels of *Cyp2f2*, while the cells that lack this enzyme, such as PNEC/NEB remain intact [47]. Following extensive airway epithelial cell injury induced by naphthalene, regeneration occurs from the cells that reside within the NEB cell clusters. Two populations of LRC and potential stem cells were identified in association with NEB cells, one represented by CGRP immunoreactive cells and the other with a Clara cell-like phenotype (variant Clara cells) that are naphthalene resistant [49]. In a related experiment, CGRP expressing PNEC proliferated but were not able to replace the entire airway epithelium after depletion of CC10-expressing cells in a transgenic mouse model with herpes simplex virus thymidine kinase under the control of CC10 promoter [51]. Finally, lung side population (SP) cells, a potential source for stem cells, were shown to be equivalent to the NEB associated variant Clara cells [52].

These data suggests a critical role for NEB cell microenvironment in the maintenance of a reservoir for toxin-resistant stem and progenitor cells that are required for airway epithelial repair following injury. Further studies are required to define the role of NEB cell niches in lung carcinogenesis and as potential stem cells for PNEC related small cell lung carcinoma (SCLC). With respect to small cell lung carcinoma (SCLC) a significant SP cell fraction in SCLC cell lines has been identified [53]. Thus the relationships between variant Clara cells, SP cells and SCLC remain to be determined.

PULMONARY NEUROEPITHELIAL BODIES (NEB) AS AIRWAY CHEMORECEPTORS: MECHANISMS OF O₂ SENSING AND CHEMOTRANSMISSION OF HYPOXIA STIMULUS

The key morphological features of NEB, suggestive of a function as a chemoreceptor include: (a) formation of “organoid” structures preferentially localized at airway bifurcation, a position ideally suited to monitor airway gas concentration; (b) expression of 5-HT and peptide mediators that are released from NEB cells upon exposure to hypoxia; (c) NEB cell innervation by vagal afferent sensory fibers that transmit the hypoxia stimulus to the CNS [54, 55]. Studies of Lauweryns and coworkers using a neonatal rabbit model were the first to provide evidence showing that NEB may represent hypoxia sensitive airway chemoreceptors modulated by the CNS [56]. In a series of experiments they demonstrated that exposure to acute airway hypoxia but not hypoxemia caused degranulation of DCV and reduced amine fluorescence in NEB cells, implying hypoxia-induced neurotransmitter release [57]. Further support for this hypothesis was obtained from recent electrophysiological studies that have confirmed the role of NEB as airway O₂ sensors and the actual transducers of hypoxia stimulus [58]. Using *in vitro* models and the patch-clamp technique the presence of voltage-activated Na⁺, Ca²⁺, and K⁺ currents was identified in NEB cells- a key feature of excitable cells [54, 55, 58]. Thus it was confirmed that NEB cell response to hypoxia is mediated by ion channels, a mechanism similar to other O₂ sensitive cells (i.e. carotid body glomus cells, adrenal chromaffin cells, pulmonary artery smooth muscle cells) [59, 60]. In these cells, including NEB, the primary event is the inhibition of O₂ sensitive K⁺ channel causing membrane depolarization, activation of voltage gated Ca²⁺ channels followed by influx of extra cellular Ca²⁺ triggering exocytosis of various neurotransmitters [55, 59, 60] (Fig. 2). Although the precise nature and the identity of the “O₂ sensor” in various O₂ sensitive cells is at present a matter of debate, findings in NEB cells supports the idea of a membrane bound O₂ sensing molecular complex comprised of a heme-linked NADPH oxidase closely associated with an O₂ sensitive K⁺ channel [58, 61]. According to this hypothesis, under normoxia, the oxidase tonically generates superoxide from ambient O₂, which is then rapidly converted to hydrogen peroxide (H₂O₂), used as a second messenger modulating the activity of O₂-sensitive K⁺ current (see below) [58, 61]. Hypoxia leads to decreased generation of H₂O₂ resulting in closure of O₂ sensitive K⁺ channel that triggers downstream events leading to neurotransmitter release as noted above.

The evidence in support of NADPH oxidase (similar to the one found in neutrophils [62]), as the principal O₂ sensor in NEB cells includes: (a) coexpression of mRNAs for both membrane components of NADPH oxidase (gp91phox, p22phox) demonstrated in native NEB cells as well as in the related tumor cell model, SCLC cell lines [61, 63]; (b) immunohistochemical localization of membrane (gp91phox, p22phox) and cytosolic components of the oxidase (p47phox, p67phox, rac2) in the membrane or submembrane regions respectively in NEB cells and in H-146 SCLC cell line [64]. The direct evidence for the critical role of the oxidase in O₂ sensing by NEB cells is derived from studies using a mouse model with NADPH oxidase deficiency [OD; gp91phox k/o]

[65]. In OD mice, hypoxia failed to reduce the O₂ sensitive K⁺ current in NEB cells (i.e. NEB failed to respond to hypoxia), and neonatal OD mice exhibited an abnormal pattern of respiration compared to wild type controls [66]. Interestingly, in studies on other O₂ sensing cells in the same OD mouse model (i.e. carotid body, adrenal medullary cells, pulmonary smooth muscle cells) responses to acute hypoxia were not affected [67, 68]. In other O₂ sensing cells, particularly in the pulmonary artery smooth muscle cells, the redox O₂ sensor appears to be located in the mitochondria [67-69]. In contrast in H-146 cells, used as a surrogate for NEB cells, specific inhibitors of the mitochondrial electron transport chain have no effect on O₂ sensing, and cells depleted of mitochondria (po H-146, cells genetically manipulated to be free of mitochondria) retained their O₂ sensing properties indicating that mitochondria are not directly involved in O₂ sensing by NEB cells [70]. Taken together, the above studies provide strong evidence for the hypothesis of a membrane delimited, oxidase dependent mechanism for O₂ sensing by NEB cells which may differ from other O₂ sensing cells.

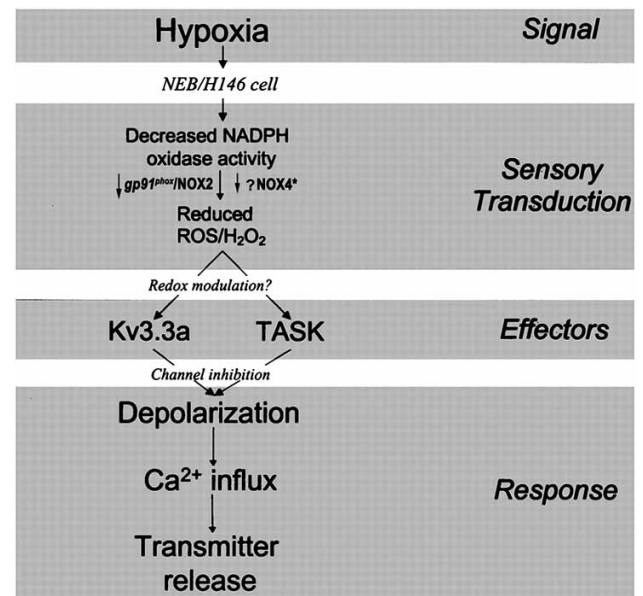


Fig. (2). The proposed transduction pathway for NEB's and their immortalized human counterparts, H146 cells. Native NEB cells and H146 tumor cell line share a common primary O₂ sensor, multicomponent NADPH oxidase including membrane bound gp91 phox/NOX2. Recent studies suggest that NOX 4 could represent a specific partner for TASK- 1 (see text). Hypoxia results in substrate-delimited production of reactive oxygen species and H₂O₂. Reduction in the cellular redox potential results in closure of O₂ sensitive K⁺ channels (Kv3. 3a, TASK 3). In both cell types, the resultant depolarization would lead to Ca²⁺-dependent release of bioactive amine and peptides. (Modified from Kemp *et al.* [75] with permission).

Co-expression of mRNAs encoding Shaw-related KV3. 3a channel protein with gp91phox/NOX2 and p22 phox components of NADPH oxidase has been reported in NEB cells of rabbit fetal and human neonatal lung as well as in several SCLC cell lines [61]. KV3. 3a belongs to a family of O₂ sensitive voltage activated K⁺ channels that are known to be modulated by H₂O₂ affecting the channel gating mechanism [71]. The common feature of these channels is a cysteine resi-

due in the amino terminus known to be highly sensitive to redox state [72]. This amino terminus is believed to be intracellular and contains a site that is responsible for channel inactivation acting as a tethered "ball and chain" which occludes the internal mouth of the channel [73]. It is of interest to note that H-146 cells, which are hypoxia sensitive, express an additional hypoxia sensitive K⁺ channel belonging to two-pore acid-sensitive K⁺ channel type 1 (TASK 1) [74]. It has been suggested that TASK 1, rather than KV3. 3a, mediates hypoxia-induced depolarization in H-146 cells, since TASK-like channels are non-voltage gated and are active at the resting membrane potential [75]. The possible involvement of recently identified homologues of NADPH oxidase (NOX 1, 3, 4) in O₂ sensing is at present unknown [76]. These "low output" oxidases are expressed in a variety of non-phagocytic cells and share with classical NADPH oxidase (NOX2) the membrane topology as well as some of the molecular activation mechanisms [77]. Of potential interest is a recent study using heterologous expression system, that suggest that NOX 4 could form an O₂ sensing protein partner with TASK-1 [78], an O₂ and acid sensitive, two pore domain K⁺ channel that is highly expressed in SCLC, H-146 cell line as well as in native NEB cells [61]. Therefore a possibility exists for a multitude of O₂ sensors even within the same cell type increasing the complexity of O₂ sensing mechanism, commensurate with their vital homeostatic function [60].

CHEMOTRANSMISSION OF HYPOXIA STIMULUS

The candidates for neurotransmitters mediating fast chemosensory transmission from NEB cells to CNS *via* vagal sensory afferents include 5-HT, acetylcholine (ACh), and adenosine triphosphate (ATP) (Fig. 3). Evidence for the role of 5-HT as a transmitter of hypoxia stimulus include *in vivo* and *in vitro* studies in the rabbit fetal/neonatal model [31-33]. More recent studies using carbon fiber amperometry that detect in real time the release of single 5-HT molecules have provided conclusive evidence that hypoxia causes dose-dependent 5-HT release from NEB cells within a physiological range expected in the airway (i.e. PO₂ < 95 mm Hg) [33]. In addition, NEB cells express ionotropic 5-HT₃ receptors that act as autoreceptors with positive feedback and amplification of hypoxia signaling from the airway [79] (Fig. 3). The evidence for cholinergic mechanisms in NEB includes demonstration of acetylcholinesterase activity [4], the presence of small clear vesicles in efferent-like nerve endings in contact with NEB cells [12] and immunohistochemical demonstration of vesicular acetylcholine transporter (VChat) activity [15, 16]. NEB cells also express several sub-types of functional nicotinic ACh receptors, which could mediate the effects of nicotine derived from smoking. Chronic exposure to nicotine activates these receptors leading to reduced sensitivity of O₂ sensor to hypoxia [80]. The possible involvement of ATP and purinergic mechanisms is based on the demonstration of ATP in the cytoplasmic dense core vesicles of NEB cells and expression of P2/X receptors in nerve endings innervating NEB as well as on NEB cells proper [43, 81]. The latter likely represent heteromeric P2X_{2/3} receptors that in NEB cells could possibly be function as autoreceptors modulating chemotransmission of hypoxia and other stimuli [81].

While 5-HT synthesized and released from NEB cells fulfils all the basic criteria for a neurotransmitter of hypoxia stimulus, the evidence for a similar role for ACh and ATP is

at present lacking. Hypoxia-induced 5-HT release from NEB cells can be expected to activate postsynaptic 5-HT receptors on vagal afferents innervating NEB. Previous studies have demonstrated that activation of 5-HT receptors leads to depolarization of both nodose neurons and isolated vagus nerve [82]. Furthermore, the electrophysiological properties of 5-HT receptors on nodose neurons have been well characterized [83]. Except for the studies of Kiwull *et al.* on pulmonary vagal afferents involved in hypoxic breathing [84], direct evidence for the transmission of hypoxia signal from NEB to the brain stem is at present lacking.

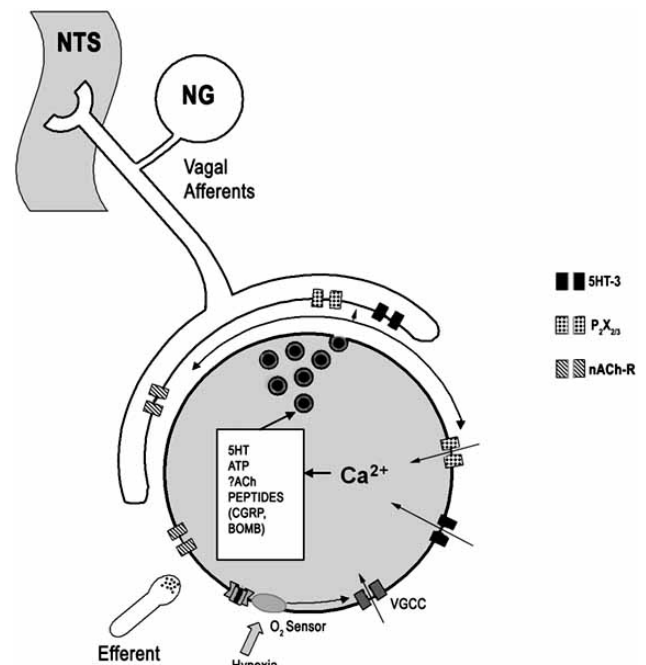


Fig. (3). Schematic diagram of neural components and pathways involved in the chemotransduction of hypoxia stimulus in pulmonary NEB. NTS: nucleus tractus solitarii in the brain stem; NG: nodose ganglion *via* sensory vagal afferent terminals in close proximity to NEB cells. Putative ionotropic neurotransmitter receptors (5-HT₃, P2X_{2/3}, nACh) are located postsynaptically as well as presynaptically. Postulated steps in hypoxia stimulated release of neurotransmitters (see text and Fig. 2) involves activation of O₂ sensor leading to membrane depolarization, opening of voltage gated Ca²⁺ channels (VGCC) followed by influx of extracellular Ca²⁺ that triggers exocytosis of dense core vesicles, the storage site for amine and peptides (boxed area). NEB cell function may be further modulated by presynaptic neurotransmitter receptors (autoreceptors) expressed on NEB cell membrane. For example activation of 5-HT₃ and P2X_{2/3} receptors that form ligand-gated ion channels may further increase intracellular Ca²⁺ providing a positive feedback, thus amplifying the hypoxia signal generated by small clusters of NEB cells widely distributed within lung parenchyma. Several nACh receptors are expressed on NEB cells but their role in hypoxia signaling is at present unknown. These are likely important in mediating the effects of nicotine (smoking) that has multiple effects on NEB cells (see text). Possible efferent nerve endings were observed by EM in NEB of rabbit lung but they have not been further characterized.

PULMONARY NEUROENDOCRINE CELL SYSTEM IN PEDIATRIC LUNG DISEASE

Abnormalities in the number and distribution of PNEC/NEB have been documented in a number of different pediatric lung disorders [6, 7, 85] (Table 2). However the clinical significance of these changes or the precise mechanisms involved are at present unknown. Pulmonary diseases, prevalent during the perinatal period, were the subject of early studies since these cells are numerous in fetal/ neonatal lungs [6, 7, 85]. Increased number of PNEC/NEB has been reported in infants with **lung hypoplasia** secondary to diaphragmatic hernia but not in those due to oligohydramnios or Potter syndrome [86]. Similar abnormalities in PNEC/NEB were also observed in a rat model of diaphragmatic hernia [87]. The proposed mechanisms include compensatory increase in PNEC/NEB secondary to impaired lung growth and/or failure of neuropeptide release during neonatal adaptation. A recent study reported "hyperfunction" of PNEC/NEB in lungs of mid to late gestation fetuses with **congenital pneumonia** [88]. The authors found significant increase in the number of bombesin, calcitonin and chromogranin immunoreactive PNEC in cases with lung inflammation compared to age matched controls without pneumonia. On the other hand, the frequency of PNEC immunoreactive for synaptophysin (a synaptic vesicle protein), a general neuroendocrine marker, was comparable between the two groups. Therefore it was concluded that the finding of synchronous increase in the three neurohormones represented a hyperfunctional state of PNEC rather than a simple hyperplasia. Altered enzymatic inactivation of neutral endopeptidase (NEP), known to be reduced in lung inflammation or increased neurohormone gene expression were suggested as possible mechanisms. The authors speculate that the hyperfunction of PNEC could contribute to the lethality of amniotic sac infection syndrome.

Table 2. Perinatal/Pediatric Disorders with Alterations in PNEC/NEB

Pulmonary hypoplasia due to diaphragmatic hernia [86]
Congenital pneumonia [88]
Bronchopulmonary dysplasia (BPD) [89, 90]
Wilson-Mikity syndrome (WMS, pulmonary dysmaturity) [89]
Congenital Central Hypoventilation syndrome (CCHS) [96]
Sudden Infant Death syndrome (SIDS) [98, 99]
Neuroendocrine hyperplasia of infancy (NEHI) [101]
Cystic fibrosis [103-105]
Pediatric asthma [108]
Pulmonary hypertension [111, 112]

Significant hyperplasia of bombesin/GRP immunoreactive PNEC/NEB has been reported in cases of **bronchopulmonary dysplasia** (BPD), a chronic lung disease of premature infants that was described in the late sixties and in the "pre-surfactant era" was characterized by ongoing airway epithelial injury and repair [85, 89, 90]. It has been postulated that chronic hypoxia and/or release of inflammatory cytokines could stimulate PNEC/NEB mitogenesis directly, or enhance recruitment from precursor cells [85, 90]. Recent

studies using a baboon model of BPD and transgenic mice lacking specific bombesin/GRP receptors revealed potential links between BLP derived from PNEC/NEB cells and pulmonary inflammatory response [91]. In a baboon model, administration of anti-BLP blocking antibodies reduced the severity of BPD changes in the lung together with reduction in number of PNEC/NEB cells and attenuation of mast cell hyperplasia [92]. Intratracheal administration of bombesin in a mouse model resulted in an increase in lung mast cells establishing a potential link between BLP and mast cell recruitment [93]. This process is facilitated by the expression of BLP family of receptors on mast cells providing a functional link with PNEC/NEB acting here as pro-inflammatory cells [93]. A potential new link between BLP and the pathogenesis of "post-surfactant" BPD, characterized by arrested acinar development and interstitial thickening, has been demonstrated recently in a neonatal mouse model [94]. Repeated intraperitoneal administration of bombesin to pregnant or newborn mice resulted in reduced alveolarization of the lung and increased proliferation of interstitial myofibroblasts with increased thickness of alveolar septae. These changes were abrogated in bombesin/GRP receptor null mice confirming the selectivity and specificity of these effects. Although BPD is a multifactorial disorder with many etiologic factors involved, these studies suggest that early overproduction of BLP by PNEC/NEB cells might be sufficient to trigger and activate multiple downstream signaling pathways leading to arrested alveolarization and interstitial fibrosis.

Infants with BPD exhibit a variety of physiological abnormalities (i.e., airway hyper-reactivity, pulmonary hypertension, and increased apneic spells) that are possibly related to PNEC/NEB cell hyperplasia and increased levels of pulmonary BLP that is detectable in the urine [95]. Both BPD and a related condition, Wilson-Mikity syndrome (pulmonary dysmaturity) show marked PNEC/NEB hyperplasia and are associated with high incidence of sudden unexpected infant death [89].

Significant hyperplasia of PNEC/NEB has been also described in cases of **congenital central hypoventilation syndrome** (CCHS) and in **sudden infant death syndrome** (SIDS), both disorders characterized by dysfunction in respiratory control. In CCHS, an inherited disorder of autonomic control of breathing, hyperplasia of PNEC/NEB was found in association with atrophy of carotid bodies (CB, the principal arterial chemoreceptors), suggesting a compensatory response and possible interaction between these two peripheral chemoreceptor systems [96]. A similar pattern was observed in cases of SIDS, where CB chemoreceptors also appear to be inhibited [97] and where lung tissue shows marked hyperplasia of PNEC/NEB [98]. Hyperplasia of PNEC/NEB cells in lungs of SIDS infants was found to be further increased/potiated by maternal smoking during pregnancy [99]. This is an important observation since maternal smoking is a recognized risk factor for SIDS and nicotine is known to induce hyperplasia of PNEC/NEB in experimental animals [100].

Recently described **Neuroendocrine Hyperplasia of Infancy** (NEHI), presents as an interstitial lung disease with persistent tachypnea, suggesting dysregulation of PNEC system [101]. The relatively benign clinical course of patients

with NEHI suggests that hyperplasia of PNEC/NEB alone does not necessarily lead to fatal outcome and that in lethal disorders (i.e. SIDS, CCHS) additional factors are involved [102].

Possible dysfunction of PNEC/NEB cells has been implicated in the pathogenesis of **Cystic Fibrosis (CF)** lung disease, since these cells express cystic fibrosis transmembrane regulator (Cftr) [103]. Experimental studies suggest that normal Cftr function is required for O₂ sensing and for amine/peptide secretion by these cells, which in turn may affect the composition of periciliary fluid eventually leading to thickened mucus accumulation and airway plugging [104]. These cells could be involved in both the early and late stage of CF lung disease due to an imbalance in the secretion of 5-HT and neuropeptides, which in turn could exacerbate the disease process. Of particular interest is a recent study of lung tissue from Cftr *-/-* mice, that showed altered distribution and frequency of PNEC/NEB, their innervation and airway smooth muscle mass during different developmental stages suggesting an intrinsic abnormality [105]. These findings correlate well with other studies in Cftr null mice that reported blunted O₂ sensing response and reduced airway tonus [106, 107]. Furthermore this data suggests an expanded role for Cftr in the lung including development and organogenesis. During infancy and childhood, PNEC/NEB may be involved (directly or indirectly) in the pathophysiology of **pediatric bronchial asthma (PBA)**. Maternal smoking has been identified as a significant etiologic factor in PBA, although the precise mechanism is unknown [108]. PNEC/NEB could provide a critical link here since these cells are numerous in developing lung, are localized in peripheral high resistance airways, and nicotine induces NEB hyperplasia *in utero* via upregulation of alpha-7 nACh receptors known to be expressed on NEB cells [80]. Furthermore BLP produced by PNEC/NEB may be involved in mast cell recruitment and activation [91]. Since NEB are extensively innervated and produce 5-HT (a bronchoconstrictor), that is released during hypoxia these cells could play a critical role in status asthmaticus. Thus these cells and bioactive molecules they produce represent novel therapeutic targets for the treatment and prevention of PBA, enhancing current therapies based on immunosuppressive and anti-inflammatory agents.

In addition to airway related abnormalities, PNEC/NEB may be also involved in the pathophysiology of **pulmonary hypertension (PH)** by virtue of their production and release of 5-HT, a potent vasoconstrictor [109], while some of their peptide products (i.e. CGRP) are known vasodilators [110]. It should be noted that PNEC/NEB, situated in small peripheral airways and at bronchial-alveolar portals are in close proximity to pulmonary arterioles that are involved in hypoxia induced vascular resistance. Immunohistochemical studies of lungs from patients with PH, both primary and secondary to congenital heart disease revealed significant hyperplasia of PNEC/NEB in early as well as late stages of pulmonary hypertensive disease [111].

Of interest is an observation of increased respiratory resistance and bronchial smooth muscle hypertrophy in patients with acute post operative PH [112]. Lung biopsies from these patients revealed significant hyperplasia of PNEC/NEB, suggesting that constriction and hypertrophy of

both bronchial and vascular smooth muscle during PH could be mediated *via* the secretory products of these cells (i.e. bombesin, endothelin, 5-HT).

Although the involvement of 5-HT in the pathophysiology of PH is well recognized, the source of 5-HT in this setting is presumed to be derived mostly from the blood platelets [113]. However it should be noted that the platelets have no capacity to synthesise 5-HT and lack O₂ sensing mechanism to respond to changes in O₂ concentration. In contrast, PNEC/NEB are not only strategically located near peripheral resistance vessels but also exhibit serotonergic and O₂ sensor phenotype [55, 79]. The potential link between PNEC/NEB, 5-HT and PH has been strengthened by a recent finding of increased incidence of persistent pulmonary hypertension of the neonate (PPHN) in infants born to mothers taking selective serotonin reuptake inhibitors (SSRIs), particularly during the second half of pregnancy [114], when PNEC/NEB are most prominent [17, 18]. The principal action of SSRIs is an inhibition of 5-HT transporter (5-HTT), an integral membrane protein that is responsible for terminating the action of 5-HT released from serotonergic neurons. Since pulmonary artery smooth muscle cells (PASM) express 5-HTT as well as a number of 5-HT receptors, the action of 5-HT on these cells relevant to pathophysiology of PH, include vasoconstriction as well as mitogenic effects that could modulate PASM hypertrophy [113]. It is of interest that hypoxia, a recognized stressor leading to PPHN is not only a potent stimulus for the release of 5-HT from PNEC/NEB cells [33] but also leads to upregulation of 5-HTT in PSMC that is expressed at low levels under basal conditions [113]. Although at present there are no studies on PNEC/NEB in cases of PPHN, this is a promising area of research that could further strengthen the links between these cells and their pivotal role during adaptation to extrauterine life, affecting both the control of respiration and pulmonary circulation.

PULMONARY NEUROENDOCRINE CELL SYSTEM IN ADULT LUNG DISEASE INCLUDING LUNG CANCER

As noted earlier, PNEC/NEB are inconspicuous in lungs of adults compared to the neonates. The estimates of PNEC frequency in normal adult lung are approximately 0.4% of all airway epithelial cells (~12.5 cells/cm airway basement membrane) with a proliferative fraction of PNEC between 1-2%, indicating a sparse cell population with a low turnover [115]. There are no estimates for NEBs that are reported to be rare in lungs of adults. A recent study that used whole mount preparations of airway epithelium viewed en face by confocal microscopy showed uneven distribution of PNEC with wider variations in cell densities (ranging from 65-250/mm²). Overall, these estimates are 3-4 times lower compared to those in fetal/neonatal lungs, perhaps reflecting higher functional activity during the perinatal period [17, 18].

Various adult pulmonary diseases with recognized alterations in the distribution and frequency of PNEC are listed in Table 3. Increased number of PNEC compared to age matched controls was reported in lung disorders characterized by chronic inflammation and/or fibrosis including **chronic bronchitis and emphysema** [116]. Of interest is a finding of higher proportion of calcitonin-containing PNEC

compared to bombesin/GRP immunoreactive cells and correlation with increased levels of calcitonin in the blood and urine of patients with inflammatory pulmonary disease [117]. A more recent study quantitated PNEC in various forms of **interstitial pneumonitis** including usual interstitial pneumonia (UIP), non-specific interstitial pneumonia (NISP), hypersensitivity pneumonia and interstitial pneumonia associated with collagen vascular disease [118]. Although there was almost two fold increase in the frequency of PNEC in some forms of IP (UIP, NISP) compared to the controls, there was variation in PNEC proliferative activity and expression of mRNA for hASH 1, a transcription factor necessary for PNEC differentiation. For example cases of UIP showed high expression of hASH 1 and low proliferative activity (determined by Ki-67 labelling) suggesting enhanced PNEC differentiation from precursor cells. This finding is in agreement with studies in mice that showed downregulation of Mash 1 under conditions that enhance proliferation of lung epithelial cells (i.e. exposure to EGF). Interestingly, there were no apparent effects of smoking on PNEC numbers or activity amongst the patients with various forms of IP. This is in contrast to striking hyperplasia of PNEC reported in patients with **eosinophilic granuloma** who are usually heavy smokers [119]. The significance and the mechanisms of PNEC alterations in IP and other forms of inflammatory and fibrogenic lung disease are at present unknown, but likely involve a variety of factors including effects of inflammatory cytokines, local hypoxia and various environmental toxins [40, 43] (Fig. 4). Since alterations in the secretion of various amine/peptide mediators from PNEC can be expected under pathological conditions, these cells have been implicated in the pathogenesis and progression of the disease process and hence may represent potential targets of new therapies.

Table 3. Adult Pulmonary Disorders with PNEC Alterations

Chronic bronchitis, emphysema [116]
Interstitial pneumonitis [118]
Eosinophilic granuloma [119]
Idiopathic diffuse hyperplasia of PNEC [120, 123]
Pulmonary neoplasms with neuroendocrine features (small cell lung carcinoma, SCLC) [125, 129-131]

Idiopathic diffuse hyperplasia of PNEC is a rare condition that occurs in the absence of preexisting lung disease [120]. The age of the patients with this condition varied between 22 and 76 years and they usually presented with exertional dyspnea and chronic non productive cough without a history of smoking or respiratory infection. The most common abnormality on lung function testing was irreversible airway obstruction. The lung biopsies revealed diffuse hyperplasia and/or dysplasia of PNEC involving the distal bronchi and bronchioles with prominence of NEB in the terminal airways. Numerous neuroendocrine-cell tumorlets and PNEC aggregates forming intraluminal polypoid-like structures, partially obliterating the airway lumen were also observed. In all lesions, PNEC showed immunopositivity for neuron-specific enolase as well as bombesin, calcitonin and chromogranin A. Some lesions showed associated areas of pulmonary fibrosis but there was no evidence of active in-

flammation. None of the patients developed metastatic cancer and most remained stable suggesting a relatively benign clinical course. The precise etiology and significance of idiopathic PNEC hyperplasia remains obscure and whether it is a primary or secondary phenomenon is not known. It has been suggested that the lung pathology, particularly the development of peribronchial and interstitial fibrosis could be mediated by PNEC secretory products such as bombesin [120]. High expression levels of neutral endopeptidase (NEP, CD10) that hydrolyzes small bioactive neuropeptides including bombesin and competes for its receptor has been reported in idiopathic PNEC hyperplasia [121]. The potential role for NEP is to neutralize the effects of increased local levels of neuropeptides since aerosolized administration of recombinant NEP reduced the symptoms induced by exposure to endogenous or exogenous tachykinins [122].

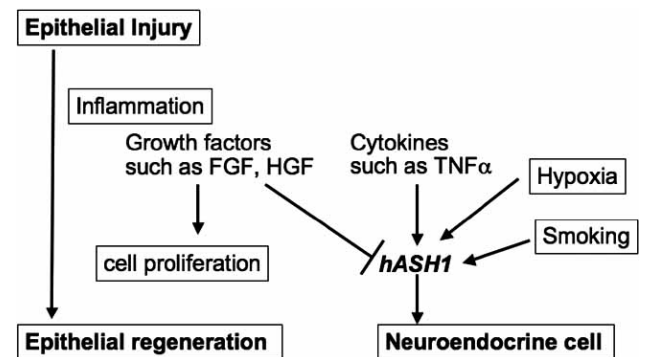


Fig. (4). Hypothetical mechanisms of PNEC kinetics in lung diseases. Epithelial injury results in activation of cell proliferation, and cell proliferation signals could suppress neuroendocrine cell differentiation. On the other hand some cytokines, growth factors, hypoxia and smoking can increase neuroendocrine cells, which could depend on hASH 1 upregulation.

A variant of idiopathic PNEC hyperplasia, presenting as interstitial lung disease with predominantly intraepithelial proliferation of PNEC in bronchioles and within alveolar septae has also been reported [123]. In this case, as in the classical form, benign PNEC hyperplasia was favored over a neoplastic process. However, the possibility that these lesions represent a premalignant condition cannot be excluded.

Pulmonary neoplasms with neuroendocrine features account for up to 20% of all lung cancers. The spectrum of neuroendocrine neoplasia in the lung includes tumorlets, typical carcinoid, atypical carcinoid, large cell neuroendocrine carcinoma, and small cell lung carcinoma (SCLC) [124]. The biological behavior and morphological appearance of pulmonary neuroendocrine neoplasms can be quite variable. The molecular mechanisms and animal models of lung carcinogenesis with neuroendocrine features have been reviewed recently [7, 40, 125]. The mechanisms of neuroendocrine differentiation in SCLC are complex and involve a network of bHLH transcription factors and their modulators similar to those in the developing lung such as hASH 1 and Hes 1. It has been suggested that while transient expression of Mash/hASH1 during lung development promotes neuroendocrine differentiation of PNEC in normal lung, malignant transformation of PNEC progenitors may lock in the proliferating, primitively differentiated phenotype of these

cells, associated with constitutive rather than transient expression of hASH 1 [125].

Various neuroendocrine neoplasms at different anatomical sites such as neuroblastoma, pheochromocytoma and medullary thyroid carcinoma express hASH 1 [126-128]. Amongst lung tumors, large proportion of SCLC express hASH 1 mRNA [41, 129]. Recent study of 238 surgically resected lung tumors examined correlations between hASH 1 mRNA expression, immunoreactivity for neuroendocrine markers and clinical outcomes [130]. hASH 1 expression was detected in 10% of adenocarcinomas, 13% of typical carcinoids, 83% of atypical carcinoids, 57% of large-cell neuroendocrine carcinomas, and 72% of SCLC, respectively, but none amongst cases of squamous cell or large-cell carcinomas. Amongst the tumors with neuroendocrine features, hASH 1 expression closely correlated with positive immunoreactivity for neuropeptides and chromogranin A, but did not correlate with immunopositivity for neural adhesion molecule, suggesting that in lung tumors hASH1 expression confers "endocrine-like" phenotype expression rather than neuronal properties. Furthermore, hASH 1 expression in lung tumors imitates its early and transient expression during fetal lung development, and it plays a critical role in the establishment but not the maintenance of cellular endocrine phenotype. Finally, hASH 1 expression correlated significantly with poor outcome in patients with SCLC. The critical role of hASH1 in molecular pathogenesis of lung neuroendocrine tumors was confirmed in experiments using SCLC cell lines that have shown suppression of neuroendocrine differentiation by antisense oligonucleotides against hASH 1 [41]. A more recent experimental study used RNA interference(RNAi) approach to significantly suppress growth of lung cancer cells with ASH 1 expression through G2-M arrest and enhanced apoptosis [131]. Lung cancer cell lines without ASH 1 expression and immortalized normal bronchial epithelial cells, used as negative controls, were not affected. An RNAi-resistant mutant ASH 1 induced rescue from G2-M arrest, suggesting target specific effect of RNAi. In addition, ASH1-RNAi adenovirus was also established and significantly inhibited not only *in vitro* cell proliferation but also *in vivo* xenograft growth of ASH1 positive NCI-H460 cells. The authors concluded that ASH 1 plays a crucial role in lung carcinogenesis and that it represents an effective therapeutic target in lung cancers with neuroendocrine features.

Animal models for SCLC have proven elusive as neither chemical carcinogens or single gene mutations were able to reproduce the pathological and clinical features of its human counterpart [7]. Recent studies by Meuwissen *et al.* using double knock -out mouse model that carried conditional alleles for both retinoblastoma, Rb-1 and p53 (Rb-1 *-/-*, p53 *-/-*) in pulmonary epithelial cells, developed highly malignant lung tumors resembling human SCLC both in its morphology and biological behavior [132]. This model, for the first time recapitulates many features of SCLC, and underlines the importance of Rb-1 and p53 tumor suppressor genes that are frequently mutated in human SCLC [133].

The aggressive clinical behavior of SCLC and its resistance to current treatment regimens has been linked to the expression of many cellular and molecular features of "neuronal" phenotype including those of excitable cells [134]. In

addition to the expression of various neuroendocrine markers, amine and peptides that act as tumor autocrine growth factors and mitogens, the membrane of SCLC cells contains many types of voltage-gated ion channels that are a hallmark of neuronal excitability. As its normal NEB cell counterpart (see section on NEB), SCLC cell lines were found to express a whole range of voltage-gated ion channels including Ca²⁺ (VGCC), Na⁺(VGSC) and K⁺(VGPC) [135]. While VGCC are mostly involved in amine/peptide secretion, VGSC have been implicated in the pathogenesis of tumor metastasis by enhancing endocytic membrane activity [136]. While up regulation of voltage-gated ion channel activity promotes cell behavior leading to formation of metastasis, inhibition of these channels was shown to reduce tumor proliferation and invasiveness *in vitro* [137]. In addition, auto-antibodies against VGSC and or VGCC are responsible for paraneoplastic syndromes (i.e. Lambert-Eaton myastenic syndrome) seen in some patients with SCLC [138]. The expression of hypoxia/acidosis sensing molecular mechanism by SCLC, particularly O₂ sensitive VGPC coupled to O₂ sensor protein (NADPH oxidase) or two-pore acid-sensitive TASK -like channels, provide an additional mechanism that allows SCLC tumor cells to adapt and survive in the hostile environment of rapidly growing tumor(severe hypoxia and acidosis). In fact both hypoxia and acidosis not only perpetuate a more aggressive cancer phenotype, they also confer therapeutic resistance to radiation and chemotherapy [139].

Thus information generated from basic cellular and molecular studies on PNEC/NEB in normal lung and in neoplasia have advanced our understanding the pathobiology of this cell system and provide opportunities to develop novel therapeutic approaches that target both neuroendocrine as well as ion channel properties of these highly malignant tumors.

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