SUMMARY AND CONCLUDING REMARKS

7.1 Quality Control

The increasing utilization of immunocytochemistry (ICC) as a research tool has resulted in a variety of techniques improving the sensitivity and specificity and broadening the applicability of this powerful device. Although ICC on frozen sections is usually regarded as the most sensitive and specific method for antigen detection, the suboptimal morphological preservation together with convenience issues inspired us to optimize and adjust alternative techniques for application in our investigation of the pulmonary diffuse neuroendocrine system (PDNES) (Chapter 2.1).

A first approach (Chapter 2.2) was to modify the fixation protocol of formaldehyde (FA). This fixator has become the gold standard for morphological preservation and is the most widely used chemical fixative in many areas of biological research, routine pathology and microbiology. Its efficacy as a preservative for cellular antigens, however, is suboptimal. Therefore, factors such as aldehyde percentage, fixation time, temperature, buffer concentration and pH, were changed over a wide range. The best results, with regard to immunoreactivity and background, were obtained with a 2% FA solution, fixation periods between 18 and 24 h, a temperature of 4 °C, phosphate concentrations between 0.01 and 0.1 M and a neutral to slightly acidic pH. Thus, in spite of the well known adverse effects of FA fixation, beautiful and reproducible results can be obtained in the lung, provided that optimal conditions are stringently observed.

A second approach (Chapter 2.3) was to compare a variety of fixatives for their ability to preserve pulmonary antigens. Different cross-linking and coagulating fixators as well as mixtures of both were applied. Bouin and especially fixation fluids containing zinc compounds were the most efficacious for the demonstration of immunoreactive pulmonary neuroendocrine cells (PNEC). The use of zinc based fixatives represents the closest approach yet to a technique, that combines optimal antigen survival with the convenience and morphological preservation of traditional FA fixed tissue embedded in paraffin.

A third approach (Chapter 2.4) was to investigate the benefits of microwave (MW) assisted fixation. Lung samples were MW heated in physiological saline (MW stabilization) or in FA fluid (MW fixation) and their effects on antigen survival compared with those of conventional FA fixation. The best results were obtained with MW stabilization, the worst with MW fixation. The latter was probably too vigorous. Thus, by virtue of the enhanced speed and the lack of chemical fluids, MW stabilization avoids several artifacts produced by standard chemical and physical fixation methods.

A fourth approach (Chapter 2.5) was to verify if dehydrating and embedding steps cause antigen impairment. Provided that lungs were optimally fixed, no or little
impact on antigenicity could be noted. So, if there is a problem in the interpretation of ICC reactivity, it is unlikely that the cause will be associated with the dehydration or embedding methods.

A final approach (Chapter 2.6) was to reconstitute pulmonary antigens with the aid of enzymatic and non-enzymatic demasking procedures. A different antigen expression for neuroendocrine markers, bioactive substances, intermediate filaments and proliferation markers was noted after both pretreatments. Our results validate the use of non-enzymatic antigen retrieval and show that evaluation of the reaction to this approach has to be included as a variable to testing of all new antibodies.

In addition, our findings demonstrate the necessity of examining several tissue preparation techniques for ICC assays on tissue sections, as there are no universal rules that can be applied for all antigens. Therefore, each laboratory must develop its own quality control program for variables which can influence the ICC outcome, taking into account the kind of tissue to be treated and the criteria put first (Chapter 2.7).

7.2 Pulmonary Diffuse Neuroendocrine System

The respiratory tract of mammals contain specialized endocrine cells, called PNEC, which are part of the diffuse neuroendocrine system (DNES). The PNEC can occur as solitary neuroendocrine cells (NEC) or in innervated organoid groups, termed neuroepithelial bodies (NEB). Both are distributed throughout the airway and alveolar epithelium. By electron microscopy (EM), these cells contain membrane bound dense cored vesicles (DCV). Several peptides, including calcitonin (CT), mammalian bombesin (MB), leu-enkephalin and the biogenic amine, serotonin (5-hydroxytryptamin or 5-HT), have been localized within PNEC. Although the physiological role of these cells is largely unknown, they are associated with sensory afferent as well as efferent nerves and actively respond to various acute respiratory perturbations, including hypoxia. In 1973, our research group theorized - based on an elegant series of experiments - that in insufficiently aerated lung areas, and as a consequence of the subsequent local hypoxia, NEB would secrete 5-HT in the surrounding lung capillaries. The resulting vasoconstriction would necessarily shunt the pulmonary blood to better ventilated lung parts, where NEB would not induce vasoconstriction. As a result, local ventilation/perfusion ratios would be normalized and the overall efficiency of the respiratory process would increase. Alongside central and peripheral (e.g. carotid body) chemoreceptors, NEB may thus constitute a third or locally inbuilt intrapulmonary chemoreceptor system: while carotid bodies ‘taste’ the composition of the blood, NEB may ‘taste’ the air. Whatever their physiology, and a great deals remains to be unraveled, NEB are clearly more than just simple transducers converting physical or chemical stimuli into nervous impulses on a one for one basis (Chapter 1).

The research described in this thesis was conducted to fill the huge gaps in our fundamental knowledge of the PDNES. Comparative anatomical, chemical coding, functional immunocytochemical and tract tracing studies were conducted to reveal the different functional strategies by means of which NEB can perform their secretory and/or chemoreceptor function. The results are summarized in the following.

A survey of the occurrence of PNEC in the lung of various non-laboratory
mammalian species was given in Chapter 3.1. Animals belonging to different families and orders were purchased from slaughterhouses, zoological gardens and private individuals. Their PDNES was outlined by means of conjunctive histochemical (hematoxylin-eosin, masked metachromasia, Grimelius' silver impregnation, amine histofluorescence) and ICC (5-HT and protein gene product (PGP) 9.5) staining techniques. Single as well as clustered PNEC were regularly found and possessed a structure, distribution and innervation pattern comparable to those of more extensively studied laboratory mammals. Moreover, the biogenic amine, 5-HT, was ubiquitously present in both types of PNEC. The strength of the study lies in the wide diversity of the lung material used. Whatever the phylogenetic origin, natural habitat, dietary pattern, mode of life or physiological adjustments (adaptation versus acclimatization) of the investigated animals are, NEC and NEB can be found, suggesting that they have a general, though subtle function in mammals.

In Chapter 3.2, a more detailed description was given of the PDNES in dog and cat. At the light microscopical level, lung sections were processed histochemically according to the hematoxylin-eosin, masked metachromasia or Grimelius technique and immunocytochemically for antigens, like 5-HT, bioactive peptides and pan-neuroendocrine markers. In addition, induced fluorescence and indirect immunofluorescence methods were applied. At the ultrastructural level, lung specimens were treated for routine transmission and scanning electron microscopical observation. Kitten as well as the smaller puppy NEB resembled those of other already described mammals, but have their own specific characteristics as well. Firstly, they were located preferentially at airway and alveolar branching points and - exclusively in the cat - above cartilage plates. Secondly, their anatomic relationship with smooth muscle cells in the airways and with capillaries in the alveolar parenchyma was striking. Thirdly, their ultrastructural features were indicative of a high metabolic activity. Fourthly, their nerve supply was more elaborate than in any other animal studied to date. And finally, the chemical coding of PNEC as well as of nerve fibers was different in both carnivores. This study demonstrates that, with respect to the architectural design, topographical location, cellular content, secretory and innervation pathways of NEB, interspecies differences exist - even between phylogenetically related mammals - and that, within each animal, NEB constitute a structurally heterogenous group of organoids.

Chapter 4 is a collection of published articles dealing with the chemical coding of the PDNES. This information is essential for correlating postulated endocrine cell functions with production of these substances. Using ICC methods, the occurrence and coexpression of diverse members of the same as well as of different peptide families were examined in NEB and/or NEC of various mammals.

In the first report (Chapter 4.1), CT and calcitonin gene-related peptide (CGRP), which are generated by alternate processing from α and β CT/CGRP genes, were located to both solitary and grouped PNEC. However, the cellular immunoreactivity for CT represented always a subset of the CGRP positive cells. Moreover, only CGRP was present in pulmonary nerve fibers and ganglion cells. The staining of the neuroendocrine cells indicates that different molecular processing of both CT/CGRP genes may be represented by different patterns in the cellular immunoreactivity of the synthetized peptides.
Next (Chapter 4.2), the usefulness of the pan-neuroendocrine marker, PGP-9.5, to reveal the dynamics of PNEC development was tested in the cat lung. The polyclonal PGP-9.5 antibody produced a diffuse cytoplasmic immunostaining in NEC and NEB, which was usually weaker and more variable than in ganglion cells and nerve fibers. Distinct changes in extent and intensity of staining were observed between the various life stages studied. Most typically was the gradual decline in number of PNEC from 3 weeks of age. In addition, NEB innervation appeared to involute rapidly soon after birth. The observed changes provide evidence that the chemoreceptor function of NEB is only of primary importance in the perinatal period. The secretory function, on the other hand, seems to remain throughout life.

In the following two articles (Chapter 4.3.1 and 2), expression patterns of diverse peptides of the endothelin (ET)/sarafotoxin (SRTX) gene family were examined. Pulmonary tissue elements had a distinct staining behavior for the investigated bioactive substances. Striking features were the restriction of epithelial reactivity to the PNEC and the discrepancy in labelling between NEC and NEB. Indeed, ET and SRTX positive clusters were numerous, but single PNEC were only rarely labelled for ET and never for SRTX. ET-3 was the most prominent isoform in NEB and coexpression with other members of the ET/SRTX family was common. The ET stained NEB, however, were always outnumbered by 5-HT and/or CGRP reactive NEB. Our results suggest that ET-3 represents the neuroendocrine form of the ET/SRTX peptide family and that NEB are storehouses of numerous and heterogeneous regulatory substances, such as amines, peptide hormones and neurotransmitters/modulators, which are functionally interacting and involved in a multitude of effects. The immunomicroscopical difference between NEC and NEB lend additional support to the hypothesis of a different functional role for both PNEC types. The demonstration of SRTX in NEB, an extremely toxic component of snake venom, is remarkable. The reason why this lethal product does not produce a toxic reaction in the lung remains questionable. One possibility is that SRTX could have different profiles of biological activity depending on the species and/or tissue.

In three cognate articles, the occurrence of 7B2, an highly conserved pituitary protein, was investigated with emphasis on (co)localization properties and interspecies differences (Chapter 4.4.1), distinct reactivities in the pulmonary neuroendocrine versus nervous system (Chapter 4.4.2) and spatiotemporal distribution patterns (Chapter 4.4.3). Immunoactivity for 7B2 was observed in NEB but never in NEC. Staining of serial sections revealed three distinct cytochemical signatures, i.e. clusters expressing CGRP, 7B2 and 5-HT, CGRP and 7B2, and CGRP only. Interspecies variations in extent and intensity of labelling were obvious. By contrast, 7B2 was undetectable in intrapulmonary ganglion cells and nerve fibers. The first three weeks after birth, 7B2 positive clusters were most abundant with a subsequent decline thereafter. This spatiotemporal distribution pattern was similar to that of PGP 9.5 on adjacent tissue sections. The specific expression features of 7B2 in the mammalian lung might indicate biochemical and functional variations between NEB and NEC, a relative importance in different organisms and the lack of a role in the pulmonary nervous system. In NEB, however, 7B2 may be an essential neuropeptide, because of its prevalent occurrence in the neonatal period, when NEB are assumed to play a crucial role. Both an intracellular role in the secretory process
(prohormone maturation or protein traffic) or, alternatively, some as yet undefined postexocytotic function as hormone or neurotransmitter/modulator have been suggested.

In the final report of this chapter (Chapter 4.5), neural cell adhesion molecule (NCAM) - a membrane protein involved in cell-cell adhesion within the central and peripheral nervous system - was demonstrated to be a sensitive and specific marker for NEB and neural tissue elements in the cat lung. During postnatal lung growth, cell surface labelling of NEB cells was shown, but in adult lung, the plasma membranes were no longer stained. NEC, on the other hand, were always negative. In contrast to the transient postnatal immunoreactivity of NEB cells, nerve fibers and ganglion cells were stained throughout all life stages studied. The distribution of NCAM in the pulmonary neuroendocrine and nervous system was similar to that of the pan-marker, neuron specific enolase (NSE), except in the adult lung. The observed variation in NCAM surface density on NEB cells during postnatal development may dynamically modulate the extent of adhesion between the corpuscular cells. Therefore, the lack of NCAM reactive deposits on the peripheral regions of NEB cells in adult lung might indicate that their presence as a functional entity has become less important. The noted difference in NCAM expression between single and grouped PNEC, on the other hand, might be the consequence of an involvement of NCAM in adhesive interactions between NEB cells and nerve terminals.

Within the framework of the initial APUD (amine precursor uptake and decarboxylation) concept, the actual or potential amine production by endocrine cells is considered to be a distinctive feature. NEB, which are members of the APUD cell series, show a primary content of 5-HT or store it after exogenous application of the appropriate amine precursor, i.e. 5-hydroxytryptophan (5-HTP) (secondary content). The investigation described in Chapter 5.1 was aimed at examining the amine handling properties of both types of NEB and the possible function of the acidic protein, chromogranin A (CGA), as amine carrier. A qualitative and semi-quantitative analysis of mirror sections, sequentially immunostained for 5-HT and CGA, was performed in control kittens and after well-characterized interventions in the 5-HT synthesis. These included inhibition of 5-HT synthesis by para-chlorophenylalanine (pCPA), exogenous application of 5-HTP and a combination of both treatments. Our results established the predominance of NEB with a primary 5-HT content and the existence of a reciprocal relationship between 5-HT and CGA immunostainings. The latter was interpreted as a masking of epitopes in the CGA molecule by electrostatically bound 5-HT. In conclusion, this study evidences the participation of CGA in the intracellular storage of resident 5-HT and in that of newly formed 5-HT after priming with precursor substance.

NEB function as hypoxia sensitive airway chemoreceptors. Under hypoxic conditions they secrete the biogenic amine, 5-HT, which plays an important though still unclarified role in the hypoxic pulmonary vasoconstriction. NEB also express several peptides, but the effect of hypoxia on these peptides has never been studied before. In Chapter 5.2, the response of ET-3 to acute experimental hypoxia was investigated as well as the extent to which the observed effect was neurogenically modulated. The experimental design consisted of analyzing the ET-3 cellular content of NEB with the optimal/supra-optimal dilution (OD/SOD) technique in intact as well
as in vagotomized rats, under conditions of normoxia and hypoxia. After incubating the sections with ET-3 antiserum at the OD, there was no statistically significant difference. At the SOD, the number of ET-3 reactive NEB in the hypoxic rats, whether vagotomized or not, was significantly lower than in the respective normoxic counterparts. Vagotomy, on the other hand, did not alter the number of stained clusters under normoxic and neither under hypoxic conditions. This study shows that short term exposure to hypoxia induces functional changes in NEB, as demonstrated by decreased intracellular ET-3 levels, and that it is not neurally controlled. Our results also support the contention that NEB are involved in sensing the composition of inspired gas and that complex dynamic changes in peptide as well as amine content may occur. Furthermore, the OD/SOD technique seems to be technically straightforward and very useful as it detects variations in antigen concentration which may be masked if the routine, optimal dilution is used.

A combination of ICC, classical nerve degeneration experiments and anterograde axonal tracing was used to investigate the outflow pathways of the nodose ganglion to the lung and in particular to the neuroendocrine component of the epithelial cell lining (Chapter 6). Two days after fluorochrome conjugated wheat germ agglutinin (WGA) was injected into the nodose ganglion, positive nerve fibers could be observed in the walls of pulmonary vessels and airways and even down to the alveolar parenchyma. The most interesting findings, however, were the offshoots of sensory vagal nerve fibers to single and clustered PNEC. In experiments with nodose ganglion injections preceded by supranodosal vagotomy, WGA reaction patterns were unimpaired. By contrast, no WGA staining was observed in the ipsilateral lung when the lectin was injected in the distal stump of the vagus nerve, transected inferior to the nodose ganglion, and neither after injection in the surrounding tissues. Combining axoplasmic transport of tracer and CGRP immunocytochemistry resulted in a similar distribution of nerve fibers, but those reactive for CGRP were the most widespread. Moreover, the sensory neuropeptide was present in both PNEC types and in intrapulmonary ganglia. Supranodosal vagotomy did not produce changes in CGRP reactive sites, whereas infranodosal transection reduced substantially the CGRP marked nerve fibers beneath and within NEB of the ipsilateral lung. This study provides direct evidence that especially sensory nerve fibers innervating PNEC originate from perikarya in the nodose ganglion, whereas additional derivations are feasible for the remainder of the sensory nerves in the lung.

7.3 Concluding Remarks

The research described in this thesis evidences that PNEC - and in particular NEB - constitute a structurally and functionally heterogenous cell community. Solitary and clustered PNEC appear to be universally present in the mammalian kingdom but with apparent interspecies differences, which surpass pure morphological variations and may range from the occupation of specific checkpoints and utilization of several routes of secretion to the enlisting of a greatly varying nerve supply.

The chemical coding reports extend evidence of PNEC heterogeneity to another level, by demonstrating that cells form subsets not only based on their
diversity of bioactive substances and combinations but also on their different intracellular concentrations and spatiotemporal occurrences within the lung. Moreover, immunostainings for structural markers of neuroendocrine cells identify a greater number of PNEC than with any other chemical or immunologic stain investigated to date, indicating that still NEC and NEB exist whose content has not yet been fully exposed and that many other - yet unidentified - substances are to come.

Heterogeneity also characterizes the amine content dynamics of NEB, notwithstanding the binding to the same carrier protein.

The vagotomy experiments, either combined with hypoxia or with neuroanatomical tract tracing, draw attention to the fact that, besides their secretory role, NEB are well-equipped to function as hypoxia sensitive airway chemoreceptors. It is also demonstrated that this secretory versus receptor behavior may fluctuate in activity and/or importance between different life times.

Taken together, it is very appealing that each NEB responds to its own specific set of requirements and that complex patterns of reactions are possible in combination with an additional nervous influence. The proven versatility may also provide the necessary mechanisms to help NEB in the fine tuning of the ventilation/perfusion ratio. By virtue of the central location of the lung in the circulation and its large surface area, NEB might be plausible contributors to the overall homeostasis. Certainly, they do not deserve their current backwater status in discussions of pulmonary innervation and regulation.

A summary of our findings on the stimulus-secretion coupling and the vagal innervation of a 'typical' NEB is given in two conceptual schemes (Figures 1 and 2).

7.4 Future Prospects

Direct studies on NEC and NEB to further define their possible function(s) have been hampered because of low numbers of these cells, thinly scattered deep within a sponge-like lung parenchyma and unapproachable by direct electrophysiological methods. In addition, PNEC represent a minute fraction within a heterogenous population of up to 40 different types of lung cells.

Hence, availability of a suitable in vitro model is highly desirable. There are currently two practical methods for the isolation and subsequent culture of PNEC, i.e. organ-type cultures/microexplants and isolated cells using single cell suspensions enriched for PNEC. The organ cultures evidently can be used to assess certain physiological responses of lung endocrine cells in an accessible, relatively organotypical setting. Cultures offer advantages over intact lungs in that stable ambient conditions are easily maintained, agonists are readily applied and tissues are more simply handled for morphometric evaluations. Therefore, organ cultures are most useful for embryonic induction studies, where maintenance of tissue integrity is important, and for assessing effects of PNEC secretion on local targets within the lungs. Cell cultures, on the other hand, permit the study of a specific cell type in a more controlled environment and avoids the problems of cell targeting. Using this culture type, specific cell function can be investigated and important new information obtained regarding cell membrane ionic currents (i.e. to define excitable versus non-excitabte cells), stimulus-secretion coupling mechanisms and responses to specific
stimuli (e.g. hypoxia). Alternate approaches to the primary cultures could include the use of transfected cells and/or PNEC tumor lines. Studies using co-culture of NEB cells with nodose ganglion cells to reinnervate NEB could be a useful model to define the role of innervation in NEB cell function.

Recently, the use of in situ hybridization (ISH) and receptor research added new dimensions to the research of regulatory peptides in the PDNES. The additional information provided by both methods can be helpful in making conclusions about the functional activity and target cells of PNEC and the implications in normal and diseased lungs.

More than likely, the next decade will witness continuing progress, based upon studies of the cultured PNEC, of its peptide receptors, of its membrane biology, of factors controlling its secretion and growth, and of its anatomy and neurophysiology. Future knowledge concerning PNEC will continue to be via the contributions of investigators of different backgrounds using diverse approaches. Armed with this knowledge, there is no doubt that the physiology and the clinical relevance of these cells will then become fully apparent.

As to the present state of the field and prospects for the future, one can be mindful of what was written on Franz Schubert's memorial, "The art of music here entombed a rich possession, but even far fairer hopes".