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MUSCARINERGIC AND NEUROPEPTIDERGIC RECEPTOR HETEROGENEITY AND SIGNAL TRANSDUCTION

D.Sc. Thesis

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Introduction and aims

The cells of multicellular organisms communicate by extracellular signals. The communication between these cells involves the following cellular events: 1. the synthesis of the neurotransmitter or hormone; 2. its release; 3. the binding of this transmitter or hormone to its receptors on the surface of the target cell membrane; 4. the change of the cellular metabolism; and 5. the removal of the signal, which often results in cessation of the response of the cell. This thesis provides details on the key elements of this communication: the perception and the transduction of the signal, the heterogeneity of the receptors and their role in the transfer of the signal. On the surface or inside the cells, the membrane receptors are complex molecular units which are able to bind neurotransmitters, hormones and other substances. These receptors are responsible for two functions. Firstly, through their high-affinity, specific binding, they are able to recognize and distinguish biologically active ligands; secondly, through their interactions with their coupling proteins, they make it possible to mediate the apropriate signals, which finally leads to the response. In neurons, the realization and joining of these two functions into one process is called neuronal signal transduction.

The muscarinergic (m1-m5), opioid (mu, delta, kappa, sigma, epsilon and lambda), and somatostatin receptors are transmembrane proteins. In the cell, when binding their high-affinity and selective agonists, they change their conformations and cause well-defined biochemical changes which can be inhibited by selective antagonists. These receptor types usually mediate well-characterized physiological functions and can be found in both the central and peripheral nervous systems and even in other non-neural tissues. They have a characteristic ontogenetic development, localization, and pharmacological and physiological features, with often a spectacularly demonstrable neuronal transport to the site of the physiological function. The number of neurotransmitter, neuromodulator and hormone receptors is currently known more than one hundred. For example, the receptor subtypes of the cholinergic

(muscarinergic M1-M5 and nicotinergic), opioidergic (mu, delta, kappa, sigma, epsilon and lambda), adrenergic (alfa1, alfa2, beta1 and beta2), dopaminergic (D1 and D2) or serotoninergic ($5HT_1$, $5HT_{1A, B, C}$, $5HT_2$ and $5HT_3$) systems are well known and also well characterized on a pharmacological basis, and the primary structures (the nucleotide sequences) of some of their genes are already known. This thesis includes discussion of the heterogeneity of the muscarinergic and opioid receptors and presents relevant results.

By the 1980s, the receptor hypothesis (relating to the conditions of binding of the ligand, the properties of the binding, and effects able to modulate the binding) and its common principles had been elaborated. The clarification of the conformational conditions of the receptor-ligand interaction started only afterwards, when the role of neuropeptides in signal transmission had been recognized. Several conformationally restricted peptide ligand are now known, and with their aid receptor-selective physiological effects can be produced. In many cases their application has made it possible to shed light on the role of the coupling proteins in signal transduction.

The elements of the endogenous opioid system are to be found in both the central and peripheral nervous systems; they mediate a number of physiological effects. By means of immunology and molecular biology, localization of the endogenous ligands of the opioid system revealed the distribution of this system in the brain. Since the first publication of the binding properties and regional distribution of the opioid receptors, twenty years ago, our knowledge has increased rapidly. On the basis of the primary structure three, and on the basis of the pharmacology six receptor subtypes can be distinguished, among them the mu, delta and kappa, which are well characterized from molecular biological and pharmacological respects, while the epsilon, sigma and lambda receptor subtypes are only sketchily known and their physiological roles have not been elucidated. Although several pharmacological and physiological investigations involving the use of classical (non-peptide) opioid ligands suggested the existence of the main subtypes (mu, kappa and delta), the results of these early findings have now been re-evaluated following the accessibility and use of highly effective and selective ligands.

With varying degrees of affinity, the endogenous opioid peptides (methionine-enkephalin [ME], leucine-enkephalin [LE], dynorphin, etc.) are capable of binding to several opioid receptors, and so they might be responsible for biological functions mediated by different receptor types. For example, the delta opioid agonist [D-Ala^2 or D-Leu^5]enkephalin is able to bind with a comparatively high affinity to the mu binding sites in the guinea-pig and rat brains. Obviously, for investigation of the physiological functions mediated by these opioid receptor subtypes, the need for highly selective ligands with high affinity and as resistant as possible to biodegradation is inevitable.

The active conformation of the linear oligopeptides (e.g. ME or LE) can at most be revealed at the moment of interaction with the receptor. However, through the use of suitable structural modifications (e.g. methylation or cyclization of the peptide sceleton), even the conformational and electrical conditions of receptor-ligand

interactions can be clarified. In the instance of the enkephalins, the application of cyclic structures proved suitable. In solution, such analogues have only a small number of conformations and, if the sceleton is rigid enough, during binding to the receptor their conformation does not change. Such a conformationally restricted cyclic antagonist is D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2 (CTOP), with high affinity and mu selectivity. This analogue has a 32,500-times higher affinity for mu than for delta receptors, which practically means preclusion of the possibility of non-receptor-specific responses. Our knowledge concerning the physiology, pharmacology, and localization of the opioid receptors is based almost exclusively on the application of receptor-selective and very often conformationally restricted opioid ligands. This thesis discusses the results of pioneering investigations in which such a delta or a mu opioid receptor-selective conformationally restricted ligand was used.

The functions and features of any cell depend on the peptides inside the cell. However, the amounts of particular proteins are regulated by the function of the genes, i. e. the concentration of the mRNA coding the protein, the frequency of the translation and, of course, the stability of the protein. These three factors determine the scale and quality of the gene expression. The different activities of the genes and the control of the gene expression determine the functions and features of the cells. In eukaryotic cells, the control of the gene expression occurs decisively at the level of the transcription. The manifestations of the genes are determined by ontogenetic programmes, while the direction and the degree of any physiological process are determined by the unique process of gene expression. Some physiological or pathophysiological stimuli can increase the activity of certain genes and even the postranslational modifications of the primary transcripts can often be modified.

In the cell, the synthesis, release and degradation of the transmitters or the hormones, and also the number of receptors are regulated, and as a result of the gene function they are under genetic control. The transmitter substances of certain transmitter systems (cholinergic, catecholaminergic, aminergic, etc.), e.g. the acetylcholine, are synthesized in several reaction steps involving collaboration with a number of enzymes; further, their degradation can occur, and thus the regulation of transmitter synthesis involves control of the gene expression of some (catabolic and anabolic) enzymes. However, the synthesis of transmitter substances in the neuropeptidergic systems is under direct genetic control (transcriptional control, posttranslational modifications), and thus the regulation of gene expression in these systems can be investigated directly. The genes of these peptides, as sensitive indicators, can rapidly react to homeostatic or pathophysiological changes, while their gene expressions can be regulated differently even in a single cell. This thesis discusses results concerning regulation of the synthesis of two neuropeptides, vasopressin (VP) and prodynorphin (PD), and also Gs_α coupling protein. In connection with the metabolic and homeostatic changes during signal transduction, an account is further given of experience regarding the regulation of some trace elements and some mono- and divalent cations at the level of the nervous system.

There are three known families of endogenous opioid peptides: 1. the proenkephalin A derivatives of which the most important are the ME and LE derivatives, and other longer fragments such as the hecta- and octapeptide fragments and peptide E; 2. PD (proenkephalin B) -derived peptides such as the dynorphin A and B fragments, α- and β-neo-endorphin and leumorphin; 3. the proopiomelanocortin (POMC) -derived peptides e.g. α-, β- and γ-endorphins. Although the nucleotide sequences of these genes are similar to a certain extent, their expressions differ, and several physiological and pathophysiological factors may be involved in their control.

Beside its essential and well-characterized effects, VP seems important in memory processes and in the development of alcohol tolerance/dependence processes. The brain distribution of the cells expressing the peptide or rather the gene is well known; its localization in the brain has been revealed by immunocytochemistry and *in situ* hybridization studies. More and more details are available on the changes in VP level during altering environmental stimuli or experimental interventions, an also the regulation of its gene expression. Various factors are involved in this regulation. It was proved that, even in cells where PD and VP are colocalized, the regulations of their gene function differ considerably, even in the case of the same stimulus. The thesis discusses the changes in neuronal gene expression in the VP and PD genes during experimental intervention.

In certain signal transduction systems, the perception of the extracellular stimulus and the generation of the intracellular response are carried out by the same peptide or peptide complex. In other cases, the relay and the intracellular effector are different proteins, which are coupled to an intermediate guanine nucleotide binding regulator protein (G protein). The G proteins have a heterotrimeric structure (α, β and γ subunits); hardly a dozen Gs_α subunits are known, but they are able to bind nearly a hundred receptors and ion channels defined by pharmacological and/or molecular biological methods. The primary structures, the localization and the role in signal transduction of the different G proteins are known, as are the changes in the regulation of their genes during physiological and pathophysiological stimuli. The thesis reports investigations by *in situ* hybridization and *in vitro* transcription methods of the changes in the Gs_α expression during chroniç ethanol treatment.

There are a numerous methods suitable for the investigation of neuronal signal transduction. In investigations of the structure and the function of the receptors, use can be made of the registration of physiological, pharmacological (tissue or organ) and biochemical (receptor-induced activation of enzymes) responses or direct receptor-binding measurements (membrane binding, autoradiography). Through the use of the high-affinity and often conformationally restricted, receptor-selective ligands developed in the last few years, the receptor-specific physiological responses can be determined, and the data acquired with the use of the early ligands, which often had cross-reactions, can frequently be re-evaluated. The use of these ligands with high specific activity made it possible to measure their binding to the receptors directly. With the aid of the methods of molecular biology (*in situ* hybridization, *in vitro* transcription, analysis of gene sequences, Northern analysis and polymerase chain

reaction), it is possible to investigate the control of the synthesis of certain peptides, including neuropeptides, at the level of the genes.

This thesis investigates the signal transduction mediated by the different muscarinergic and neuropeptidergic (primarily opioidergic, somatostatinergic and vasopressinergic) receptors in neuronal tissue under normal, pathological or experimental conditions. Data acquired through the use of receptor-binding studies and the methods of molecular biology are summarized. An account is also given of the changes in the control of the gene functions of two neuropeptides (PD and VP) and a coupling protein (Gs_{α}) during experimental interventions.

In the course of the investigations, answers were sought to the following questions:

1. What are the characteristics of the synthesis, the intracellular transport, the subcellular and autoradiographic distributions of the mAChRs in the central and peripheral nervous systems of the rat?
2. How do the binding parameters of the mAChRs in the central and peripheral nervous systems of the rat change under physiological, pathophysiological and experimental conditions?
3. How can the muscarinergic, somatostatinergic and delta opioid receptors be characterized in the course of the neurodegenerative Alzheimer's disease? Do these binding parameters change in two animal models of Alzheimer's disease?
4. What are the characteristics of the heterogeneity of the mAChRs in the tissues of the heart and brain in the rat, and in human neuroblastoma cultures?
5. What are the conditions of receptor selectivity according to the ligand conformations in the case of substance P and the delta and mu opioid receptors?
6. What are the autoradiographic distributions of somatostatin, delta and mu opioid receptors in the central nervous system of the rat?
7. What are the physiological and pharmacological characteristics of DPDPE and CTOP?
8. How do the delta opioid receptors control the level of some neuronal trace elements which are mono- and divalent cations?
9. How do the gene expressions of VP and PD alter during dehydration and chronic ethanol intoxication?

Materials and methods

Materials

Studies were carried out with adult male and female rats of the CFY, Long-Evans, Wistar and Sprague-Dawley strains, and with mice of the C57BL/6NCR and CFLP strains, using several parts of their central and peripheral nervous systems and hearts, the ileum of guinea-pigs, the lumbar stretch of the spinal cords with the descending n. ischiadicus of albino rabbits, the spinal cords of newborn pigs, the brains of carps, postmortem human brain tissues from the parietal and frontal cortex and the hippocampus from normal members of the British population (age±S.E.M.=81±8years) and from subjects with Alzheimer's disease

(age±S.E.M.=78±6years) (Newcastle General Hospital, Newcastle upon Tyne, UK), bred cells of the human neuroblastoma cell line (SH-SY5Y) which has a low passage (60-85). For the ontogenetic studies, the striatum and the cerebellum of rats from the same family but of different ages were used.

Methods

Anatomical methods: electron microscopy; enzyme histochemistry, autoradiography for light microscopical studies and histology.

Surgical procedures: electrolytic lesion; nerve ligation; nerve transection; perineural suturing; isolated ileum preparation from guinea-pig; opening of the spinal cord and the brain in order to apply substances (e.g. NVP, ibotenic acid, beta-BT and opioid substances); injection of neurotoxins (ibotenic acid and beta-BT) into the brain tissue or the ventricle in order to develop neurodegeneration; GABA microinfusion through a capillary implanted into the superior cervical ganglion.

Biochemical methods: subcellular fractionation; release of ACh by a solution with high K+ concentration; receptor solubilization; cell culturing (SH-SY5Y); measurement of enzyme activity (ChAT, AChE and BuChE) and protein content; purified membrane preparation; receptor binding.

The methods of molecular cell biology: *in situ* hybridization; *in vitro* transcription, nuclear run-on assay; Northern analysis (total RNA analysis); polymerase chain reaction; gene sequence analysis.

Experimental interventions: acute opioid treatment; chronic morphine treatment; chronic aluminum intoxication; chronic ethanol intoxication; dehydration; chronic cold stress.

Other methods: analgesia; measurement of body temperature and stereotype forms of motion.

Physical-chemical methods: atomic absorption; gas chromatography; spectrophotometry.

Mathematical methods: nonlinear regression analysis; image analysis (computer microdensitometry); statistics (ANOVA, Dunnett or Scheffe *post-hoc* tests, Student's *t*-tests).

Summary of new results and discussion

1. In the striatum, the mAChRs are already synthesized intensively during early postnatal life. The early perikaryonal synthesis of the receptors and their rapid axonal transport are responsible for the fact that the development of the striatal mAChRs precedes the development of ChAT and AChE activities. In the striatum, the B_{max} values increase in every subcellular fraction during ontogenesis, and the binding sites are highest in the microsomal and the synaptosomal fractions throughout. The number of mAChRs originating from areas outside the striatum is low. Consequently, in the subcellular fractions the measured receptor contents are due to the striatal pre- and postsynaptic receptors.

The mAChR contents of two well-defined areas of the cerebellum, the archi- and paleocerebellum, were detected during postnatal development. The distributions of these receptors in the developing cerebellum are different: in the archicerebellum, the mAChR content is higher than in the paleocerebellum, and the rate of synthesis there is faster too. Our autoradiographic studies indicated that the molecular layer of the cerebellum exhibits higher [^{3}H](-)QNB binding than the granular layer or the deep cerebellar nuclei.

The mAChRs transported in the n. ischiadicus are synthesized in the motoneurons of the spinal cord and reach their presynaptic positions by rapid migration. Our results allowed the conclusion that the mAChRs undergo two-directional (anterograde and retrograde) transport in the nerves. The anterograde transport is extensive, whereas the retrograde transport moves a much lower content of the receptors. This seems to be

proved by our autoradiographic studies. The functional role of the presynaptic mAChRs could possibly be modulation of ACh release. During neurodegenerative processes, the number of receptors transported through the axon is in direct proportion to the time of axonal regeneration; 5 months after neural transection and resuturing, the bidirectional receptor transport, which returned to 23-26% of the control value, is able to provide a basis for the function of the regenerating nerve.

2. The binding parameters of the mAChRs can be modified experimentally. We found that, as a result of *in vivo* beta-BT treatment, a nonselective, presynaptic neurodegeneration develops, which leads to decreases in both B_{max} and K_d. Repeated toxin treatment resulted in 52.2% of the control value in the case of B_{max} and 52.6% of the control in the case of Kd. During cell death, the degraded receptor content presumably has a presynaptic localization; and its deficiency might be responsible for the changes in the binding parameters. The M_2 character of the presynaptic mAChRs in the hippocampus is proved by the facts that AF-DX 116 stimulates and PZ has no effect on the release of ACh.

During cold stress, the number of hippocampal receptors increases to 166.6% of the control value, while the K_d value does not change. This change in the receptor number is remarkable since it accompanies a protein content decrease of 21.5%. It was presumed that during chronic cold stress the adaptive biochemical changes (within them the decrease in ACh content or the release can give rise to the lack of the transmitter) cause functional denervations which, through homologous regulation, stimulate mAChR synthesis.

Not all experimental interventions lead to changes in the binding parameters of the mAChRs. NVP, a specific inhibitor of ChAT, does not directly affect the mAChR content of the brain areas investigated. Although the ACh content decreases significantly in response to NVP and, can cause denervational hypersensitivity, during a short NVP treatment there is no significant *de novo* receptor synthesis. The long-lasting inhibition produced by a chronically applied GABA microinfusion into the superior cervical ganglion (although free postsynaptic thickenings of the plasma membrane appear) does not give rise to significant changes in the receptor binding parameters.

3. Binding studies on the muscarinergic radioligands ([^{3}H](+)CD, [^{3}H]PZ and [^{3}H](-)QNB) suggest that there is no significant difference between the mAChR contents in human brain tissues of patients with Alzheimer's disease and controls, but the use of [^{3}H]MCC to measure the binding of the nAChRs indicates a lower number in the disease. During our studies, we first reported the [^{3}H]PZ binding in the human frontal cortex imaged by light microscopic autoradiography, which was compared with the [^{3}H](-)QNB binding in consecutive sections. In the course of the disease, the number of somatostatin receptors decreases significantly, but there is no change in the number of delta opioid receptors. Selective lesioning of the nBM does not yield any changes either in the receptor number of the forebrain or the portion of the presumed individual receptor types. In the muscarinergic system as with other transmitter systems, we were not able to simulate faithfully all the neurochemical changes

observed in Alzheimer's disease by selective lesioning of the nBM. In the animal model in which an increased Al level characteristic of Alzheimer's disease was set up serious symptoms of cholinergic hypofunction were observed. In response to chronic Al intoxication, beside the increased endogenous Al content of the brain, significant decreases in ChAT activity and in the numbers of mAChRs and nAChRs were observed.

4. During the direct and indirect receptor binding studies of selective and nonselective muscarinergic ligands prepared in rat brain and heart tissues, human neuroblastoma cell lines and carp brain, we described mAChR heterogeneity, in part confirming the previous findings, and in part providing new results on mAChR heterogeneity. Pharmacological verification of this receptor heterogeneity was greatly promoted by our investigations and data. We first described the direct binding properties of the new cardioselective antagonist, [^{3}H]AF-DX 116 in brain and heart tissues and also in the human neuroblastoma SH-SY5Y cell line.

5. Investigations of the neuropeptide systems, their receptors, their heterogeneity and the conformational requirements of their peptide ligands led to the development of a peptide antagonist with the greatest mu opioid receptor selectivity and affinity so far. D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2, a cyclic somatostatin analogue octapeptide (CTOP), has more than 32,500-fold mu/delta selectivity, but practically no somatostatin activity. Investigations of the binding of [^{3}H]CTOP to membranes or tissue slices reveals the receptor population indicated by this radioligand to be homogeneous; selective mu opioid ligands can inhibit it with high affinity, selective delta and kappa ligands have very low inhibitory activities, and non-opioid ligands fail to inhibit at all. On the other hand, the binding of [^{3}H]DPDPE to membranes or tissue slices has high affinity towards delta receptors, and delta/mu selectivity. The specific binding can be inhibited with high affinity by using selective delta agonists or antagonists; CTOP and other mu selective ligands have low affinity to bind to these receptors.

6. In acute and chronic experiments, the physiological effects of DPDPE and CTOP were investigated through the analgesia, withdrawal effects (e.g. hypothermia and loss of body weigh) or the ability to antagonize the effects. We established that CTOP, which according to the applied test has 10-400 times greater potencies as an antagonist than naloxone, inhibits the analgesic effects of morphine effectively, in a dose dependent manner, and produces withdrawal effects rapidly in morphine-dependent animals. CTOP induces withdrawal hypothermia and loss in body weight in morphine-dependent animals. I.c.v. application of CTOP after development of a morphine-induced dependence decreases the body weight (by increasing salivation, urination and diarrhoea) in a dose dependent manner. This drug alone does not cause analgesia and does not have any effects on body weight or temperature. The fact that peripherally administered CTOP does not affect the symptoms of morphine-induced chronic dependence proves that the drug is virtually unable to cross the blood-brain barrier. This observation may be of great importance: in the development of the morphine dependence, the central and not the peripheral mu receptors play the key

role. We were among the first in the literature to publish the physiological effects of an opioid antagonist with high receptor selectivity.

In parallel with the above studies in order to elucidate the actions realized through the delta receptors, we looked into certain physiological effects of DPDPE, which has a high delta opioid receptor selectivity. Dose-dependent analgesia antagonized by naloxone was observed in mice receiving i.c.v. DPDPE. Acute tolerance developed in response to the drug, and to a lesser degree physical dependence also appeared (withdrawal hypothermia and loss in body weight). DPDPE did not end the serious symptoms of morphine withdrawal. It was concluded from our experiments that the central delta opioid receptors play a role in the processes of analgesia. In a dose-dependent manner DPDPE also increased the stereotype scratching and scenting of rats.

7. After characterizing [^{125}I]CGP 23,996, [^{3}H]DPDPE, [^{3}H]PLO17 and [^{3}H]CTOP binding to cryostat slides, we investigated the autoradiographic distribution of their specific binding in the rat brain. The distributions of the somatostatin, mu and delta receptors were examined with these conformationally restricted cyclic ligands by means of computer densitometry analysis. The autoradiographic characterization of the mu and delta opioid receptors with high selectivity and affinity was performed for the first time.

8. We observed dose- and time-dependent effects (antagonized by naloxone) of the delta opioid receptor-selective agonist DPDPE in the levels of cations and trace elements in the rat brain. A subanalgetic dose of DPDPE transiently decreased in a time- and dose-dependent manner, the levels of Ca^{2+}, Mg^{2+}, Zn^{2+} and Al^{3+} , but it did not influence the levels of Na^{+}, K^{+} and Mn^{2+}. These effects of the drug on the ions can in all cases be inhibited by naloxone pretreatment. We observed that the high Al content developed by chronic Al intoxication, which also led to a cholinergic hypofunction, can be decreased by DPDPE treatment. In the future, medical application through the delta opioid receptors could possibly decrease the endogenous Al content observed in Alzheimer's disease and probably in casual relation with it.

9. During experimental interventions, we investigated the changes in the gene expression of the neuropeptide systems in the case of VP, PD and Gs_{α} in the mouse brain. During dehydration, the VP gene expression increased in all examined hypothalamic and extrahypothalamic structures (except the n. suprachiasmaticus), and during chronic ethanol intoxication decreased in all areas. The PD gene expression also altered during these stimuli; it increased in the course of both experimental paradigms. In the areas where VP and PD can be colocalized (n. paraventricularis and n. supraopticus), these two genes produced different controls for the same stimuli, indicating that the nervous system reacts in a transmitter- and not stimulus-specific manner to the changed environment. In connection with this study, we first defined the partial sequence of the main, translating exon of the mouse PD gene, which also involves the mature hormones. During chronic ethanol intoxication, the expression of the Gs_{α} gene altered, depending on the brain areas.

Possible applications of the results

Our knowledge concerning the biochemical and receptor binding data on the mAChR types, which play an important role in the muscarinergic cholinergic transmission of the mammalian central and peripheral nervous systems, was quite defective, and thus the results reported in this thesis added further data to the previous studies, accordingly mostly having a basic research character. The realization that, in certain human diseases or as a result of some environmental stimuli, the binding parameters of the mAChRs could change, turned our attention to the importance of the receptors and the possibility of regulation by environmental factors through the receptors. The high-affinity and selectivity muscarinergic agonists and antagonists developed for the different receptor types may possible be suitable for the diagnosis or even cure of certain diseases.

With our animal models (e.g. for Alzheimer's disease or paraplegia), we were able to simulate neuropathological alterations which are almost impossible to investigate in human subjects. Although the limits of these models are obvious, they still make it possible to perform several pharmacological, biochemical and molecular biological studies.

The studies regarding the neuropeptidergic systems described in the thesis have already provided results exploitable in practice. Our studies concerning the neuropeptide receptors and the conformational requirements of their ligands resulted in the production of opioid ligands with high affinity and selectivity, and some of them can now be purchased commercially. The conformationally restricted, high-affinity ligands with selectivity for a unique receptor and high resistance against biodegradation (we participated in their development and the first tests in biological systems) are much in demand for both basic and clinical research all over the world. These drugs lack the cross-reactivity often observed in the case of the opioid receptors, which was characteristic of the previously available opioid substances and consequently they have receptor specificity. In our other studies, e.g. on the role of the delta opioid receptors in the regulation of ion movement, we followed the control of the physiological processes based on this receptor heterogeneity. Using opioid ligands in our studies, we also provided the basis for clinical research in which the aim was the restoration of the neuronal trace element content or the ion surroundings damaged during an illness or a trauma. Investigations of the gene expressions of neuropeptide systems led to the first observations of basic research, e.g. partly sequencing the main exon of the PD gene coding the mature hormones, and we increased the knowledge concerning the function of the genes in respect of molecular biology, thereby turning attention to alcoholism and the results on the level of the gene function of the development of alcohol tolerance and dependence.