

The History of Neuropeptides IV

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STUDIES OF NEUROPEPTIDES AS A SPECIFIC GROUP OF BIOLOGICALLY ACTIVE COMPOUNDS (1974-)

The current period of studies on neuropeptides may be characterized by the appearance of a unifying neuropeptide concept. Various separate pieces of knowledge have been assembled and, finally, the idea of neuropeptides as a novel class of highly specific, physiologically active compounds possessing a number of specific properties has become widely recognized. Two major events in the mid and late 1970s gave powerful impetus to the studies of biologically active peptides: the discovery of the family of opioid peptides and the demonstration of the localization in the central nervous system of the several peptides otherwise known to be produced in peripheral organs and tissues. These discoveries not only laid the groundwork for the development of new research areas, but also led to other important findings. The fact that substance P is present in both the brain and the viscera (gut and lungs) suggested that this type of distribution may be common for some other peptides as well. In 1974 it was established that somatostatin, known to be produced in the brain, is also produced in the pancreas. This finding was followed by a search for alternate localizations of other peptides, which resulted in the discovery of a number of biologically active peptides in the CNS. Studies on the physiological role of these peptides in the central nervous system revealed that they possessed neurotropic activities.

Although this paper deals with the most recent period in the history of neuropeptides, it is not meant to present a review of all recent findings in this huge and rapidly expanding research area. Instead, it seeks to reconstruct the development and sequence of major events which contributed to it.

DISCOVERY OF ENDOGENOUS OPIOID PEPTIDES

The demonstration of the existence of opiate receptors raised the question of the presence of their endogenous ligands within the animal organism since it seemed highly improbable that such highly specific structures would exist only to interact with poppy alkaloids. In 1974, L. Terenius and A. Wahlstrom from

the University of Uppsala reported the demonstration of endogenous ligands of stereospecific opiate receptors (240). They showed that a peptide was present in brain extracts and in the cerebrospinal fluid which inhibited the binding of labeled opiates to opiate receptors. At a meeting held in May 1974, J. Hughes also reported progress toward the isolation of endogenous opiates. He had found that crude brain extracts contained small peptides which inhibited electrically stimulated contractions of the mouse *vas deferens* (101).

The clinical and pharmaceutical possibilities resulting from the discovery of endogenous opiates were obvious and competition in this field became fierce. In 1975 five groups reported simultaneously on the isolation of endogenous morphine-like compounds. J. Hughes and associates from the Hans Kosterlitz laboratory at Aberdeen University isolated from acidic-acetone brain extracts a substance which they named enkephalin. Enkephalin, like opiate drugs, inhibited contractions of the isolated mouse *vas deferens* and guinea pig ileum, and these effects could be abolished by naloxone. Enkephalin could be destroyed by incubation with peptidases and had a molecular mass of about 700 (101, 103). L. Terenius and A. Wahlstrom isolated a 1000-Da peptide from human brain and cerebrospinal fluid (241). G. M. Pasternak, S. H. Snyder, and R. Goodman of John Hopkins University found a similar proteinase-sensitive factor in rat and calf brain (177), and H. Teschemacher, K. E. Opheim, B. M. Cox, and A. Goldstein demonstrated the presence of a morphine-like peptide in acetone-acetic acid extracts of bovine pituitaries and crude ACTH preparations (242). The factor obtained by Goldstein's group had a higher molecular weight than the similarly acting compounds isolated by the three other groups and was resistant to the action of proteolytic enzymes (42).

Kosterlitz' group was the first to purify the endogenous ligands of opiate receptors. Determination of the amino acid composition of enkephalin isolated from porcine brain extracts and purified on ion-exchangers (99) revealed six amino acid residues: Tyr, 2Gly, Phe, Met, and Leu. L. Fothergill established that the sequence of the four amino-terminal residues was Try-Gly-Gly-Phe and both Met and Leu could be detected at the C-terminus. The primary structure of enkephalin was finally established using mass spectrometry. It was found that enkephalin was a mixture of the two pentapeptides, Tyr-Gly-Gly-Phe-Met and Tyr-Gly-Gly-Phe-Leu, in a 3-4:1 ratio (104). Hughes *et al.* pointed out that the structure of Met-enkephalin is identical to that of the (61-65) fragment of beta-lipotropin. The structural analogy among beta-lipotropin, α - and β -MSH, and ACTH had by then been recognized. β -MSH was shown to be contained within the β -lipotropin sequence and α -MSH was identified as an acetylated N-terminal fragment of ACTH. Hughes proposed the idea of a structural relationship between β -lipotropin and opioid peptides, and particularly with the peptide purified by Goldstein's group. He also proposed that lipotropin might be a natural precursor of opioid peptides.

The enkephalins were shown by Hughes and associates to be active in the mouse *vas deferens* and guinea pig ileum preparations and the effect was shown to be naloxone-reversible, inferring that it is mediated by opiate receptors. In 1976 A. W. Waterfield, J. Hughes, and H. W. Kosterlitz demonstrated

that morphine-tolerant rats exhibited cross-tolerance to enkephalins (253). Systemic administration of even large doses of synthetic enkephalins did not produce analgesia, presumably due to rapid degradation of enkephalins in blood. At the same time several groups were able to show the ability of centrally administered enkephalins to reduce pain in rats and mice (15, 31, 81). Candace Pert and associates were the first to obtain a peripherally active proteolysis-resistant synthetic enkephalin analogue by introducing a D-amino acid residue in its structure (189).

The exciting discovery of endogenous opioid peptides was immediately recognized to be of major importance. The first paper by Hughes *et al.* (99) on the discovery of an endogenous morphine-like compound appeared in the list of the most cited articles of the year in 1975 (73). Many other groups joined the search for new endogenous opiate ligands. Guillemin's group had the advantage of their large supplies of pituitary and hypothalamic extracts. Very soon they detected in these extracts a number of peptides possessing opiate activity. These were called endorphins (from "endogenous morphines"), a term suggested by E. Simon. Elucidation of the primary structure of these peptides revealed that α - and γ -endorphin sequences are identical to those of the (61–76) and (61–77) fragments of β -lipotropin molecules (85, 127, 135).

β -Endorphin, the most potent endogenous opioid, was discovered in 1976 by several groups. C. H. Li, whose work on the determination of the primary structure of polypeptide hormones was mentioned earlier, and D. Chung found a β -lipotropin (–91) fragment in camel pituitary extracts which possessed very high opiate activity (133). The same year the primary structures of β -lipotropin and β -endorphin from human pituitary glands were determined in the same laboratory, and β -endorphin was shown to be a potent analgesic on intravenous injection (132, 134, 243). A. F. Bradbury *et al.* (22) isolated a "C fragment" from pituitary extracts and a mixture of fragments obtained after the treatment of β -lipotropin with trypsin. The opiate activity of (61–91) lipotropin was also demonstrated by a Hungarian group headed by L. Graf (80).

The discovery of endogenous opioid peptides spawned a number of studies on the localization of opioid peptides and opiate receptors in the brain and peripheral tissues, on their functions and mechanisms of action, on the pathways for production, and metabolism of these compounds in the organism, i.e., their biosynthesis and processing. Pioneering studies on neuropeptide biosynthesis were performed in 1975–1977 by R. E. Mains and B. A. Eipper at the University of Oregon and the University of Colorado and by S. Nakanishi and associates at Kyoto University.

Mains and Eipper studied ACTH biosynthesis in mouse pituitary tumor cell cultures. Using the double immunoprecipitation technique they were able to detect four forms of ACTH differing in molecular weight: 4.5, 13, 23, and 31 (61, 141). The data so far accumulated suggested that ACTH and β -lipotropin, and presumably β -endorphin, are secreted simultaneously. Mains and Eipper added another stage to the existing technique, immunoprecipitation of the different forms of ACTH with antiserum developed against β -endorphin. As a result, three additional forms of β -endorphin, 3.5, 17.5, and 31 kDa, were found (140).

The majority of fragments obtained after the treatment of "big" ACTH (31 kDa) could be precipitated with anti- β -endorphin antiserum, which suggested the presence of β -endorphin antigenic determinants in their sequences. The 17.5- and 3.5-kDa fragments seemed identical to β -lipoprotein and β -endorphin, respectively. Mains *et al.* (140) concluded that ACTH and endorphins are formed as a result of cleavage of a common precursor with a molecular weight of 31 kDa. Eventually, using various methods of peptide chemistry, Eipper and Mains established the structure of the precursor molecule: they showed that it included a C-terminal portion containing a lipotropin sequence, an ACTH-containing portion, and an N-terminal 16-kDa glycopeptide (62). The fragment of a precursor molecule corresponding to the ACTH sequence could also be found in a glycosylated form.

The conclusions of Mains and Eipper were confirmed in 1977–1978 by S. Udenfriend's group. They found that rat pituitary contained a 30-kDa polypeptide chain, the hydrolysis of which led to the generation of fragments possessing opiate activity (204, 205). The common precursor of these opioid peptides and ACTH was named pro-opiocortin (205). Similar results concerning the biosynthesis of ACTH-related peptides in isolated cells of the pars intermedia of the rat were obtained by R. Crine *et al.* (43, 44) in M. Chrétien's laboratory in Montreal. In 1978–1979 three groups provided immunohistochemical evidence that both ACTH and β -endorphin are present in the same hypothalamic neurons (19, 169, 254).

Novel methods of DNA cloning and sequencing developed in the 1970s (38, 143, 207) made it possible to determine the primary structure of eukaryote genes. These methods had been successfully applied by J. L. Roberts *et al.* (199, 200), working in the laboratory headed by E. Herbert (University of Oregon), and by S. Nakanishi and associates at Kyoto University to the characterization of the common ACTH and β -lipotropin precursor. In 1976 S. Nakanishi *et al.* (162) initiated studies on the translation of mRNA coding for ACTH in a heterologous cell-free system. They were able to demonstrate the translation of a large (35 kDa) ACTH precursor. In 1977 they demonstrated that the translation product of ACTH mRNA contains the β -endorphin sequence (161). In 1978–1979 Nakanishi's group isolated mRNA from bovine pituitary, and the double-stranded cDNA complementary to this mRNA was synthesised *in vitro*. The cDNA thus obtained was inserted into an *Escherichia coli* plasmid, and the nucleotide sequence of the inserted cDNA was determined. Reconstruction of the corresponding amino acid sequence confirmed the conclusions of Mains and Eipper concerning the structure of the precursor molecule that had been named pro-opiomelanocortin and allowed certain regularities in its structure to be identified (159, 160). It was found that pro-opiomelanocortin (POMC) contains repetitive sequences of α - and β -MSH chains, as well as a third MSH-like sequence, γ 3-MSH, that is contained in the N-terminal portion of the POMC molecule. All the fragments of POMC corresponding to the individual hormones were separated by pairs of basic amino acid residues, Lys and Arg.

Later it was established that processing of POMC is tissue-specific. The cells of the anterior pituitary and of the intermediate lobe convert POMC into two

completely different sets of peptides: β -LPH, ACTH, and the 16-kDa N-terminal fragment are the main products in the anterior pituitary. Part of β -LPH is processed further to γ -LPH and β -endorphin. In the intermediate lobe, ACTH is processed to α -MSH and CLIP. This latter tissue was shown to produce more γ -LPH and β -endorphin than the anterior pituitary, and γ 3-MSH was found in both tissues (30, 92, 188, 198).

The search for the new opioid peptides ran in parallel to studies on the biosynthesis of ACTH-related hormones and endorphins. In 1979 K. Kangawa, H. Matsuo, and M. Igarashi isolated a novel opioid peptide from porcine hypothalamus which they called α -neo-endorphin (113), and Goldstein's group discovered another pituitary opioid peptide, dynorphin (77). Both peptides contained the N-terminal Leu-enkephalin sequence. In 1980 N. Minamino *et al.* (149) isolated an octapeptide from porcine hypothalamus, which was identical to (1-8)-dynorphin.

In 1978 a group of researchers, including Terenius and T. Hokfelt, studied the distribution of enkephalin-like immunoreactivity in sympathetic ganglia and adrenal glands of several species (212, 214). They found that the adrenal medulla and nerve terminals arising mainly from fibers in the splanchnic nerve were rich in enkephalins. They proposed that enkephalins might be released from the gland cells into the blood as hormones and from nerve terminals as modulators or transmitters. In 1978–1980 a number of low- and high-molecular weight peptides containing enkephalin sequences were isolated from bovine adrenal medulla chromaffin granules and striatum by A. Beaumont and associates and by the laboratories of S. Udenfriend (Roche Institute) and H. Matsuo (Miyazaki Medical College, Kiyotake), using high-performance liquid chromatography (13, 117, 118, 130, 151, 152, 203, 226, 227). In 1980 Udenfriend's group isolated a 50-kDa protein which released one Leu-enkephalin and seven Met-enkephalin molecules as a result of treatment with proteases (131). The primary structure of this pre-proenkephalin was determined from the structure of its cDNA obtained from bovine adrenal medulla (82, 170) and a human adrenal tumor, pheochromocytoma (39), by three independent groups: those of Nakanishi (170), Udenfriend (82), and Herbert (39). The proenkephalin sequence was found to consist of 263 amino acid residues and to include four Met-enkephalin sequences and one copy of each of the following sequences: Leu-enkephalin, Arg6, Phe7-Met-enkephalin, and Arg6,Gly7,Leu8-Met-enkephalin. The latter was soon detected in the adrenal medulla. Thus the mechanism of biosynthesis of enkephalins and endorphins was established. The structures of α - and β -neo-endorphin (115, 148) and the dynorphin precursor from porcine hypothalamus were soon determined by the Japanese group (110) using the same technique. Proenkephalin B (as they called the precursor to distinguish it from medullary proenkephalin A) was shown to consist of 256 amino acid residues, with β -neo-endorphin and dynorphin sequences in positions 175–183 and 209–225, respectively. A third sequence of Leu-enkephalin was present at the 228–232 site, surrounded by Arg–Arg pairs.

The discovery of the existence of precursors common for several peptides had a strong impact on further developments in the studies on neuropeptides. The

pattern was established, which was important, and soon the structure of several other precursors (those for oxytocin, vasopressin, somatostatin, substance P, calcitonin, GRF, TRH, etc.) was determined (78, 94, 108, 144, 166, 196, 238). Studies on precursors were followed by the studies on the structure of genes encoding them, and conclusions were drawn concerning the evolution of neuropeptides and their genes (34, 58, 119, 163). The peptides whose sequences were revealed after determination of the structure of precursors (γ 3-MSH, substance K) were shown to be present in the brain and peripheral organs (27, 165).

Studies on opiate receptors proceeded in parallel with studies on their endogenous ligands. By the mid-70s, what was known about diverse pharmacological effects of opioids suggested that they might be mediated through more than one type of receptor. In 1976, based on data on behavioral and neurophysiological actions of morphine- and nalorphine-like drugs in naive, morphine-dependent, and cyclazocine-dependent chronic spinal dogs, W. R. Martin and associates (75, 142) arrived at the conclusion that at least three distinct patterns could be identified. They maintained that these were the result of drug interaction with three different types of opiate receptors: mu, kappa, and sigma, the agonists of which were morphine, ketocyclazocine, and SKF-10,047, respectively. Typically morphine-like drugs interact with the mu receptor, certain benzomorphanes (ketocyclazocine and ethylketocyclazocine), which do not substitute for morphine in morphine-dependent monkeys but are potent analgesics, act through kappa receptors, and drugs like *N*-allylnorcyclazocine interact with sigma receptors. The hypothesis of opiate receptor heterogeneity was supported by results obtained by Kosterlitz and his group (136). They compared the affinity of opioid peptides to opiate receptors in four test systems (depression of electrically induced contractions of the guinea pig ileum and of the mouse vas deferens, and the inhibition of binding of labeled naloxone and Leu-enkephalin in brain homogenates) and concluded that the opioid peptidergic system acted through different agonists interacting with more than one type of receptor. They argued that enkephalins and endorphins interact with delta receptors in the mouse vas deferens while, in the guinea pig ileum, opioid peptides were assumed to act on mu receptors. Analysis of the effects of enkephalins and β -endorphin led the authors to the conclusion that β -endorphin might have a less specialized function than the enkephalins. Comparing the opioid peptidergic system with the catecholamine system, they proposed that like adrenalin, which participates in the fight and flight reactions, β -endorphin might be of similar importance as a hormone producing, for example, analgesia on the battlefield or reducing pain during childbirth, while enkephalins, like noradrenalin or dopamine, might act as neurotransmitters or neuromodulators. Enkephalins would subserve transmission of a rapid and more or less transient character, while the longer chain peptides would mediate more lasting neuronal and possibly endocrine changes (136). In 1977 G. Urca *et al.* (244), in the course of studies on analgesia induced by intracerebroventricular injections of Met-enkephalins, discovered that this opioid peptide could also cause EEG and behavioral epileptic phenomena in rats and proposed that these

effects involve an interaction with receptors different from those mediating analgesia.

Further research on opiate receptors included studies on the pharmacological actions of synthetic enkephalin analogues resistant to proteolysis. By 1981 more than 300 enkephalin analogues were synthesized (88). Designing of synthetic analogues of various neuropeptides and the study of their pharmacological action became a vast research area involving many laboratories and institutes.

In 1976 J. K. Chang and associates showed that the regional distribution of mu and delta agonist binding in the brain is different (35). The concept of opiate receptor heterogeneity was further confirmed in several other studies in the late 1970s and early 1980s (220, 221, 223, 255, 262). Other types of opiate receptors were detected, for example, epsilon receptors in rat vas deferens with high affinity for β -endorphin (259), kappa receptors in guinea pig ileum interacting with dynorphin (37), and even iota receptors in rabbit ileum insensitive to morphine but sensitive to enkephalins (172). Some authors doubted whether every opiate peptide had a corresponding specific receptor, arguing that such organization would be too specialized and unreliable. G. W. Pasternak, A. Zhang, and L. Tecott, in particular, reasoned that the effects of opiate drugs and opioid peptides should be regarded as a consequence of their interaction with the whole spectrum of opiate receptors (178), and J. Hughes held a similar point of view (100, 102). The idea implied that small doses of the ligand would interact with only high-affinity receptors, while large doses would interact with both high- and low-affinity binding sites and perhaps have effects overlapping with or masking those mediated through high-affinity receptors. Thus higher amounts of opioid peptides are released during stress than under normal conditions, producing analgesia and euphoria via the mu receptor. Dynorphin was shown to interact with several types of opiate receptors as a mixed agonist-antagonist; it did not itself produce analgesia but it attenuated the analgesia induced by morphine and large doses of β -endorphin and potentiated the analgesia caused by small doses of β -endorphin (68).

The idea of the heterogeneity of the opiate receptor population was not shared by all workers (124, 218). Thus, E. J. Simon maintained that only one receptor existed that could change its conformation in different ways depending on the ligand with which it was interacting (218).

Studies on the localization of opiate receptors initiated in 1973 added information on their neurotropic functions. Receptors were found on both pre- and postsynaptic membranes (36), and much evidence suggested a neuromediator role for opioid peptides. These included release of opioid peptides upon depolarization, their ability to affect neuronal activity, synaptic localization, and the influence of opioid peptides on nonpeptide neuromediators (41).

Studies of behavioral effects of opioid peptides have been carried out by several groups since 1976 when F. Bloom *et al.* (20) and Y. F. Jacquet and N. Marks (109) simultaneously demonstrated neurotropic actions of opioid peptides. Guillemin's team reported on various behavioral effects of intracerebroventricularly injected high doses of endorphins in rats (20). β -Endorphin was

shown to be most potent at inducing the disappearance of corneal reflexes and responsiveness to pain-producing stimuli, as well as depression of general motor activity, finally resulting in a catatonic state. These effects were naloxone-reversible. Smaller opioid molecules could not induce the catatonic state and caused naloxone-reversible wet-dog shakes, which were regarded as typical symptoms of opiate withdrawal. Bloom *et al.* (20) proposed that the endorphins are involved in maintaining behavioral homeostasis. Jacquet and Marks obtained similar results (sedation and catalepsia) when they injected endorphins into the periaqueductal grey of the rat (109). They suggested that β -endorphin might be an important neuroleptic-like neuromodulator in the central nervous system. The neuroleptic-like actions of endorphins suggested an involvement in the etiology of schizophrenia, and the same year L. Terenius and associates found increased levels of opioids in the cerebrospinal fluid of schizophrenic patients (239). The possible involvement of opioid peptides in schizophrenia was investigated by Terenius in his other studies. In 1977 B. J. Meyerson and Terenius demonstrated that β -endorphin affected sexual behavior in male rats (147).

In 1976, de Wied, whose group was occupied at the time with investigations on the mechanism of behavioral effects of ACTH/MSH peptides, received a call from Guillemin. Guillemin informed him of their discovery of α - and γ -endorphins and asked if de Wied might be interested in studying the influence of these peptides on avoidance behavior (49). "The time had come to join the race," as de Wied put it, and his group began to do experiments based on active and passive avoidance behavior. Met-enkephalin appeared to be nearly as active as ACTH in delaying the extinction of pole-jumping avoidance behavior, when injected subcutaneously, and β -LPH- (31–39) and α - and β -endorphins were more potent (51). These behavioral effects of opioid peptides were not found to be mediated by opiate receptors. They could not be prevented by opiate antagonists. Since these effects were found with peptide amounts much smaller than those necessary to induce analgesia, it was proposed that endorphins were more involved in pain motivation than in pain perception.

In these experiments, β -endorphin was less active than α -endorphin. Further experiments with γ -endorphin and pole-jumping avoidance behavior revealed that, in contrast to the other opioid peptides tested, γ -endorphin facilitated rather than delayed extinction. This was consistent with the findings of Bloom *et al.* (20) that the effect of γ -endorphin on temperature (hyperthermia) was opposite to that of β -endorphin (hypothermia). It was exciting for de Wied's group to find that the two closely related peptides, α - and γ -endorphin, differing by only one amino acid residue, exerted opposite actions on extinction. γ -Endorphin appeared to be a most promising neuroleptic-like neuropeptide, and since de Wied assumed that its behavioral effects were not mediated by the opiate receptors, he decided that it might be worth testing a des-Tyr analogue for behavioral activity. The choice of the structure modification was made according to the same logic as had been applied to ACTH-related peptides devoid of hormonal activity, since it was known that N-terminal Tyr is essential for opiate activity. The results of experiments with the new analogue revealed that des-Tyr- γ -endorphin was even more potent than γ -endorphin in facilitating the

extinction of pole-jumping and passive avoidance behavior (54). These and other findings were interpreted by de Wied as indicating that des-Tyr- γ -endorphin is an endogenous neuroleptic-like peptide. He could thus propose that a reduced availability as a result of an "inborn" deficiency of this peptide might be an etiological factor in the psychopathological state for which neuroleptic drugs are beneficial (54). Should the result of an "inborn" error be the generation of α -endorphin-like peptides from β -endorphin, their "amphetamine-like" effects might be responsible for the psychotic symptoms. In 1978, after the group had tested des-Tyr- γ -endorphin effects on animals for toxicity, it was decided to try the peptide in schizophrenic patients. This work was performed by W. Verhoeven of the Utrecht University Psychiatric Clinic and J. van Ree of the Rudolf Magnus Institute. All six patients in the group selected for the trial, who were partly or totally resistant to neuroleptic drugs, responded to the treatment with des-Tyr- γ -endorphin by a decrease in their psychotic symptoms, although the effect was short-lived in three (249). The experiments were repeated with the same results in a double-blind crossover study in eight patients. Another double-blind study with des-enkephalin- γ -endorphin (which had been shown to be the shortest chain retaining behavioral activity) demonstrated that this neuropeptide also possessed antipsychotic effects (49). The beneficial effects of γ -type endorphins in schizophrenia that de Wied and his associates had observed, together with their finding that all the γ -type endorphins they tested and α -endorphin are present in rat brain (248), plus results from several other laboratories, laid a solid foundation for further studies of γ -type endorphins in schizophrenia (246). The idea that des-Tyr-endorphins play an important physiological role was also supported by the discovery of an aminopeptidase responsible for the generation of this type of neuropeptides (93, 215).

Further studies on α -endorphin at the Rudolf Magnus Institute carried out in the late 1970s revealed that this peptide, as well as its des-Tyr-derivative, exert behavioral effects which resemble those of the psychostimulant drug amphetamine (49) in certain aspects. It was shown subsequently that the site and mode of action of α - and γ -type endorphins on the brain dopamine system are quite different from one another.

Another direction followed in the studies on opioid peptides was the question of their hormone-like effects on endocrine and other physiological functions, such as thermoregulation, digestion, and circulation (155). Studies on opioid peptides covered vast areas. The list of key terms from a recently published review by G. A. Olson, R. D. Olson, and A. J. Kastin (174) illustrates the topics it now covers: stress, tolerance, dependence, eating, drinking, depression, learning, memory, cardiovascular responses, temperature, respiration, epilepsy, activity, aging, mental illness, aggression, sex, immunology.

DISCOVERY OF THE LOCALIZATION OF PEPTIDES IN THE BRAIN AND OF THEIR NEUROTROPIC PROPERTIES

Soon after the discovery of somatostatin, K. G. M. M. Alberti *et al.* (2) in 1973 and D. J. Koerker *et al.* (120) in 1974 demonstrated its ability to inhibit the

release of insulin and glucagon from the pancreas, and in 1975 Schally's group (5), M. P. Dubois (60) and T. Hokfelt and associates (95) found immunoreactive somatostatin in the pancreas. A series of studies on somatostatin was performed in Schally's laboratory using a somatostatin antibody specifically developed for the purpose. The group showed somatostatin to be present in all regions of the brain with only about 30% of the total brain content in the hypothalamus (28). Somatostatin appeared to be widely distributed in the viscera and could be found not only in the pancreas, but also in different portions of the gastrointestinal system, where it was shown to inhibit the secretion of gastrin, secretin, and HCl (5). Dubois, using an immunofluorescent technique, established that, in the pancreas, somatostatin is produced by specific D-cells of the endocrine pancreas (60). The same year P. C. Goldsmith *et al.* (76) demonstrated that somatostatin is present in secretory cells in the pancreatic islets, using electron microscopic immunocytochemistry. In 1975 Schally's group and in 1978, Y. C. Patel and S. Reichlin (179), discovered "big" somatostatin, presumably a somatostatin precursor, and in 1980 the big somatostatin chain of 28 amino acid residues was isolated by three groups: Schally's, Guillemin's, and V. Mutt's (64, 193, 208). The same year C. Patzelt *et al.* (180), D. Schields (217), and R. H. Goodman *et al.* (79), using the pulse-labeling technique, demonstrated the biosynthesis and processing of somatostatin precursor in the islets of Langerhans, and in 1981 the primary structure of preprosomatostatin of different vertebrate species was discovered by three groups, all using a recombinant DNA technique (78, 94, 238).

The distribution of TRH in the brain was reported by A. Winokur and R. D. Utiger (258), M. J. Brownstein *et al.* (29), I. M. D. Jackson, and S. Reichlin (106), and C. Oliver *et al.* (173) in 1974. To study the distribution of TRH in specific nuclei of rat brain, Brownstein *et al.* (29) used a technique that involved pinching out the nuclei from frozen brain slices which had been developed by M. Palkovits in 1973 (176). This method, which allowed the assay of peptides in discrete nuclei, proved to be quite useful for the studies on localization of hypothalamic-releasing hormones and other neuropeptides in specific brain nuclei. Three years later J. E. Morley and associates detected immunoreactive TRH in the gut and in pancreatic extracts (157). Specific TRH receptors were found in a brain synaptosomal fraction, and in 1977 it was established by McCann's group that this peptide specifically inhibits feeding behavior (250). Subsequently, it was shown that TRH exerts various other neurotropic effects (194).

The presence of LH-RH in neurons of extrahypothalamic brain regions was shown in 1976. The demonstration by Moss and MacCann of the action of LH-RH on female copulatory behavior in rats was discussed earlier. In 1979 LH-RH was found in murine heart and visceral organs.

The finding of somatostatin in the pancreas, gut, and brain, and the fact that the substance P is widely distributed in the central nervous system as well as in internal organs, raised the question of whether other hormones and biologically active peptides produced in endocrine glands of higher and lower animals might be also present in the central nervous system. Thus, in 1974, it was

shown that insulin receptors are present in the brain (192). Also in brain, in 1975 J. J. Vanderhaegen, J. C. Signeau, and W. Geptes (247) detected immunoreactive gastrin. In 1976, immunoreactive cholecystokinin was found in the brain by G. J. Dockray (55), and VIP by S. I. Said and R. N. Rosenberg (206). Concurrently, J. H. Walsh and A. L. Holmquist reported the presence of immunoreactive bombesin in vertebrate gut extracts (252) and, in 1977, all peptides of the ACTH family were shown to be present in the brain (121, 122), as were prolactin (69), bombesin, vasopressin, oxytocin, thyrotropin, growth hormone, and insulin (57, 89, 175, 251). In 1979 immunoreactive bradykinin and glucagon were found in brain and thus could be added to the group of brain peptides (40, 229) and, in 1980, immunoreactive physalaemin was found in higher vertebrates (128).

Thus, it was established that most peptide hormones, and many other biologically active peptides, are produced not only in organs where they were traditionally produced, but also in the central nervous system where they presumably play a role different from that already recognized. One of the most striking discoveries in the course of immunohistochemical studies on neuropeptide localization was the finding of peptides colocalized in the same neurons with classical neurotransmitters, and the presence of more than one neuropeptide in the same neuron (67, 138). The leader in the immunohistochemical mapping of neuropeptides that developed in the late 1970s was T. Hökfelt of the Karolinska Institute, Stockholm. Hökfelt and his associates performed numerous studies of the central and the peripheral nervous systems, localizing peptides such as substance P, somatostatin, VIP, gastrin/cholecystokinin, angiotensin II, enkephalin, and neurotensin. The abundance of peptide-containing neurons in the central and particularly the peripheral nervous systems suggested that several peptides might occur in the same neuron (98). Demonstration of the coexistence of ACTH and β -endorphin in the same neurons by three groups has been mentioned earlier and, in 1980, Hökfelt and associates found that medullary raphe neurons projecting to the spinal cord contain both substance P and TRH. Some gastrointestinal neurons were found to contain both somatostatin- and gastrin-cholecystokinin-like peptide (97, 98, 213). The coexistence of peptides with classical "small" neurotransmitters was demonstrated by Hökfelt's group in several studies. The first evidence of such coexistence was obtained in 1977 when somatostatin-like peptide was shown to be present in noradrenergic neurons of the guinea pig peripheral autonomic ganglia, followed by the demonstration of the presence of substance P-like peptides in 5-hydroxytryptamine neurons by Chan-Palay *et al.* (33) and Hökfelt *et al.* (96) in 1978. Recognition that peptides initially isolated from extraneural systems can be produced in the brain and, vice versa, that brain peptides are also present in internal organs upset traditional notions of the differentiation of functions of endocrine and neural cells, as well as the classical one neuron one neurotransmitter concept.

Further studies uncovered previously unknown actions of neuropeptides. Angiotensin II, for example, is generated not only in blood, but also in the pituitary and in the brain. In addition to increasing blood pressure, stimulating

aldosterone and vasopressin secretion, and inducing drinking behavior, angiotensin II participates in the paracrine and neuroendocrine regulation of secretion of anterior pituitary and hypothalamic hormones (72). Many peptides were shown to interact with specific receptors in the CNS, and it was shown that they possess neurotropic activities, i.e., cause certain biochemical and electrophysiological changes in neuronal systems and affect behavior. It thus appeared that polylocalization of neuropeptides, which is a typical feature of a neuropeptide, is closely related with their polyfunctionality.

DISCOVERY OF NEW NEUROPEPTIDES

A number of new neuropeptides have been discovered since 1973. In 1974 Erspamer's group isolated a novel physaleimin-like peptide, uperolein (4). The primary structure of proctolin, an insect neuropeptide, was determined in 1975 by N. A. Starrat and B. E. Brown (224), and in 1977 D. Price and M. J. Greenberg elucidated the primary structure of a mollusc cardioexcitatory neuropeptide, FMRF-amide (201). Another neuropeptide, head activator, was isolated from a coelenterate hydra and its structure was sequenced by H. C. Schaller and H. Bodenmuller in 1981; this undecapeptide was shown to be present in rat intestine and bovine and human hypothalamus (21).

In 1977 G. A. Schoenenberger and M. Monnier elucidated the amino acid sequence of δ sleep-inducing peptide (DSIP), discovered in 1964 (210). Further studies of its effects, using a synthetic peptide and the antiserum developed against it, revealed that although DSIP is present in the CNS and the gut (7, 211) its role in the organism appeared to be other than sleep induction. This latter action was shown to be one of the effects of substance P (171) and other neuropeptides (105). The same year K. Uchizono's group isolated substance S, which they claimed to be one of the sleep-inducing factors, and Pappenheimer's group purified a sleep-promoting factor S, which caused excess slow-wave sleep in rabbits (105, 123).

It was shown in 1974–1978 that immunoreactive bombesin is present in the gut and brain of mammals and that it stimulates gastrin and cholecystokinin release and affects thermoregulatory functions (26, 154, 229). In 1979 a peptide was isolated from porcine stomach and intestines using the antiserum developed against bombesin by T. J. McDonald *et al.* (146) from Mutt's laboratory. Determination of its primary structure revealed that it is not identical to bombesin but only structurally related to it. The peptide was named gastrin-releasing peptide, GRP, because of its ability to induce gastrin secretion.

Urotensin II, a peptide structurally related to somatostatin, was isolated from the fish caudal neurosecretory system in 1980 (187). The same year K. Tatemoto and V. Mutt developed a new approach to the isolation of new biologically active peptides (158). Traditionally, detection of a new biological activity preceded the isolation of its active principle, and during the isolation procedure bioassays based on this biological activity were used for screening fractions for the presence of the active principle. Tatemoto and Mutt proposed

first to isolate new peptides from the tissues and then to study their biological effects. Taking into consideration the fact that C-terminal amide is present in the structure of many known peptides, the authors developed a technique for the isolation of C-amidated peptides. Using this method they discovered a number of C-amidated peptides in porcine intestine extracts, including the one they called PHI, which was structurally related to secretin, VIP, and glucagon, and another peptide, PYY, the structure of which resembled that of pancreatic polypeptide (236). The sequence of a 27-amino-acid residue chain of PHI which exhibited biological activities similar to that of vasoactive intestinal peptide was determined in 1981 (241), and subsequently PHI was also found in brain and thus joined the family of gut-brain peptides. In 1982 Tatemoto and Mutt isolated from porcine brain extracts a neuropeptide Y (NPY) which consisted of 36 residues and resembled PYY (235) and pancreatic polypeptide. Neuropeptide Y, detected by such an unconventional technique, was found to be one of the most widespread of neuropeptides. In the CNS it was found to be localized in neurons ranging from the cerebral cortex to the spinal cord, while outside the CNS, NPY-containing neurons were found to constitute a distinct group in the enteric nervous system, and throughout the periphery NPY was found in adrenergic nerves innervating smooth muscle. It was shown that NPY behaves as a potent vasoconstrictor (63), and it was subsequently established as an important neuromessenger in the regulation of anterior pituitary hormone secretion (111).

Galanin was discovered in the same laboratory in 1983 (237). This 29-amino-acid peptide originally isolated from porcine gut extracts was found to be widely distributed in the mammalian central nervous system, where it is colocalized with other neuropeptides. It exerts a wide spectrum of hormonal and neurotropic effects, including regulation of the release of pituitary hormones, growth promotion, and modulation of nociception. Galanin was shown to inhibit insulin release, hippocampal acetylcholine release, and firing of locus coeruleus cells and to stimulate feeding and the release of growth hormone (11). Subsequently galanin became implicated in the pathophysiology of Alzheimer's disease.

Neuroendocrine control of egg-laying in *Aplysia* was the object of extensive studies for several years. By 1981, the work of D. H. Schlesinger, S. B. Babirak, and J. E. Blankenship had demonstrated a complicated and peculiar mechanism of regulation of egg-laying, which includes the release of several peptide neurohormones in a particular order (209). The structure of neurohormones was determined.

After several unsuccessful attempts by other groups, another releasing factor, GRF (growth hormone-releasing factor), was isolated, using HPLC. Its primary structure was determined in 1982 by two independent research groups, both from the Salk Institute for Biological Studies, La Jolla. One group was from the Laboratory of Neuroendocrinology headed by Guillemin and another was from the Laboratory of Peptide Biology, where two of Guillemin's ex-associates, J. Rivier and W. Vale, worked. Both groups isolated GRF from the same source, a human pancreatic islet tumor which caused acromegaly. Guille-

min obtained the tissue from the Center of Nuclear Medicine, Lyon, and Rivier, Vale, and associates from the University of Virginia School of Medicine. GRF characterized by Guillemin's group was a polypeptide which consisted of 44 amino acid residues and possessed high structural homology with the peptides of the "secretin-glucagon" family, especially PHI (84). The peptide isolated by J. Rivier *et al.* (197) was identical to the (1-40)-fragment of GRF characterized by Guillemin's group. It was shown that the (1-29)-fragment retains full intrinsic growth hormone-releasing activity *in vitro*. The same year Vale and associates (245) succeeded in the isolation of another peptide, the existence of which had been demonstrated many years before—the famous, elusive CRF ("constant research frustration" as Guillemin once called it). CRF was purified from the old fractions of ovine hypothalamic extracts remaining after the isolation of LH-RH. It was found to be a 41-amino-acid residue peptide with a structure highly homologous to that of sauvagine, a frog skin peptide isolated by Erspamer's group (65). Like sauvagine, CRF stimulated ACTH and β -endorphin release. It was shown that CRF also releases MSH (195).

The fact that many peptides were found to be structurally homologous with other peptides allowed the development of yet another approach to the search for new neuropeptides, based this time on the possible affinity of unknown peptides to antisera developed against the known ones. Thus G. J. Dockray and associates isolated a novel hypertensive pentapeptide from chicken brain and identified it using antibodies to FMRF-amide (56). Simultaneously, R. E. Carraway and C. F. Ferris isolated a hypotensive neurotensin-related hexapeptide, Lys8-Asn9-neurotensin-(8-13) (LANT-6), from chicken intestine (32).

In addition to enkephalin-containing opioid peptides, several analgesic peptides with a different structure have been isolated. In 1979 H. Takagi *et al.* (232, 233) of Kyoto University isolated a dipeptide, Tyr-Arg, which they named kyotorphin, from bovine brain. This peptide could induce naloxon-reversible analgesia, but had no inhibitory effect on electrically induced contractions of the guinea pig ileum. In 1982 Takagi's group isolated a new analgesic pentapeptide from bovine brain, neokyotorphin, and showed that it was an N-terminally extended kyotorphin (231).

Exogenous (food) opioid peptides were discovered in 1979. In 1976 M. M. Singh and S. R. Kay reported that wheat gluten might act as a pathogenic factor in schizophrenia (219). Maintaining schizophrenic patients on a wheat-free and milk-free diet had a benign effect on their condition and adding wheat to their food affected it adversely. The discovery of neurotropic effects of opioid peptides, together with the above-mentioned observations, allowed the suggestion that certain substances might be present in wheat and milk which can interact with opiate receptors, producing opiate-like effects. C. Zioudrou, R. A. Streaty, and W. A. Klee reported that peptic hydrolysis of wheat gluten and α -casein generates peptides with opioid activity as measured by naloxone-reversible adenylyl cyclase inhibition *in vitro*, naloxone-reversible inhibition of electrically stimulated contractions of mouse vas deferens, and inhibition of labeled dihydromorphine and Met-enkephalin-amide binding to opiate receptors (261). These compounds were called exorphins (exogenous morphins). At

the same time V. Brantl and H. Teschemacher discovered a material with opioid activity in bovine milk and milk products (24). Subsequently, it was found that bovine casein peptone contains peptide fragments with opioid activity, which were named β -casomorphins (91, 137). In 1984 Brantl isolated human β -casomorphins, opioid peptides derived from human β -casein (23). It was shown that milk also contains several neuropeptides at concentrations higher than those found in plasma. These neuropeptides can be synthesized or processed in the mammary gland or excreted into milk through various pathways. In suckling mammals, hormones and neuropeptides, including casomorphins, are absorbed through the gastrointestinal tract and appear intact in the plasma. This absorption is age-dependent and could be of physiological significance in neonatal development (90).

In 1981 Erspamer's group made a surprising discovery: they isolated from the skin of *Phyllomedusa sauvagei* a unique class of opioid peptides, dermorphins, containing a D-alanine residue in the 2 position (25, 153). The heptapeptide dermorphin was much more potent than Met- and Leu-enkephalins, β -endorphin, and morphine in guinea pig ileum and mouse vas deferens assays and induced analgesia in mice injected intravenously and in rats injected intracerebroventricularly. Higher doses of intracerebroventricularly injected heptapeptide caused catalepsy (25). It was soon shown that dermorphin is also present in rat brain (168).

A group of biochemists from Merck together with E. Costa of NIMH, Washington, isolated a big polypeptide, 11 kDa, with chemical and physiological affinity to benzodiazepine receptors (83), the existence of which had been demonstrated in 1976.

In 1982 another neuropeptide was discovered when an approach originating in molecular biology was followed. Studies of alternate tissue-specific RNA processing in calcitonin gene expression revealed the generation of mRNAs encoding different polypeptide products. Calcitonin-encoding mRNA is formed predominantly in the thyroid parafollicular cells, while in the nervous system, the main product of calcitonin gene expression is an mRNA coding for a novel neuropeptide named calcitonin gene-related peptide, CGRP (3, 202). CGRP has potent cardiovascular actions in humans and in animals. It acts as a powerful vasodilator and has a direct positive inotropic effect on the heart. It was demonstrated later that CGRP has specific effects on the human heart. These findings suggested an important physiological and therapeutic role for CGRP in cardiovascular diseases. Immunoreactive CGRP was found in the spinal cord, where it was colocalized with substance P and galanin (257), and it was shown to possess neurotropic actions in the CNS (59). In 1984 S. Nakanishi's group, using alternative RNA splicing, demonstrated a similar tissue-specific generation of the two different preprotachykinin mRNAs from one gene (167).

Atrial natriuretic factor, ANF, is another important regulatory peptide discovered in the 1980s. Its discovery received widespread attention and aroused much research interest. Blaine and Rosenblatt (17) described the events preceding this finding. As they put it, "one of the reasons for the marked enthusiasm in this area is that the discovery of ANF culminated a quarter century search for an elusive hormone postulated to increase urinary sodium

excretion." Atrial natriuretic factor was isolated and characterized by several groups almost simultaneously. In 1981, A. J. de Bold *et al.* (48) demonstrated that rat atrial extracts cause a rapid and profound natriuretic response in rats. Blaine and Rosenblatt (17) noted that this discovery "produced a veritable explosion of research that within the last five years has led to remarkable advances in this field." In 1983 de Bold's group announced the amino acid sequence of ANF, which consists of 28 amino acid residues and differs from other known peptides. In 1984, within months of the publication of the paper by de Bold's group (66), six other laboratories reported almost simultaneously the primary structure of this cardiac hormone obtained using microsequencing (9, 45, 114, 150, 164, 216). Determination of the ANF amino acid sequence was followed by cloning and sequencing of cDNA corresponding to ANF mRNA, and later by cloning and sequencing rat, mouse, and human genes for ANF.

The discovery of ANF dramatically changed the idea of a heart as only a pump, indicating that it is also an endocrine organ. Secreted ANF helps to maintain cardiovascular homeostasis; it stimulates excretion of excess salt and water by acting directly on the kidneys. Former notions of the regulation of hemodynamics via the renin-angiotensin/aldosterone/noradrenalin (norepinephrine) vasoconstrictor system had to be revised, as well as views on the pathogenesis and therapy of various cardiovascular diseases.

ANF receptors have been identified in all putative target tissues (vascular smooth muscle, kidney, adrenal zona glomerulosa cells, posterior pituitary), as well as in the brain. In 1985, evidence for the presence of ANF in rat brain was obtained by D. M. Jacobowitz *et al.* (107). In 1988, T. Sudoh *et al.* (228) isolated another peptide, the 26-amino-acid brain natriuretic peptide, BNP, from rat brain. BNP has marked structural homology to ANF. This peptide, as well as its bigger forms, was subsequently found in porcine, human, and rat heart.

Endothelin (ET), a vasoconstrictor 21-amino-acid peptide, was found during the search for the long-postulated endothelial vascular smooth muscle activator. Endothelin was isolated in 1988 by M. Yanagisawa and associates at the University of Tsukuba (260) from the supernatant of porcine aortic endothelial cells. It is one of the most potent and long-lasting vasoconstrictor agent known to date. Subsequently, it was shown that the endothelin is synthesized as a prohormone which is cleaved by unusual proteolytic processing. The different isoforms of endothelin (endothelin-1, -2, and -3), originally predicted from the finding of three separate genes, appeared to differ in their localization and biological activity. Distribution patterns and pharmacological functions of endothelin isoforms have now been defined. Vascular endothelial cells were shown to produce only endothelin-1. Endothelin-1 and endothelin-2 are mainly distributed in the CNS, including spinal cord and peripheral tissues, while endothelin-3, although present in other tissues, is mainly concentrated in the pituitary. It was found that endothelins exert a number of different effects, such as positive inotropic and chronotropic actions on the heart, release of other regulatory agents and pituitary hormones, regulation of regional cerebral blood flow, and renal and mitogenic actions. Endothelins have been implicated in the pathophysiology of a number of cardiovascular, renal, and respiratory diseases. The

widespread distribution of endothelin within the central nervous system suggests that endothelin might also have an important function as neuromodulator (86).

Another strategy for detecting novel neuropeptides consists of analyzing the structure of mRNA or cDNA encoding peptide precursors and screening for nucleotide sequences corresponding to putative biologically active peptides. Peptides, the sequences of which were revealed after determination of the structure of the precursors of other peptides, include γ 3-MSH (originally found as a POMC fragment) and substance K (part of one of the two of bovine brain substance P precursors). These were tested for biological actions and subsequently were identified as such in the organism (27, 165). Analysis of procholecystokinin indicated that it exists as a number of peptide fragments. Examples are CCK58, CCK39, CCK33, CCK8, and CCK4 (14, 87). Analysis of the neuropharmacological actions of the CCK fragment V-9-M (sedative action and prevention of experimental amnesia in both passive and active avoidance behavior) allowed A. Takashima and S. Itoh to conclude that this compound might be a potent behaviorally active neuropeptide (234).

Thus, by the mid 1980s it was established that a variety of biologically active peptides are present in both the CNS and the internal organs. Several families can be distinguished among these peptides from their structural homology and a number of similar pharmacological and physiological activities (Table 1). Analysis of the structure and functions of neuropeptides in different classes of the animal kingdom provided the material for studies of neuropeptide evolution (14, 46, 125).

EMERGENCE OF NEUROIMMUNOENDOCRINOLOGY

Neuroimmunology, a new interdisciplinary research area, emerged in the late 1970s, with its birth officially certified by the appearance of the *Journal of*

TABLE 1

Families of Structurally and Functionally Related Peptides

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- (1) VIP, secretin, glucagon, GIP, PHI, GRF
 - (2) Neurotensin, pancreatic polypeptide
 - (3) Cholecystokinin, gastrin, cerulein, phyllocerulein
 - (4) Vasopressin, oxytocin, vasotocin, etc.
 - (5) a. Bombesin, alytesin
b. Ranatensin, litorin
 - (6) Tachykinins: eledoisin, physalaemin, uperolein, phyllomedusin, kassinin, chylambatin, substance P
 - (7) a. ACTH, MSH, LPH, endorphins, Met-enkephalin
b. Leu-enkephalin, dynorphin, alpha- and beta-neo-endorphins, etc.
 - (8) Kinins
 - (9) Angiotensins
 - (10) Sauvagine, CRF
 - (11) Somatostatin, urotensin II
 - (13) Kyotorphin, neo-kyotorphin
 - (14) Atrial and brain natriuretic peptides
 - (15) Growth hormone, prolactin
-

Neuroimmunology in 1981 and the first international conference on neuroimmunology with 150 attendees in 1982. A number of clinical observations (12, 181, 222, 225) and experimental studies performed by B. D. Jancovic and K. Isakovic, R. J. Cross, W. H. Brooks, H. O. Besedovsky, A. Del Rey, E. Sorkin, and others suggested that the nervous and endocrine systems are directly linked to each other (16, 47). The results of numerous studies indicated that behavioral conditioning, stress, exercise, and sexual activity can have major effects on immunity, while the immune system, through the production of immunoregulators (immunomodulators and immunopeptides), can regulate specific nervous system functions. Discovery of the interrelationship of the nervous and immune systems was followed by the finding that neuropeptides participate in the communications between these two systems. For decades, the neuroendocrine and the immune systems have been viewed as being two autonomous networks functioning to maintain a balance between host and environment. It was established that the neuroendocrine system responds to external stimuli such as temperature, pain, and stress, whereas the immune system responds to exposure to viruses, bacteria, and trauma. Within the past 20–30 years, evidence from several sources has pointed to a direct link between the functions of the two systems, especially with respect to the influence of the neuroendocrine system on the function of the immune system. It was demonstrated by D. G. Payan, E. J. Goetzel, J. Wibran, J. E. Blalock, and many others that a number of peptides, including ACTH, opioid peptides, TSH and TRH, growth hormone, oxytocin and vasopressin, substance P, VIP, and HCG exert immunoregulatory effects *in vitro* (18, 145). Specific and functionally relevant receptors for neuropeptides have been demonstrated on the surfaces of different types of lymphocytes (work of J. Wybran, N. P. Plotnikoff, J. E. Blalock, B. D. Jancovic, and others). Moreover, leukocytes were shown to produce neuropeptides such as ACTH and endorphins (18, 74). Immunoreactive TSH, growth hormone, chorionic gonadotropin, FSH, LH, prolactin, VIP, somatostatin, and other polypeptides have also been detected as lymphocyte products. Neuropeptides have been implicated in immunopathogenesis (arthritis, asthma, allergic rhinitis, urticaria, hepatic coma, chronic arachnoiditis, cystic fibrosis, etc.), and elevated concentrations of neuropeptides have been demonstrated in fluids and tissues of affected systems.

The systemic immune response, which involves a general activation of the immune system throughout the host, and the local immune response, which is tissue-specific and involves immune responses localized to a particular tissue of the host, were both shown to be under neuropeptide influence (74, 181, 182, 190).

Having analyzed current knowledge of neuropeptide implication in immune reactions, D. A. Weigent and Y. E. Blalock proposed that, in the immune system, neuropeptides act both as endogenous regulators of this system and as messengers transmitting the information from the immune system to the neuroendocrine system. Weigent and Blalock maintained that translation of such information to the neuroendocrine system reflects the sensory function of the immune system, in which leukocytes recognize stimuli that are unrecognizable for both central and autonomous nervous systems. Such stimuli, which they named

"noncognitive" stimuli, include bacteria, tumors, viruses, and antigens. These noncognitive stimuli are recognized by immunocytes and translated into the peptide "language" understood by neuroendocrine system, which then responds by producing physiological changes (256).

STRUCTURING THE PROBLEMS IN NEUROPEPTIDE RESEARCH AND ELABORATION OF A UNIFYING CONCEPT

During the period from the early 1970s to the early 1980s different pieces of the neuropeptide mosaic were finally assembled, and the study of neuropeptides became an established, vast research area. Extensive research in this area allowed a number of their typical properties to be distinguished, including multiple localizations and multiple functions. Apart from being in the central nervous system, neuropeptides have been located in the heart, lungs, gastrointestinal tract, placenta, gonads, etc. Attempts to explain this phenomenon were made by A. G. E. Pearse in his concept of APUD cells. In 1966, Pearse advanced the idea that polypeptide hormone-producing cells possess a number of common cytochemical and ultrastructural properties. He argued that chromaffin and enterochromaffin adrenal cells might be ontogenetically derived from the neuroectoderm (183). Such cells, which were supposed to contain enzymes for the synthesis of amines, were termed "amine precursor uptake and decarboxylation," or APUD cells. Further studies of these cells revealed that incorporated amines were stained by the formaldehyde immunofluorescence (FIF) technique. Using this technique, Pearse and J. M. Polak demonstrated that endocrine polypeptide (APUD) cells of the chicken gastrointestinal tract and pancreas are derived from the neuroectodermal stem cells originating from the neural crest (186, 191). As studies on neuropeptides advanced, especially on discovery of their localization in the brain and internal organs and colocalization with amines and amino acid neurotransmitters, Pearse reformulated his concept so that it became a central tenet that APUD cells are neuroendocrine cells (184, 185). The presence of neuropeptide-producing cells in the brain of both higher and lower animals suggested that these cells played an important role in the regulation of nerve function, perhaps even before their endocrine and metabolic functions had developed.

It has already been demonstrated that most neuropeptides can play multiple roles in the organism. This property is closely related to their multiple localization. The existence of several different mechanisms of action involving different types of receptors was proposed to account for the multiple functions of neuropeptides. Apart from their specific endocrine-like effects in the internal organs, neuropeptides exert neurotropic effects and may act as neuromediators and/or neuromodulators. Neuropeptides participate in such complex reactions as the regulation of appetite and thirst, memory, thermoregulation, sleep, pain perception, and sexual, feeding, and drinking behavior (10, 52, 53, 116, 139, 156). Neuropeptides appear to play a central role in the adaptive responses of the organism.

Taking into consideration recent findings concerning the biochemistry and physiology of neuropeptides, de Wied reformulated the neuropeptide concept as follows: "Neuropeptides are endogenous substances present in nerve cells, and involved in nervous system function. Neuropeptides are synthesized in large precursor proteins, and several are formed in the same precursor such as ACTH and β -endorphin in proopiomelanocortin (POMC). A cascade of processes evolve in peptidergic neurons to express the genetic information into biologically active neuropeptides. These processes control the quantities of neuropeptides synthesized as well as the nature of their biological activity, through size, form, and derivatization of the end product. In this way sets of neuropeptides with different, opposite, and more selective properties are formed from the same precursor" (50, p. 100).

Another pioneer in neuroendocrinology, William F. Ganong, expounded his ideas of the system of neuroendocrine communications as a whole (70, 71): "There are four modes of chemical transmission, three involving the release of chemical messengers into the interstitial fluid. Two different types of cells, neurons and endocrine cells, secrete these chemical messengers. In some instances, it has been proved that the same chemical messenger is secreted by neurons as a neurotransmitter, by neurons as a paracrine mediator, by neurons as a neural hormone, by endocrine cells as a paracrine mediator, and by endocrine cells as a typical hormone" (71, p. 236). Examples of such messengers are somatostatin, noradrenalin, dopamine, and LHRH. Ganong concludes that "in this sense there is no clear separation between the nervous system and the endocrine system and there is instead a neuroendocrine system with neural and endocrine subdivisions. . . . Neuroendocrine cells that secrete chemical messengers are widely distributed in the body. In some locations, these cells develop the morphological features of gland cells and in others they develop the morphological features of neurons even though they secrete the same chemical messengers" (72, pp. 237, 238).

Based on the analysis of opiate receptor gradients in the monkey cerebral cortex, M. E. Lewis *et al.* (129) proposed a hypothesis which regarded brain opioidergic pathways as the system for filtering out currently unnecessary information.

Another general hypothesis was proposed by I. P. Ashmarin (Moscow State University and P. K. Anokhin Institute of Normal Physiology) in 1986–1987. One of the central topics of the experiments carried out in his laboratory was behavioral (learning and memory) and neurotropic actions of peptides. In the early 1980s Ashmarin's group showed that tuftsin, an immunostimulating tetrapeptide, Thr–Lys–Pro–Arg (which has a tripeptide sequence in common with neurotensin), possesses direct neurotropic, behavioral, and psychostimulatory actions, including stimulation of motor activity and aggressiveness in rats, induction of analgesia, etc. (112, 126). The central idea of Ashmarin's hypothesis based on a comparative analysis of the spectra of biological activities of peptides was that neuropeptides form a continuum in the organism. In certain combinations, numerous neuropeptides make up a continuous set of the spectra of biological actions, i.e., functionally continuous integrity (continuum)

which provides a pathway for complex influences on organism functions, while each peptide retains its own qualitative properties in which it differs from other neuropeptides (8). Ashmarin illustrated his hypothesis with available data underlying complex chain reactions of the entire neuropeptide continuum in response to changes in concentration of one single peptide. Analysis of the reciprocal effects of neuropeptides on their release allowed Ashmarin to distinguish a group of neuropeptides including VIP, cholecystokinin, neurotensin, and bombesin, characterized by their action on secretion of other neuropeptides (including hypothalamic peptides), while their own secretion was not significantly affected by other neuropeptides. This observation together with the finding that VIP and cholecystokinin are present in cerebral cortex in particularly high quantities allowed Ashmarin to propose a hypothesis that these peptides belong to a higher category in the hierarchy of neuropeptides (6).

CONCLUSION AND FUTURE PERSPECTIVES

The following main directions can be seen in current studies on neuropeptides:

- (1) studies on neurotropic and behavioral actions of neuropeptides;
- (2) studies on physiological and pharmacological actions of neuropeptides;
- (3) studies on the mechanisms of action (including studies on receptors; receptor solubilization; the most promising technique is cloning of receptor cDNAs);
- (4) structure-activity studies, construction of synthetic analogs with predicted properties;
- (5) studies on neuropeptide localization and colocalization;
- (6) studies on neuroendocrine tumors;
- (7) molecular biology of neuropeptides and their life cycles, including processing by specific peptidases;
- (8) studies on neuropeptide-specific peptidases, including search for their specific activators and inhibitors as possible tools of regulation of the level of neuropeptides in the organism;
- (9) studies on neuropeptides in lower animals and in evolution;
- (10) neuroimmunoendocrinology.

The decade which followed the announcement of the primary structure of hypothalamic neurohormones virtually exploded with new discoveries. It was expected that all the problems associated with neuropeptides would be solved in the 1990s, the complex role of the individual neuropeptides then being understood, along with the existence of a whole peptidergic system in the organism. The result was expected to be the elaboration of a new generation of drugs based on neuropeptides. However, this did not happen. Instead, the process of complication of relatively simple structure (endocrine system versus nervous system), which began with the emergence of neuroendocrinology, was

further confounded with each new discovery. Most of the classical physiological doctrines have been proved too simplistic and have had to be revised. The finding that neuropeptides participate in the communication between the immune and the neuroendocrine systems and the rise of neuroimmunoendocrinology have put the final touch to this process of boiling all branches of animal physiology together in one cauldron. Studies on neuropeptides are still in the phase of extensive accumulation of data. Assembling all the pieces of neuropeptide mosaics has become a far more complicated task since the number and the diversity of pieces have grown so much. It has become clear that many more of these pieces still have to be found and that the whole pattern may change. But, whatever new discoveries and insights in this area will come in the future, it is clear that the studies on neuropeptides have revolutionized physiology.

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