110 Warembourg, M., Tranchant, O., Perret, C., Desplan, C., and Thomasset, M., In-situ detection of vitamin D-induced calcium binding protein (9-kDa CaBP) messenger RNA in rat duodenum. J. Histochem. Cytochem. *34* (1986) 277–280.
111 Wilcox, J. N., Gee, C. E., and Roberts, J. L., In-situ cDNA:mRNA hybridization: development of a technique to measure mRNA levels in individual cells. Meth. Enzymol. *124* (1986) 510–533.
112 Wintour, E. M., The role of the adrenal in control of foetal-developmental gene-switches in haemoglobin. Proc. XXXth Congress I.U.P.S. (1986) 121 (abstr.).
113 Wintour, E. M., Smith, M. B., Bell, R. J., McDougall, J. G., and Cauchi, M. N., The role of fetal adrenal hormones in the switch from fetal to adult globin synthesis in sheep. J. Endocr. *104* (1985) 165–170.
114 Zajac, J. D., Penschow, J. D., Mason, T., Tregear, G. W., Coghlan, J. P., and Martin, T. J., Identification of calcitonin and calcitonin gene-related peptide messenger ribonucleic acid in medullary thyroid carcinomas by hybridization histochemistry. J. clin. Endocr. Metab. *62* (1986) 1037–1043.
115 Zawatsky, R., De Maeyer, E., and De Maeyer-Guignard, J., Identification of individual interferon-producing cells by in-situ hybridization. Proc. natl. Acad. Sci. USA *82* (1985) 1136–1140.

0014-4754/87/070741-10$1.50 + 0.20/0

Regulatory peptide receptors: visualization by autoradiography

by J. M. Palacios and M. M. Dietl

Preclinical Research, SANDOZ LTD, CH 4002–Basel (Switzerland)

Summary. The receptors for regulatory peptides have been extensively characterized using radioligand binding techniques. By combining these binding techniques with autoradiography it is possible to visualize at the light and electron microscopic levels the anatomical and cellular localization of these receptors. In this review we discuss the procedures used to label peptide receptors for autoradiography and the peculiarities of peptides as ligands. The utilization of autoradiography in mapping peptide receptors in brain and peripheral tissues, some of the new insights revealed by these studies particularly the problem of 'mismatch' between endogenous peptides and receptors, the existence of multiple receptors for a given peptide family and the use of peptide receptor autoradiography in human tissues are also reviewed.
Key words. Receptors; multiplicity; radioligand binding; quantitative autoradiography; light- and electron microscopy; peptidases; endogenous ligands; brain; gut.

Introduction

The physiological and pharmacological effects of the regulatory peptides, like those of other hormones and neurotransmitters, are mediated by the interaction of these substances with specific recognition sites named 'receptors'. In parallel with the steadily growing number of peptides being discovered, multiple receptors for these peptides have been characterized[7,50,54,55,116,117]. Pharmacological and biochemical techniques have provided evidence for the existence of multiple receptors for the different peptide families. Historically the characterization of the opioid receptor opened the path for the isolation and characterization of the opiate peptides[12,51,114,118]. The use of the so-called high-affinity radioligand binding techniques was instrumental in beginning the search for endogenous ligands. By using radiolabeled molecules at high specific activity like, for example, the same peptides, it is possible to study the binding of these ligands to membrane preparations. These techniques allow the characterization of sites with high affinity (in the low nanomolar range) and low capacity (fmol/mg of protein) and presenting selectivity and specificity for a given peptide and its analogues with similar physiological effects. In recent years this relatively simple approach has allowed the detailed study of the structure-activity relationship and the biochemical mechanism involved in peptide action and progress is being made with solubilization and purification and towards the ultimate goal of the elucidation of the molecular characteristics of these receptors[115,123].

One main limitation of these biochemical techniques is, however, the lack of sufficient anatomical resolution to answer questions such as: where are these sites localized at the microscopic level? Are there specific cell populations in a given tissue or organ enriched in a particular peptide receptor? Finally; which subcellular structures bear these receptor sites?

Techniques with high anatomical resolution are required to answer these questions. Because of the wide use of radioligand binding assays in the study of peptides and other receptors it is not surprising that *autoradiography* has been one of the techniques most widely used in the localization of peptide receptors at the microscopic level. In particular the in vitro autoradiographic procedure originally developed by Young and Kuhar[134] is specially well suited for the study of peptide receptors. Organ and tissue barriers encountered in in vivo labeling are overcome, as is the problem of ligand metabolism, a very important problem when dealing with peptide ligand both in vivo and in vitro[59,60,89]. In this paper we will review the use of this technique in the visualization of peptide receptors both in the central nervous system and peripheral tissues at the light and electron microscopic levels of resolution as well as other alternatives for the visualization of peptide receptors.

Methodology

The procedures for the labeling of peptide receptors for autoradiography are essentially identical with those used for the labeling of other hormone, drug or neurotransmitter receptors[59,60,89]. Some of the particularities encountered in peptide receptor labeling are related to the use, in most cases, of radiolabeled peptides as ligands[123]. These ligands present, in general 1) a high susceptibility to degradation by peptidases present on the tissue, and 2) a tendency to bind in a non-specific way to tissue, glass and gelatine in the histological

preparations. An outline of the procedure is summarized in table 1.

Table 1. Autoradiographic visualization of regulatory peptide receptors: outline of the procedure

1) *Tissue preparation*
   a) Slight in vivo fixation followed by in vitro prefixation or not depending on desired anatomical resolution or tissue preservation.
   b) Regular in vivo fixation after in vivo labeling of receptors.
2) *Labeling of receptor*
   a) In vivo, systemic administration of ligand followed by perfusion/fixation.
   b) In vitro
      I) *Preincubation* with guanylnucleotides and ions, washing of endogenous ligands.
      II) *Incubation* with peptidase-inhibitors, ions, displacers, at different temperatures, addition of chemicals to prevent chemical degradation of ligand and non-specific binding to supports.
      III) *Washing* of unbound ligand.
      IV) *Postfixation*, cross-linking.
3) *Generation of autoradiograms*
   a) 'wet' (liquid) emulsions; dipping of labeled sections.
   b) 'dry' emulsion-coated coverslips, films.
   c) standards.
4) *Quantification of autoradiograms*
   a) Manual counting of autoradiographic grains.
   b) Automatic, computer-assisted microdensitometry or grain-counting.

Tissue preparation

Different procedures have been used to prepare tissues for peptide receptor localization depending on the method used to label the receptor, i.e., in vivo or in vitro and the type of resolution desired, i.e., light or electron microscopy.

When in vivo labeling procedures are used the ligand is administered systemically, for example, intravenously or subcutaneously and after a given time (chosen to favor a maximal level of specific binding) the animal is sacrificed, generally by perfusion with a fixative. This procedure has been widely used to label brain peptide receptors with non-peptide ligands (such as opiates[1-3]) and peripheral receptors, for example somatostatin receptors[78].

The in vivo labeling procedure is limited to the use of metabolically stable analogues or non-peptide ligands, because of the poor ability of the peptide-ligand to penetrate through barriers such as the blood-brain barrier, and the high cost resulting from the use of large amounts of ligand.

These limitations are overcome by using the in vitro approach initially developed by Young and Kuhar[134]. In this procedure tissues are obtained post-mortem from laboratory animals after perfusion, or not, of the anesthetized animal with buffers containing low concentrations of fixative (routinely 0.04% paraformaldehyde in PBS); higher concentrations might be detrimental to the receptor[134]. The tissues are then sectioned in a cryostat-microtome normally at a 10 to 20 µm thickness and thaw-mounted onto gelatine-coated glass-slides. These procedures have been found to preserve receptors quite adequately: no differences have been found between either the density or the characteristics of receptors in these mounted sections and those in standard membrane preparations from fresh tissues[134]. The stability of peptide receptors is also remarkable even in human postmortem tissues, particularly in brain[18,19,74,91,105].

In some cases a better tissue preservation is desired, as in studies oriented towards the electron microscopic localization of receptors[40,41], or the preservation of antigens for correlative immunohistochemical studies. A compromise has then to be found between the loss of receptor binding following the use of fixatives, and tissue structure and antigen preservation[64].

Ligands

[^{3}H]- and [^{125}I]-labeled ligands are generally used in autoradiographic studies of peptide receptors. Many of these ligands are commercially available. Most of the peptides can be iodinated, using the chloramine T method when the peptide has a tyrosine or a histidine residue[37] or in the Bolton-Hunter method[8] when there is a lysine and a terminal NH_2 group. The labeling sites should preferably occur only once in the primary sequence and should represent amino acids of lesser importance in the binding of the ligand to the receptor. In all cases, radiolabeled peptide ligands have to be carefully checked for purity and stability. The biological activity of the radiolabeled peptide, although not always easy to test, is also an important criterion before any binding experiment is attempted. The selection of the isotope is linked to criteria such as stability and purity of the ligand, specific activity requirements, resolution desired, etc. Table 2 lists the most commonly used ligands for the labeling of peptide receptors.

Labeling of receptors for autoradiography

As already mentioned, two procedures can be used to label peptide receptors in vivo and in vitro. The limitations of the in vivo procedure have already been discussed above, and we will now refer in more detail to the in vitro procedure. In labeling peptide receptors in mounted tissue sections (see table 1) the following parameters have to be taken into account 1) the presence of endogenous ligand; 2) the presence of peptidases in the tissue; 3) the possibility of binding of the ligand to sites unrelated to the receptor itself; 4) the nature of the binding of the ligand to the receptor, which is generally reversible and thus presents possibilities of dissociation; 5) the chemical stability of the ligand.

In general endogenous ligands are eliminated by preincubating the tissues for different amounts of time, in conditions in which the released peptide should be accessible to degradation by endogenous peptidases, or is washed out by changing the preincubation buffer. Dissociation of the endogenous ligand is favored by adding to the incubation medium guanylnucleotides or ions[80,123,134].

Routinely a cocktail of several peptidase-inhibitors is included during the incubation period to prevent the degradation of the ligand, if this is a peptide. Anti-oxidants are also generally used to protect the ligand from chemical degradation. Examples of a peptidase inhibitor mixture are those

Table 2. Regulatory peptide receptors: autoradiographic studies

Endogenous peptide	Receptor subtypes	Ligands	References
Opioid peptides			
Met-enkephalin (MET)	µ	[^{3}H]diprenorphine	1, 2, 3 (rat, in vivo), 33 (rat),
Leu-enkephalin (LEU)		[^{3}H]etorphine	40, 41 (rat), 46 (rat), 64 (rat),
Dynorphin (DYN)		[^{3}H]bremazocine	74 (human), 76 (rat, in vivo),
β-endorphin (βEND)		[^{3}H]morphine	90 (rat, guinea pig),
		[^{3}H]naloxone	94 (rat, in vivo), 130 (monkey)
		[^{3}H]dihydromorphine (DHM)	
		[^{3}H]-[D-Ala^2, MePhe^4,	

Table 2 (cont.)

Endogenous peptide	Receptor subtypes	Ligands	References
		Gly-ol^5]enk. (DAGO)	
		[^{3}H]FK 33824	
	δ	[^{3}H]bremazocine	33 (rat), 36 (rat, guinea pig),
		[^{3}H]-[D-Ala^2, D-Leu^5]enk. (DADL)	90 (guinea pig)
		[^{125}I]-[D-Ala^2, D-Leu^5] enk. (DADL)	
		[^{3}H]DPDPE	
	κ	[^{3}H]bremazocine	22, 23 (rat, guinea pig), 34 (rat),
		[^{3}H]ethylketocyclazozine	65 (guinea pig), 74 (human),
		[^{3}H]dynorphin A	83 (rat), 90 (guinea pig)
	ε	[^{3}H]-β-endorphin	35 (rat)
	σ	[^{3}H]-3-PPP	39 (rat, guinea pig),
		[^{3}H]phencyclidine	62 (guinea pig), 96 (rat)
		[^{3}H]SKF 10047	
Gut-brain peptides			
Tachykinins			
Substance P (SP)	SP-P (NK1)	[^{3}H]SP	13 (rat), 68 (rat), 79 (rat), 98 (rat)
		[^{125}I]BH-SP	5 (rat), 10 (rat gastrointestinal tract), 70 (rat gastrointestinal tract), 100 (guinea pig), 108 (rat)
Physalaemin (PHY)	SP-P (NK1)	[^{125}I]PHY	133 (rat)
Substance K = Neurokinin A (SK)	SP-K (NK2)	[^{125}I]BH-SK	10 (rat gastrointestinal tract), 69 (rat), 100 (guinea pig)
Eledoisin (ELE)	SP-E (NK3)	[^{125}I]BH-ELE	5 (rat), 13 (rat), 109 (rat)
Kassinin (KAS)	SP-E (NK3)	[^{125}I]BH-KAS	69 (rat)
Gastrins			
Gastrin			–
Pentagastrin (G-5)		[^{3}H]CCK-5/pentagastrin	26 (rat)
Cholecystokinin (CCK-4)		[^{3}H]CCK-8	126 (rat, pancreas)
Cholecystokinin (CCK-8)		[^{125}I]BH-CCK-8	18 (human), 71 (rat)
Cholecystokinin (CCK-33)		[^{125}I]CCK-33	42 (rat), 137 (rat, guinea pig)
Vasoactive intestinal polypeptide (VIP)		[^{125}I]VIP	17 (rat), 73 (rat)
Motilin (MOT)			–
Pancreatic polypeptide (PPP)			–
Secretin (SECR)			–
Pituitary peptides			
Adrenocorticotropin (ACTH)			–
Growth hormone (GH)			–
Lipotropin			–
α-Melanocyte-stimulating hormone (α-MSH)			–
Arginin-Vasopressin (AVP)		[^{3}H]AVP	4 (rat), 6 (rat), 9 (rat), 15 (rat), 127 (rat)
Oxytocin (OXT)		[^{3}H]OXT	15 (rat), 24 (rat), 128 (rat)
Circulating hormones			
Angiotensin II (AII)		[^{125}I]-[Sar^1, Ile^8]AII	29, 30 (rat, brain, kidney), 43 (rat adrenal gland), 52 (rat), 82 (rat), 119 (dog)
Calcitocin (CT)		[^{125}I]CT	21 (human), 44, 45 (rat), 75 (rat), 85 (rat)
Calcitocin-gene-related peptide (CGRP)		[^{125}I]CGRP	14 (rat), 45 (rat), 53 (rat, human), 112 (rat)
Insulin (INS)		[^{125}I]INS	49 (rat), 135 (rat)
Hypothalamic-releasing hormones			
Thyrotropin-releasing hormone (TRH)		[^{3}H]methyl-TRH	66 (rat), 72 (rat), 92 (rat, guinea pig), 95 (rat)
Corticotropin-releasing factor (CRF)		[NLe^{21}, ^{125}I-Tyr^{32}]CRF	16 (rat)
Luteinizing-hormone-releasing hormone (LHRH)		[^{125}I]LHRH	103 (rat), 106 (mouse, guinea pig, hamster, rabbit, rat, human)
Somatostatin (SS)		[^{125}I]CGP 23996	38 (rat)
		[^{125}I-Tyr^{11}]SS-14	63 (rat), 77 (rat),
		[^{125}I-LTT]SS-18	63 (rat)
		SMS 204-090	104 (rat, pituitary), 105 (human), 122 (rat, human)
		[^{3}H]SS-14	131 (human)
Growth hormone releasing factor (GRF)			–
Others			
Bradykinin (BK)		[^{3}H]BK	67 (guinea pig ileum)
Bombesin (BN)		[^{125}I-Tyr^4]BN	132 (rat)
Neuropeptide (NPY)		[^{125}I]BH-NPY	see 111 (rat)
Galanin (GAL)		[^{125}I]GAL	81 (rat)
Atrial natriuretic factor (ANF)		[^{125}I]ANF	99 (rat), 129 (rat, peripheral tissues)
Atriopeptin III (APIII)		[^{125}I]APIII)	31 (rat, adrenal), 61 (rat, thymus and spleen), 84 (rat kidney)
Neurotensin (NT)		[^{3}H]NT	97 (rat), 86 (rat), 120 (human, rat), 121 (human), 136 (rat)
		[^{125}I]NT	110 (human)

The table lists the most important endogenous peptides known until now, the receptors localized and the ligands used for autoradiography in vertebrate tissues (when only the species is mentioned refers to *brain*). Numbers correspond to references; – indicates that no receptor for this peptide has been yet localized.

used in the labeling of substance P (SP) receptors or cholecystokinin (CCK) receptors. For SP a 50 mM Tris-HCL buffer at pH 7.4 is used with 200 mg/l BSA to which is added bacitracin 40 mg/l, chymostatin 2 mg/l and leupeptin 4 mg/l, then $MnCl_2$ 5 mM; some investigators like to add polyethylenimine at 0.005%[68,69]. In contrast, for CCK the same buffer is used but at pH 7.7 and with 200 mg/l of bacitracin, $MgCl_2$ 5 mM and dithiothreitol 1 mM[71,126,137].

Other ways to deal with the problem of peptidase activity in the tissue sections are 1) the use of peptide analogues designed to be resistant to catabolic activity, as for example opioid or somatostatin analogues[40,41,77,78] and 2) the use of low incubation temperatures, although this technique also results in slower association kinetics and consequently longer incubation periods. An example is the procedure used to label TRH receptors which decrease peptidase activity[72,92,95] (see however Manaker et al.[66]).

Several procedures have been used to decrease or block the non-specific binding of peptide ligands to a glass surface or to gelatine. One of the most commonly used, which is also postulated to provide protection against peptidase activity, is the addition of bovine serum albumin to the incubation medium. Compounds such as polyethylenimine are reported to decrease the non-specific binding of peptides to glass and have been used in membrane and section labeling of such peptide receptors as those for SP[68,133].

The influence of all these compounds on the characteristics and density of the receptor being studied have to be carefully monitored in preliminary experiments to ensure that no negative influence occurs. The different ligands and receptors behave in a very unpredictable way in response to the parameters mentioned above. As no general rule can be proposed it is advisable to check carefully in each individual situation for all the conditions listed.

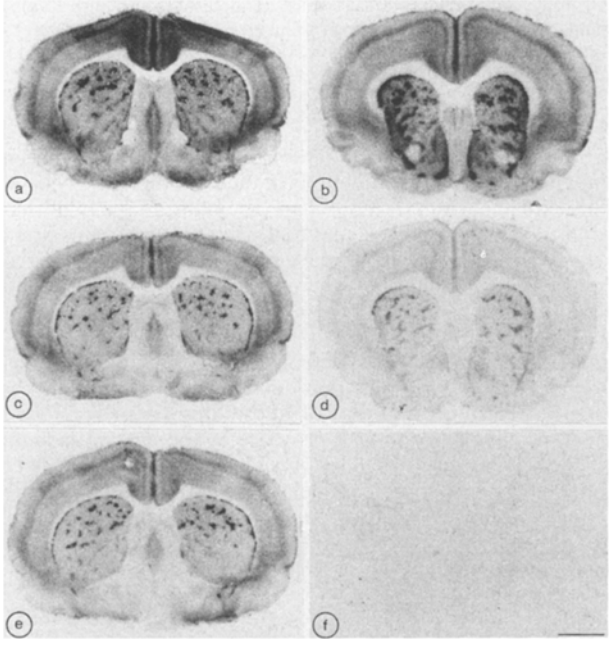

Figure 1. Effects of fixation and dehydration on the topographical distribution of opioid binding sites labeled with [^{125}I]FK 33-824 (left) or [^{125}I]FW 34-569 (right). In *a* and *b* the autoradiographic films were generated before, in *c* and *d* after glutaraldehyde fixation. In *e* and *f*, fixation was followed by dehydration. [^{125}I]FK 33-824 is covalently cross-linked to tissue proteins by aldehydes; no modification in the distribution of this ligand is observed after glutaraldehyde fixation and tissue dehydration (e). In contrast, [^{125}I]FW 34-569 do not cross-link and is completely washed out in the course of dehydration (f). Bar = 2.0 mm. (Figure kindly provided by A. Beaudet[41], reproduced with permission.)

Autoradiogram generation

Autoradiograms are generated by putting the labeled tissue sections in close contact with emulsions. This can be done in two ways: 1) dipping the tissues in liquid emulsion[40,41,46]; 2) using a dry emulsion in the form of an emulsion-coated coverslip[134] or a film, such as a tritium-sensitive or X-ray film[87,93,102,125]. The use of the dipping procedure requires that the ligand is irreversibly fixed to the receptor site because emulsions are used in liquid form at relatively high temperature (around 40 °C). The use of reversibly bound ligands will lead to the dissociation of the ligand from the receptor, and will produce erroneous autoradiographic pictures. This method has been called by Hamel and Beaudet[40,41] the 'wet' autoradiographic technique in contrast to the 'dry' autoradiographic technique in which emulsion-coated coverslips or films are used (fig. 1). The emulsion-coated coverslip procedure introduced by Young and Kuhar and has been extensively used in the mapping of receptors for regulatory peptides. Finally the use of ^{3}H-sensitive films such as the [^{3}H]Ultrofilm from LKB, Sweden or the recently developed ^{3}H-film from Amersham[87,93,102,125] allows an easier handling and quantification of the autoradiograms by computer-assisted image analysis systems. The advantages and disadvantages of the use of nuclear tracing emulsions or ^{3}H-sensitive films have been extensively discussed by other authors and the reader is referred to these discussions for more detailed information[59].

Briefly, the main advantage of using the 'wet' procedure or emulsion-coated coverslips is the possibility of visualizing the autoradiographic grains together with the tissue. In the case of the 'wet' emulsion procedure the autoradiographic grains and the tissue are located in the same optical plane, while in case of the coverslip a slight difference in the optical plane of the autoradiographic grains and the tissue exists. On the other hand, the use of film leads to a loss of anatomical register between the localization of autoradiograms and the actual tissue structure although it allows much easier quantification.

Analysis of autoradiograms

Autoradiograms once generated can be analyzed using many different procedures. In recent times the use of computer-assisted image analysis systems has gained widespread popularity because of the feasibility of performing complete quantitative autoradiographic studies[89]. On the market a large variety of image analysis systems are now available and we will not discuss the differences between the different systems. Basically the autoradiographic films are digitalized using a TV camera and analyzed by microcomputers. The response of the film to radioactivity can be calibrated using appropriate standards and optical densities transformed into receptor densities in terms of fmol/mg protein or area unit[32,58,60,102,125].

One of the most studied problems using receptor autoradiography with tritiated ligands has been 'quenching'[27,28] caused by the differential absorption of β-radiation by different tissue elements[58]. A number of procedures have been designed to overcome the problem in the use of tritium labeled ligands[47]. This difficulty is not encountered with [^{125}I]iodine labeled compounds; this is an important advantage of these ligands, which are the most popular in peptide receptor studies. Regarding the quantification of autoradiograms generated using the 'wet' or 'dry' emulsion procedure there are also some automatic devices for the counting of autoradiographic grains, but in contrast to the devices for the study of films these are expensive, and they are still subject to a number of technical problems. Most commonly the quantification of autoradiographic grains in emulsions is carried out by visual counting of grains[124]. As in the case of the films the use of appropriate standards allows for the full transformation

of grain density into receptor density. Of course this procedure is much more time-consuming than fully automatic image analysis.

Uses of peptide receptor localization by autoradiography

Mapping of peptide receptors and the 'mismatch' problem

The most extensive use of peptide receptor autoradiography is the detailed analysis of the regional and anatomical distribution of peptide receptors in different tissues, particularly in the brain. This is the so-called 'mapping of receptors'. Table 2 is a summary of the peptide receptors which have been visualized in different mammalian species and, in some cases, in other vertebrates. Table 2 lists the subtypes of receptors mapped, the ligand used and some key references. The references are, however, not exhaustive because of space limitations; however, the most relevant studies have been cited.

Historically the opioid receptor was the first one to be mapped in detail. Early studies were done in the rat brain after in vivo labeling of these receptors using non-peptide opioid ligands[1–3,94]. The results obtained in these studies are an example of the type of analysis and insights provided by peptide receptor mapping in brain and other tissues. The detailed description of the distribution of opioid receptors in the mammalian brain was instrumental in understanding the neuronal mechanisms involved in the physiological actions and pharmacological effects of opioid drugs. Opioid receptors were found to be highly concentrated in some specific areas of the central nervous system, for example, the substantia gelatinosa of the spinal cord, an anatomical locus known to be involved in the processing of sensory information. In addition, other areas of enrichment in opioid receptors were found to be the periaqueductal gray and the medial thalamus. All these localizations provided a clear anatomical substrate for the known analgesic effects opioids exert at both spinal and supra-spinal levels. In addition, the euphoric effects of opiates can now be attributed to the localization of substantial numbers of receptors for these drugs in the limbic system[114,117].

An important consequence of the studies on the localization of opioid receptors in the brain was a search for 'endogenous morphine'[12,51,113]. The development of antibodies against the enkephalins and the immunohistochemical studies of the enkephalins soon provided a detailed mapping of the presynaptic components of the opioid system in the mammalian brain. It was clear from these studies that a relatively good overlap existed between the distribution of endogenous enkephalins and opioid receptors. This led to the proposal that enkephalins were the endogenous ligands for the opioid receptor. More detailed studies have, however, demonstrated that not in every case is there a good overlap between the distribution of endogenous peptide and the distribution of the receptors for this peptide. This has been called the 'mismatch' problem and is the subject of much speculation[48,57,88]. Herkenham and McLean have recently reviewed in detail 'mismatches' between the distribution of the receptors for peptides and their endogenous peptide ligands[48]. Thus, while it was originally proposed that the distribution of endogenous enkephalins and the μ-opioid receptor were in good accordance, an examination of the distribution of opioid receptors in other brain areas such as the diencephalon and forebrain has concluded that 'mismatches' are rather the rule. The hypothalamus is another good example of 'mismatch'. The presence of high densities of opioid peptides as well as opioid-mediated functions on the one hand have to be reconciled with a low density of receptors. This has been explained by the presence of high densities of opioid-receptors of the kappa-type. Dynorphine which is postulated to be the endogenous ligand for the kappa-receptor, because of its high affinity for this binding site, has been shown to influence the functions of the hypothalamic system. This explains why κ-receptors are abundant in the hypothalamus while only sparse μ and δ sites are present in this brain areas. On the other hand the opposite situation has been described in the thalamus where very low densities of opioid peptides have been found to correlate with high densities of the μ- and κ-subtypes of the opioid receptors.

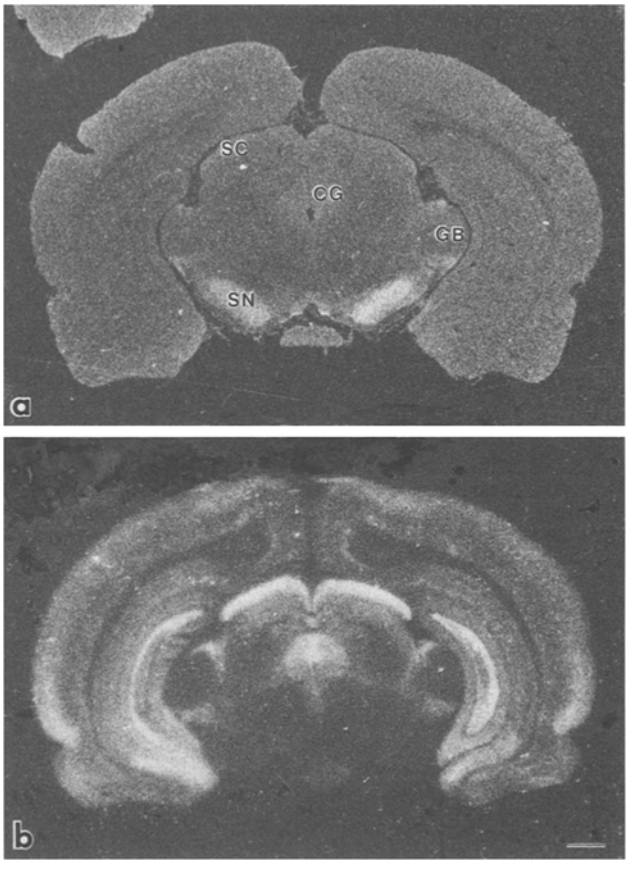

Figure 2. The distribution of substance P localized by immunocytochemistry using monoclonal internal labeled antibodies to substance P (a) is compared to substance P binding sites in an equivalent level localized by autoradiography using [^{125}I]Bolton-Hunter substance P (b). Bar = 1.0 mm.

Similar mismatches have been observed for many other peptides in the rat brain. A particularly good example is the distribution of SP and that of SP binding sites (fig. 2). The most spectacular example is provided by the substantia nigra where the highest densities of endogenous SP have been found and yet no significant density of SP receptors has been reported. This will lead us to a second problem that we will discuss later, the problem of subtypes of receptors and the relation of peptide receptor subtypes to the presence of multiple endogenous peptides.

Other examples of mismatches are provided by the localization of receptors for the peptides neurotensin, somatostatin, angiotensin II, CCK, thyrotropin-releasing hormone (TRH) and corticotropin-releasing factors (CRF). The problem of the mismatch between endogenous peptides and peptide receptors is further complicated by species differences. Very variable patterns of distribution for peptide receptors are found in different species. Here again the case of the opioid receptors has been a paradigm of the multiplicity of distributions in different species. We have also observed species differences in the distribution of receptors for several

regulatory peptides. A particularly striking example of both the mismatch problem and the species differences is illustrated in figure 3 where the localization of CCK receptors in the cerebellar cortex is illustrated. The cerebellum is supposed to contain no measurable density, or a very low level, of the endogenous peptide CCK. However, in the guinea pig and the primates including man, we have found a rich supply of CCK receptors in the cerebellar cortex, while in the rat cerebellum no significant binding was observed.

Several explanations have been proposed for the interpretation of the 'mismatch problem'.

The first classical explanation is that a mismatch is produced by the differences in the fineness of the techniques used. Thus, the lack of resolution of the current immunocytochemical methods being used to visualize endogenous peptides on the one hand and/or the presence of occupied receptors, non-functional spare receptors, and the presence of low affinity receptors which are not visualized using the current high affinity binding techniques on the other hand can lead to an apparent mismatch between neuropeptides and their receptors.

Another explanation is based on the fact that neurotransmitter molecules and receptor molecules are contained often within two completely different neurons which have completely different anatomical distributions[57,60]. In addition, the presence of receptors on non-neuronal elements can also lead to such a mismatch. It has furthermore been suggested that many receptors are not located at synapses and may be sites of action of neuropeptides released from a distance, which would mean a hormone-like transmission of information[40,41,48,80]. Herkenham and McLean[48,80] include the possibility that receptors identified in one region might actually be in transit to another region.

Finally, Schultzberg and Hökfelt[111] have recently proposed a further possibility, based on the finding of the coexistence of neurotransmitters and neuropeptides in the same synapses. They suggest that receptors for two types of co-localized/co-released transmitters may be expressed in a 'coupled' form. Such a coupled expression may occur in regions where only one of the transmitters is present, thus providing a further explanation for the mismatch between transmitters and receptors.

At the present time a full explanation for the mismatch problem is not yet available. The mismatch problem reveals our lack of understanding of the correlation between innervation and receptor density and how this problem relates to the physiological responses of peptides in different tissues and organs.

Multiple peptide receptor subtypes

The existence of more than one pharmacologically characterized receptor type for a given peptide has already been mentioned in the context of the mismatch between opioid peptides and opioid receptors. It appears to be a general rule that multiple subtypes or receptors exist for a given peptide family. Again the historical and foremost example is the case of opioid receptors[117,118]. At least 5–6 putative opioid receptor subtypes (table 2) have been proposed in the literature and attempts have been made to correlate the affinity of different opioid peptides to these receptors in order to establish the most probable endogenous ligand for each receptor subtype. Receptor autoradiography has revealed that these subtypes present very different regional distributions in the mammalian brain and has suggested the most appropriate regions to study the different receptor subtypes. Many other examples of multiplicity of peptide receptors have been reported and have been illustrated by receptor autoradiography.

The most recent example of peptide receptor multiplicity is provided by the tachykinins[101]. At least three subtypes of receptors for the peptides of the family of SP have been proposed and named SP-P, SP-K and SP-E (recently renamed NK-1, NK-2 and NK-3 respectively), depending on the preferential affinity of the different peptides of the tachykinin familiy for these subtypes. In figure 4 we illustrate the different distribution of receptors for SP (SP-P) and receptors for eledoisin (SP-E receptors) in the rat brain. The presence of very distinct regional distributions for these ligands clearly suggests that these receptors are different not only in terms of pharmacology but probably in terms of structure. This is an illustration of the power of receptor autoradiography to provide information on the presence of different subtypes of receptors for peptides and information that can be used to explore their different physiological functions.

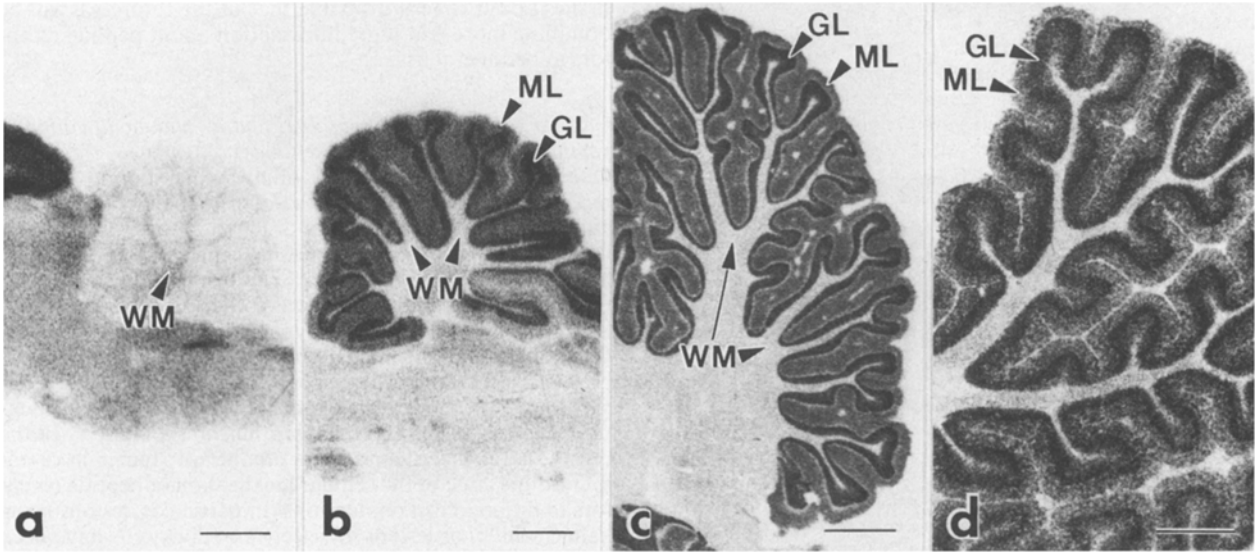

Figure 3. Distribution of [^{125}I]Bolton-Hunter cholecystokinin-8 binding in the cerebellum of 4 different vertebrate species is shown. In the rat (a) no cholecystokinin-8 binding sites can be detected in this structure, while guinea pig (b), cat (c) and human (d) show a high density of cholecystokinin-8 binding sites particularly in the granular cell layer. ML, molecular cell layer; GL, granular cell layer; WM, white matter. Bar = 2.0 mm.

Peptide receptors in the human brain. Implications for pathology

As mentioned above, the technique of receptor autoradiography can be used in the study of human postmortem material[91]. We have analyzed a large series of neurotransmitter and drug receptors in human postmortem material and found that these molecules survive the chemical changes in the postmortem human brain quite well. We have been able to localize and map receptors for a number of peptides in human postmortem tissues including opioid, neurotensin, somatostatin, TRH, CCK, SP and other receptors. In addition to the already mentioned differences in species distributions and pharmacological characteristics it has been observed that receptors for peptides are changed under some neuropathological conditions[91,120,121]. One of the first reported receptor changes in the human brain was the loss of neurotensin receptors in the substantia nigra of patients dying with Parkinson's disease[121]. This observation followed the observation of high densities of neurotensin receptors in the rat substantia nigra[86] and the loss of these receptors after chemical lesion of the dopaminergic cells using the neurotoxin 6-hydroxydopamine (fig. 5).

Other neuropeptide receptors have also been found to be modified in some neuropathological states of the human brain. For example, somatostatin receptors have been found to be decreased in the cortex of patients dying from senile dementia of the Alzheimer type. We have used autoradiography to study these changes and observed selective decreases of somatostatin receptors in areas such as the hippocampus and the neocortex of patients with senile dementia while in parkinsonian patients somatostatin receptors appear to be more preferentially decreased in the nuclei caudatus and putamen and the substantia nigra (Cortés et al., unpublished).

The ability of autoradiography to detect subtile changes in pathology offers the possibility of using peptide receptor autoradiography as a tool in neuropathological investigations. This type of investigation will probably be extended to pathologies outside the brain where peptide receptor mechanisms appear to be involved, and in particular diseases of the gut where peptides play an important regulatory role.

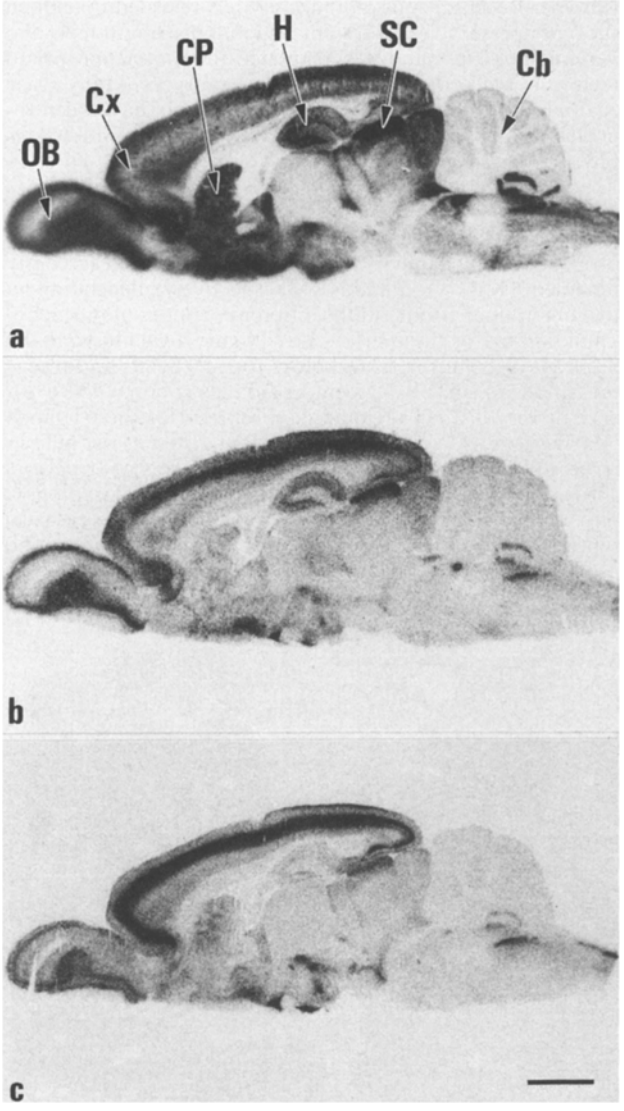

Figure 4. Three sequential sagittal rat sections processed for autoradiography to visualize the three tachykinin receptor subtypes. Three different ligands were used, [^{125}I]Bolton-Hunter substance P (a) for the SP-P or NK1 subtype, [^{125}I]Bolton-Hunter substance K (b) for the SP-K or NK2 subtype and [^{125}I]Bolton-Hunter eledoisin (c) for the SP-E or NK3 subtype were used. Note the differential localization of these receptors in areas such as the neocortex (Cx), caudate-putamen (CP), hippocampus (H) and olfactory bulb (OB). Bar = 1.0 mm.

Peptide receptors in peripheral tissues

Using in vitro autoradiography, peptide receptors have also been localized to peripheral tissues and some of the results in these tissues are listed on table 2. The localization of SP receptors in the guinea pig ileum is illustrated in figure 6. These results clearly demonstrate the possibility of using autoradiography for the study of receptors in peripheral tissues, allowing the microscopic localization of receptors in these tissues and the correlation of the known function of regulatory peptides in the regulation of their functions. Problems similar to those already discussed above, such as the mismatch problem and the presence of receptor subtypes for related peptides have been examined using autoradiography. An example is the presence of the different subtypes of the tachykinin receptors in peripheral organs[10,70].

The technique of receptor autoradiography, however, has not yet been extensively exploited in the study of peripheral tissues and it is very probable that in the future we will be obtaining more and more information about peptide receptors in peripheral tissues.

Cellular localization of peptide receptors and modification of peptide receptors after drug treatment

One of the main limitations of the most commonly used receptor autoradiography techniques is the lack of cellular and subcellular resolution. In order to compensate for this lack of resolution the technique of receptor autoradiography has been combined with selective lesions of different cellular types. As mentioned above it was the lesion of the dopaminergic cells in the substantia nigra with the neurotoxin 6-hydroxydopamine which led to the discovery of the presence of high densities of neurotensin receptors in these cells[86] and eventually to the finding of a selective decrease of neurotensin receptors in the parkinsonian substantia nigra[121]. In the central nervous system a large number of studies have addressed the issue of the cellular localization of peptide receptors to different cell populations. In particular, lesions using kainic acid[107], or lesions of selective pathways[13a], have been used to illustrate the preferential localization of receptors in different types of neurotransmitter-identified neurons and pathways in the central nervous system of the rat[107] and the cat[13a].

One of the most important conclusions of all these studies is

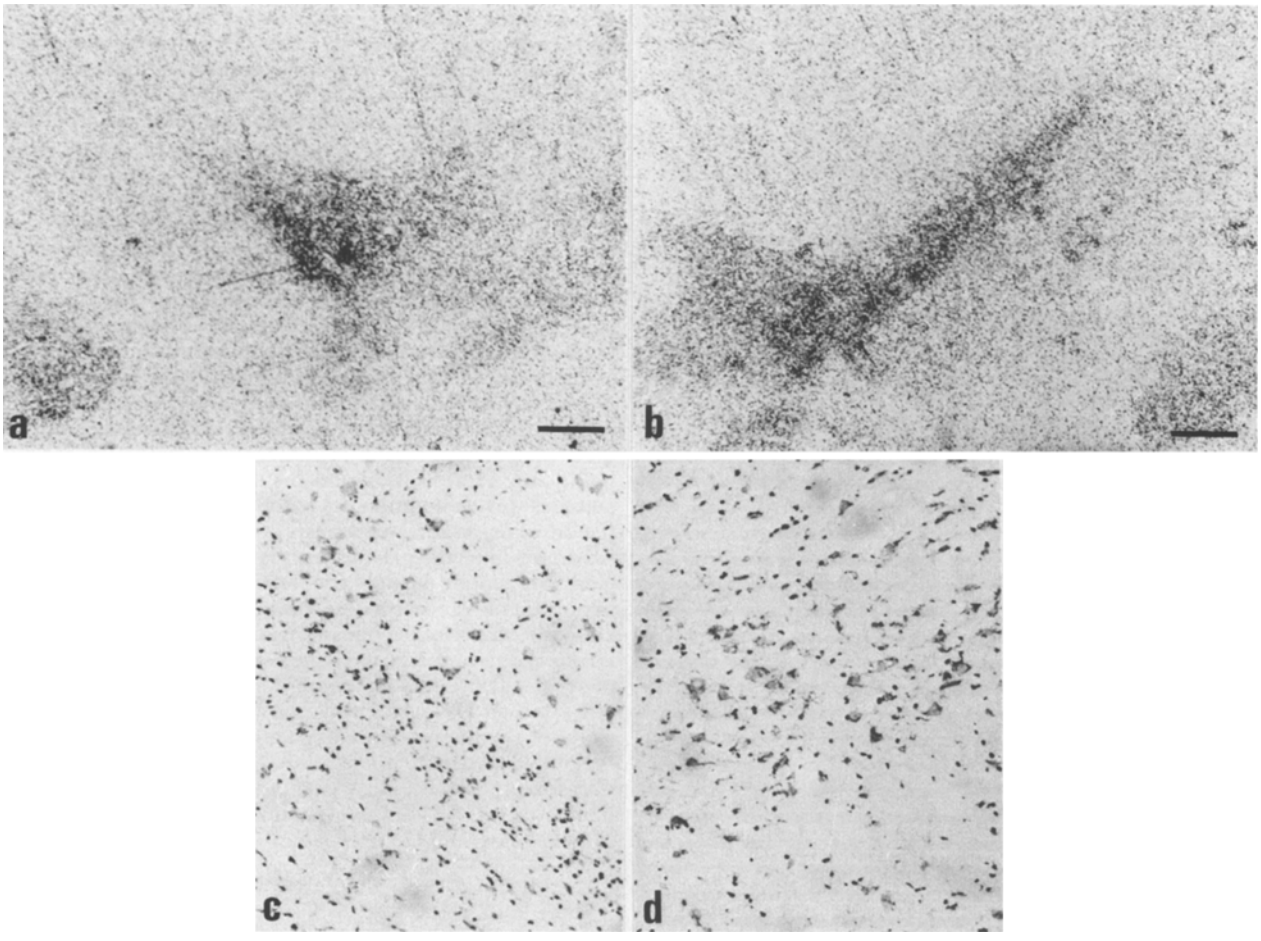

Figure 5. Autoradiographic localization of neurotensin receptors to dopaminergic neurons in rat substantia nigra zona compacta (a). Injection of 6-hydroxydopamine results in a loss of neurotensin receptors and dopamine-containing cells in the substantia nigra zona compacta (b). Higher power photomicrographs of the same sections colored with cresyl violet (c, d). Note the loss of cell bodies in the zona compacta in *c* (corresponding to the injected left side, see *a*) compared with the opposite uninjected side in *d*. Bar = 250 μm (a, b).

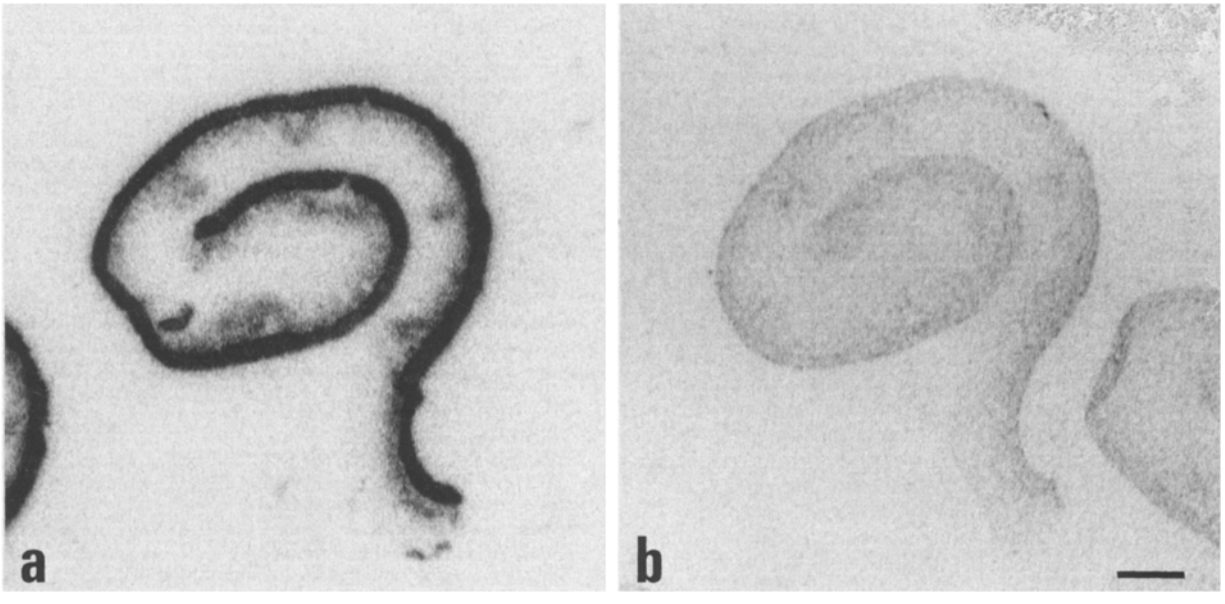

Figure 6. [^{125}I]Bolton-Hunter substance P binding in the guinea pig ileum is mainly localized on the muscle layer (a). Non-specific binding in an adjacent section obtained by adding 1 μM of cold substance P to the incubation medium (b). Bar = 1.0 mm.

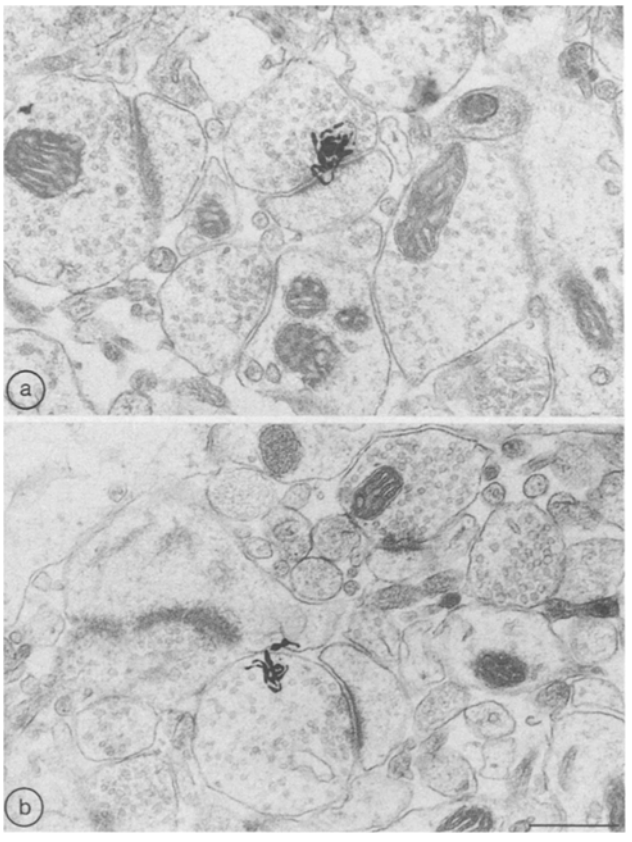

Figure 7. Electron microscopic autoradiographs processed from a vibratome-cut, prefixed section of rat striatum and incubated with [^{125}I]FK 33-824. The radioactivity is detected in the form of isolated silver grains overlying in both instances the apposed plasma membranes of an axon terminal and a dendritic process. Synaptic localization shown in *a*, and non-synaptic localization shown in *b*. Bar = 0.5 μm. (Reproduced with permission from A. Beaudet[41].)

that peptide receptors appear to be preferentially localized in neurons and selected pathways in the mammalian central nervous system.

Interestingly, also in the human brain peptide receptors appear to be preferentially localized in neurons. We have analyzed the effects of localized small vascular lesions in the hippocampus of patients dying without a reported neurological disease and found that the loss of neurons was correlated with the loss of binding for peptides, as for example somatostatin[105]. However, the lack of cellular resolution of light microscopic autoradiography is an important issue which has led several groups to attempt to develop procedures for the electronmicroscopic localization of peptide receptor sites[40,41]. As mentioned in the methodology section this has been facilitated by the fact that peptides can be cross-linked to their receptors. Electronmicroscopic autoradiography, however, is also limited in its resolution by the fact that in the central nervous system membranes of more than one type co-exist inside the so-called '50% probability circle'[56]. However, the results obtained so far with the μ-subtype[40,41] of the opioid receptor (fig. 7) and neurotensin (Beaudet et al., unpublished) have resulted in a number of interesting conclusions regarding the localization of peptide receptors. The majority of these receptors appear to be extrasynaptic, that is they are localized outside the synaptic terminals as visualized at the electronmicroscopic level and this had led to the speculation that opioids can act at distance in a hormonal type of transmission.

Conclusions and future trends

The results briefly reviewed in this work illustrate the possibility of using receptor autoradiography in the analysis of the mechanisms of action of regulatory peptides in the brain and other tissues. We have also mentioned a number of limitations of the technique, particularly the relatively low level of subcellular resolution. In the future it is probable that new types of probes will be developed to allow a clearer definition of the cells making peptide receptors. Recently a number of receptors for growth factors, oncogenes and hormones and some neurotransmitters have been cloned and their molecular structure elucidated from the sequence of the genes coding for these proteins. Although until now no receptor for a regulatory peptide has been cloned it is not too speculative to assume that we will soon see published the sequences of DNA coding for opioid, SP and other peptide receptors. This will allow the development of genetic probes for the study of the localization of receptors at the microscopic level, including in situ hybridization and the development of synthetic peptides to which antibodies will be raised, and will expand the use of immunohistochemistry in receptor localization.

Advances in medicinal chemistry are also providing us with new non-peptide ligands for peptide receptors. This is an important advance because of the limitations mentioned above on the use of peptides as ligands, particularly for the in vivo imaging of receptors in the living man using the technique of positron emission tomography. Opioid receptors have already been visualized using a carfentanil and used in the study of the human opioid system in vivo[25]. It is probable that we will see more non-peptide ligands developed for peptide receptors. The most dramatic example is the development of a CCK and non-peptide CCK antagonist by the Merck, Sharp and Dohme Group[11,20].

Acknowledgments. We would like to thank Dr A. Beaudet for providing us with illustrations of electronmicroscopy localization of peptide receptors, Drs M. Herkenham and S. McLean for their unpublished materials and Dr C. R. Jones for the critical reading of the manuscript.

1 Atweh, S.F., and Kuhar, M.J., Autoradiographic localization of opiate receptors in rat brain. I. Spinal cord and lower medulla. Brain Res. *124* (1977) 53–67.
2 Atweh, S.F., and Kuhar, M.J., Autoradiographic localization of opiate receptors in rat brain. II. The brainstem. Brain Res. *129* (1977) 1–12.
3 Atweh, S.F., and Kuhar, M.J., Autoradiographic localization of opiate receptors in rat brain. III. The telencephalon. Brain Res. *134* (1977) 393–405.
4 Baskin, D.G., Petracca, F., and Dorsa, D.M., Autoradiographic localization of specific binding sites for [^{3}H][Arg^{8}]vasopressin in the septum of the rat brain with tritium-sensitive film. Eur. J. Pharmac. *90* (1983) 155–157.
5 Beaujouan, J.C., Torrens, Y., Saffroy, M., and Glowinski, J., Quantitative autoradiographic analysis of the distribution of binding sites for [^{125}I]Bolton Hunter derivatives of eledoisin and substance P in the rat brain. Neuroscience *18* (1986) 857–875.
6 Biegon, A., Terlou, M., Voorhuis, Th.D., and De Kloet, E.R., Arginine-vasopressin binding sites in rat brain: A quantitative autoradiographic study. Neurosci. Lett. *44* (1984) 229–234.
7 Björklund, A., and Hökfelt, T., Eds, GABA and Neuropeptides in the CNS. Handbook of Chem. Neuroanatomy, vol. 4. Elsevier, Amsterdam 1985.
8 Bolton, A.E., and Hunter, W.M., The labeling of proteins to high specific radioactivities by conjugation to a [^{125}I]-containing acylating agent. Biochem. J. *133* (1973) 529–538.
9 Brinton, R.E., Gee, K.W., Wamsley, J.K., Davis, T.P., and Yamamura, H.I., Regional distribution of putative vasopressin receptors in rat brain and pituitary by quantitative autoradiography. Proc. natn. Acad. Sci. USA *81* (1984) 7248–7252.
10 Burcher, E., Shults, C.W., Buck, S.H., Chase, T.N., and O'Donohue, T.L., Autoradiographic distribution of substance K-

binding sites in rat gastrointestinal tract: a comparison with substance P. Eur. J. Pharmac. *102* (1984) 561–562.
11 Chang, R.S.L., and Lotti, V.J., Biochemical and pharmacological characterization of an extremely potent and selective nonpeptide cholecystokinin antagonist. Proc. natn. Acad. Sci. USA *83* (1986) 4923–4926.
12 Cox, B.M., Opheim, K.E., Teschemacher, H., and Goldstein, A., A peptide-like substance from pituitary that acts like morphine. 2. Purification and properties. Life Sci. *16* (1975) 1777–1782.
13 Danks, J.A., Rothman, R.B., Cascieri, M.A., Chicchi, G.G., Liang, T., and Herkenham, M., A comparative autoradiographic study of the distributions of substance P and eledoisin binding sites in rat brain. Brain Res. *385* (1986) 273–281.
13a Dashwood, M.R., Gilbey, M.P., and Spyer, K.M., The localization of adrenoceptors and opiate receptors in regions of the cat central nervous system involved in cardiovascular control. Neuroscience *15* (1985) 537–551.
14 Dawbarn, D., Gregory, J., and Emson, P.C., Visualization of calcitonin gene-related peptide receptors in the rat brain. Eur. J. Pharmac. *111* (1985) 407–408.
15 De Kloet, E.R., Rotteveel, F., Voorhuis, Th.A.M., and Terlou, M., Topography of binding sites for neurohypophyseal hormones in rat brain. Eur. J. Pharmac. *110* (1985) 113–119.
16 De Souza, E.B., Perrin, M.H., Insel, T.R., Rivier, J., Vale, W.W., and Kuhar, M.J., Corticotropin-releasing factor receptors in rat forebrain: autoradiographic identification. Science *224* (1984) 1449–1451.
17 De Souza, E.B., Seifert, H., and Kuhar, M.J., Vasoactive intestinal peptide receptor localization in rat forebrain by autoradiography. Neurosci. Lett. *56* (1985) 113–120.
18 Dietl, M.M., Probst, A., and Palacios, J.M., On the distribution of cholecystokinin receptor binding sites in the human brain: An autoradiographic study. Synapse *1* (1987) 169–183.
19 Dietl, M.M., Probst, A., and Palacios, J.M., Mapping of substance P receptor sites in the human brain, In preparation.
20 Evans, B.E., Bock, M.G., Rittle, K.E., DiPardo, R.M., Whitter, W.L., Veber, D.F., Anderson, P.S., and Freidinger, R.M., Design of potent, orally effective, nonpeptidal antagonists of the peptide hormone cholecystokinin. Proc. natn. Acad. Sci. USA *83* (1986) 4918–4922.
21 Fischer, J.A., Tobler, P.H., Kaufmann, M., Born, W., Henke, H., Cooper, P.E., Sagar, S.M., and Martin, J.B., Calcitonin: Regional distribution of the hormone and its binding sites in the human brain and pituitary, Proc. natn. Acad. Sci. USA *78* (1981) 7801–7805.
22 Foote, R.W., and Maurer, R., Autoradiographic localization of oiate K-receptors in the guinea-pig brain. Eur. J. Pharmac. *85* (1982) 99–103.
23 Foote, R.W., and Maurer, R., Distribution of opioid binding sites in the guinea-pig hippocampus as compared to the rat: A quantitative analysis. Neuroscience *19* (1986) 847–856.
24 Freund-Mercier, M.J., Stoeckel, M.E., Palacios, J.M., Pazos, A., Reichhart, J.M., Porte, A., and Richard, Ph., Pharmacological characteristics and anatomical distribution of [^{3}H]oxytocin-binding sites in the Wistar rat brain studied by autoradiography. Neuroscience *20* (1987) 599–614.
25 Frost, J.J., Wagner, H.N. Jr., Dannals, R.F., Ravert, H.T., Links, J.M., Wilson, A.A., Burns, H.D., Wong, D.F., McPherson, R.W., Rosenbaum, A.E., Kuhar, M.J., and Snyder, S.H., Imaging opiate receptors in the human brain by positron tomography. J. comp. ass. Tomog. *9* (1985) 231–236.
26 Gaudreau, P., Quirion, R., St. Pierre, S., and Pert, C.B., Tritium-sensitive film autoradiography of [^{3}H]cholecystokinin-5/pentagastrin receptors in rat brain. Eur. J. Pharmac. *87* (1983) 173–174.
27 Geary, W.A., and Wooten, G.F., Regional tritium quenching in quantitative autoradiography of the central nervous system. Brain Res. *336* (1985) 334–336.
28 Geary, W.A., Toga, A.W., and Wooten, G.F., Quantitative film autoradiography for tritium: Methodological consideration. Brain Res. *337* (1985) 99–108.
29 Gehlert, D.R., Speth, R.C., Healy, D.P., and Wamsley, J.K., Autoradiographic localization of angiotensin II receptors in the rat brainstem. Life Sci. *34* (1984) 1565–1571.
30 Gehlert, D.R., Speth, R.C., and Wamsley, J.K., Autoradiographic localization of angiotensin II receptors in the rat brain and kidney. Eur. J. Pharmac. *98* (1984) 145–146.
31 Gibson, T.R., Wildey, G.M., Manaker, S., and Glembotski, Ch.C., Autoradiographic localization and characterization of atrial natriuretic peptide binding sites in the rat central nervous system and adrenal gland. J. Neurosci. *6* (1986) 2004–2011.
32 Goochee, C., Rasband, W., and Sokoloff, L., Application of computer-assisted image processing to autoradiographic methods for studying brain functions. Trends Neurosci. *6* (1983) 256–260.
33 Goodman, R.R., Snyder, S.H., Kuhar, M.J., and Young III, W.S., Differentiation of delta and mu opiate receptor localizations by light microscopic autoradiography. Proc. natn. Acad. Sci. USA *77* (1980) 6239–6243.
34 Goodman, R.R., and Snyder, S.H., Kappa opiate receptors localized by autoradiography to deep layers of cerebral cortex: Relation to sedative effects. Proc. natn. Acad. Sci. USA *79* (1982) 5703–5707.
35 Goodman, R.R., Houghten, R.A., and Pasternak, G.W., Autoradiography of [^{3}H]β-endorphin binding in brain. Brain Res. *288* (1983) 334–337.
36 Gouardères, C., Cros, J., and Quirion, R., Autoradiographic localization of mu, delta and kappa opioid receptor binding sites in rat and guinea-pig spinal cord. Neuropeptides *6* (1985) 331–342.
37 Greenwood, F.C., Hunter, W.M., and Glover, J.S., The preparation of [^{131}I]-labeled human growth hormone of high specific radioactivity. Biochem. J. *89* (1963) 114–123.
38 Gulya, K., Wamsley, J.K., Gehlert, D., Pelton, J.T., Duckles, S.P., Hruby, V.J., and Yamamura, H.I., Light microscopic autoradiographic localization of somatostatin receptors in the rat brain. J. Pharmac. exp. Ther. *235* (1985) 254–258.
39 Gundlach, A.L., Largent, B.L., and Snyder, S.H., Autoradiographic localization of sigma receptor binding sites in guinea-pig and rat central nervous system with (+)[^{3}H]3-(-3-hydroxyphenyl)-N-(1-propyl)piperidine. J. Neurosci. *6* (1986) 1757–1770.
40 Hamel, E., and Beaudet, A., Electron microscopic autoradiographic localization of opioid reaceptors in rat neostriatum. Nature *312* (1984) 155–157.
41 Hamel, E., and Beaudet, A., Localization of opioid binding sites in rat brain by electron microscopic radioautography. J. Electron Microsc. Techn. *1* (1984) 317–329.
42 Hays, S.E., Beinfeld, M.C., Jensen, R.T., Goodwin, F.K., and Paul, S.M., Demonstration of a putative receptor site for cholecystokinin in rat brain. Neuropeptides *1* (1980) 53–62.
43 Healy, D.P., Maciejewski, A.R., and Printz, M.P., Autoradiographic localization of [^{125}I]angiotensin II binding sites in the rat adrenal gland. Endocrinology *116* (1984) 1221–1223.
44 Henke, H., Tobler, P.H., and Fischer, J.A., Localization of salmon calcitonin binding sites in rat brain by autoradiography. Brain Res. *272* (1983) 373–377.
45 Henke, H., Tschopp, F.A., and Fischer, J.A., Distinct binding sites for calcitonin gene-related peptide and salmon calcitonin in rat central nervous system. Brain Res. *360* (1985) 165–171.
46 Herkenham, M., and Pert, C.B., In vitro autoradiography of opiate receptors in rat brain suggests loci of 'opiatergic' pathways. Proc. natn. Acad. Sci. USA *77* (1980) 5532–5536.
47 Herkenham, M., and Sokoloff, L., Quantitative receptor autoradiographic tissue defatting eliminates differential self-absorption of tritium radiation in grey and white matter of brain. Brain Res. *321* (1984) 363–368.
48 Herkenham, M., and McLean, S., Mismatches between receptor and transmitter localization in the brain, in: Quantitative Receptor Autoradiography, pp. 137–171. Eds C.A. Boast, E.W. Snowhill and C.A. Altar. Alan R. Liss, New York 1986.
49 Hill, J.M., Lesniak, M.A., Pert, C.B., and Roth, J., Autoradiographic localization of insulin receptors in rat brain: Prominence in olfactory and limbic areas. Neuroscience *17* (1986) 1127–1138.
50 Hökfelt, T., Johansson, O., Ljungdahl, A., Lundberg, J.M., and Schultzberg, M., Peptidergic neurons. Nature *284* (1980) 515–521.
51 Hughes, J., Smith, T.W., Kosterlitz, H.W., Fothergill, L.A., Morgan, B.A., and Morris, H.R., Identification of two related pentapeptides from the brain with potent opiate agonist activity. Nature *258* (1975) 577–559.
52 Hwang, B.H., Harding, J.W., Liu, D.K., Hibbard, L.S., Wieczorek, C.M., and Wu J.-Y., Quantitative autoradiography of [^{125}I]-[Sar^1, Ile^8]-angiotensin II binding in the brain of spontaneously hypertensive rats. Brain Res. *16* (1986) 75–82.
53 Inagaki, S., Kito, S., Kubota, Y., Girgis, S., Hillyard, C.J., and Macintyre, I., Autoradiographic localization of calcitonin gene-related peptide binding sites in human and rat brains. Brain Res. *374* (1986) 287–298.
54 Iversen, L.L., Neuropeptides – what next? Trends Neurosci. *4* (1983) 293–294.
55 Krieger, D.T., Brownstein, M.J., and Martin, J.B., Eds, Brain Peptides. John Wiley & Sons, New York 1983.
56 Kuhar, M.J., Taylor, N., Wamsley, J.K., Hulme, E.C., and Birdsall, N.J.M., Muscarinic cholinergic receptor localization in brain by electron microscopic autoradiography. Brain Res. *216* (1981) 1–9.

57 Kuhar, M.J., The mismatch problem in receptor mapping studies. Trends Neurosci. *8* (1985) 190–191.

58 Kuhar, M.J., and Unnerstall, J.R., Quantitative receptor mapping by autoradiography: Some current technical problems. Trends Neurosci. *8* (1985) 49–53.

59 Kuhar, M.J., De Souza, E.B., and Unnerstall, J.R., Neurotransmitter receptor mapping by autoradiography and other methods. A. Rev. Neurosci. *9* (1986) 27–60.

60 Kuhar, M.J., Quantitative receptor autoradiography: An overview, in: Quantitative Receptor Autoradiography, pp. 1–12. Eds C.A. Boast, E.W. Snowhill and C.A. Altar. Alan R. Liss, New York 1986.

61 Kurihara, M., Shigematsu, K., and Saavedra, J.M., Localization of atrial natriuretic peptide, ANP-(99-126) binding sites in the rat thymus and spleen with quantitative autoradiography. Reg. Peptides *15* (1986) 341–346.

62 Largent, B.L., Gundlach, A.L., and Snyder, S.H., Pharmacological and autoradiographic discrimination of sigma and phencyclidine receptor binding sites in brain with (+)-[^{3}H]SKF 10,047, (+)-[^{3}H]-3-[3-hydroxyphenyl[-N-(1-propyl)piperidine and [^{3}H]-1-]1-(2-thienyl)cyclohexyl]piperidine. J. Pharmac. exp. Ther. *238* (1986) 739.

63 Leroux, P., Quirion, R., and Pelletier, G., Localization and characterization of brain somatostatin receptors as studied with somatostatin-14 and somatostatin-28 receptor radioautography. Brain Res. *347* (1985) 74–84.

64 Lewis, M.E., Khachaturian, H., and Watson, S.J., Visualization of opiate receptors and opioid peptides in sequental brain sections. Life Sci. *31* (1982) 1347–1350.

65 Lewis, M.E., Young, E.A., Houghten, R.A., Akil, H., and Watson, S.J., Binding of [^{3}H]dynorphin a to apparent K-opioid receptors in deep layers of guinea-pig cerebral cortex. Eur. J. Pharmac. *98* (1984) 149–150.

66 Manaker, S., Rainbow, T.C., and Winokur, A., Thyrotropin-releasing hormone (TRH) receptors: Localization in rat and human central nervous system, in: Quantitative Receptor Autoradiography, pp. 103–135. Eds C.A. Boast, E.W. Snowhill and C.A. Altar. Alan R. Liss, New York 1986.

67 Manning, D.C., and Snyder, S.H., Bradykinin receptor-mediated chloride secretion in intestinal function. Nature *299* (1982) 256–259.

68 Mantyh, P.W., Hunt, S.P., and Maggio, J.E., Substance P receptors: Localization by light microscopic autoradiography in rat brain using [^{3}H]SP as the radioligand. Brain Res. *307* (1984) 147–165.

69 Mantyh, P.W., Maggio, J.E., and Hunt, S.P., The autoradiographic distribution of kassinin and substance K binding sites is different from the distribution of substance P binding sites in rat brain. Eur. J. Pharmac. *102* (1984) 361–364.

70 Mantyh, P.W., Goedert, M., and Hunt, S.P., Autoradiographic visualization of receptor binding sites for substance P in the gastrointestinal tract of the guinea-pig. Eur. J. Pharmac. *100* (1984) 133–134.

71 Mantyh, C.R., and Mantyh, P.W., Differential localization of cholecystokinin-8 binding sites in the rat vs. the guinea-pig brain. Eur. J. Pharmac. *113* (1985) 137–139.

72 Mantyh, P.W., and Hunt, S.P., Localization by light microscopic autoradiography in rat brain using [^{3}H][3-Me-His^2]TRH as the radioligand. J. Neurosci. *5* (1985) 551–561.

73 Martin, J.L., Dietl, M.M., Hof, P., Palacios, J.M., and Magistretti, P.J., Autoradiographic localization of [Mono(^{125}I)Iodo-Tyr^{10}MetO^{17}]vasoactive intestinal peptide. Neuroscience (1987) in press.

74 Maurer, R., Cortés, R., Probst, A., and Palacios, J.M., Multiple opiate receptor in human brain: an autoradiographic investigation. Life Sci. *33* (1983) 231–234.

75 Maurer, R., Marbach, P., and Mousson, R., Salmon calcitonin binding sites in rat pituitary. Brain Res. *261* (1983) 346–348.

76 Maurer, R., Comparative in vitro / in vivo autoradiography using the opiate ligand ^{3}H-(-)-bremazocine. J. Receptor Res. *4* (1984) 155–163.

77 Maurer, R., and Reubi, J.C., Brain somatostatin receptor subpopulation visualized by autoradiography. Brain Res. *333* (1985) 178–181.

78 Maurer, R., and Reubi, J.C., Somatostatin receptors in the adrenal. Molec. cell. Endocr. *45* (1986) 81–90.

79 Maurin, Y., Buck, S.H., Wamsley, J.K., Burks, T.F., and Yamamura, H.I., Light microscopic autoradiographic localization of [^{3}H]substance P binding sites in rat thoracic spinal cord. Life Sci. *34* (1984) 1713–1716.

80 McLean, S., Rothman, R.B., Jacobson, A.E., Rice, K.C., and Herkenham, M., Distribution of opiate receptor subtypes and enkephalin and dynorphin immunoreactivity in the hippocampus of squirrel, guinea-pig, rat and hamster. J. comp. Neurol. (1987), in press.

81 Melander, T., Hökfelt, T., Nilsson, S., and Brodin, E., Visualization of galanin binding sites in the rat central nervous system. Eur. J. Pharmac. *124* (1986) 381–382.

82 Mendelsohn, F.A.O., Quirion, R., Saavedra, J.M., Aguilera, G., and Catt, K.J., Autoradiographic localization of angiotensin II receptors in rat brain. Proc. natn. Acad. Sci. USA *81* (1984) 1575–1579.

83 Morris, B.J., and Herz, A., Autoradiographic localization in rat brain of kappa opiate binding sites labeled by [^{3}H]bremazocine. Neuroscience *19* (1986) 839–846.

84 Murphy, K.M.M., McLaughlin, L.L., Michener, M.L., and Needleman, P., Autoradiographic localization of atriopeptin III receptors in rat kidney. Eur. J. Pharmac. *111* (1985) 291–292.

85 Olgiati, V.R., Guidobonon, F., Netti, C., and Pecile, A., Localization of calcitonin binding sites in rat central nervous system: evidence of its neuroactivity. Brain Res. *265* (1983) 209–215.

86 Palacios, J.M., and Kuhar, M.J., Neurotensin receptors are located on dopamine-containing neurons in rat midbrain. Nature *294* (1981) 587–589.

87 Palacios, J.M., Niehoff, D.H., and Kuhar, M.J., Receptor autoradiography with tritium-sensitive film: Potential for computerized densitometry. Neurosci. Lett. *25* (1981) 101–105.

88 Palacios, J.M., and Wamsley, J.K., Receptor for amines, amino acids and peptides: Biochemical characterization and microscopic localization, in: Chemical Transmission in the Brain, Progress in Brain Research, vol. 55, pp. 265–278. Eds R.M. Buijs, P. Pévet and D.F. Swaab. Elsevier Biomedical Press 1982.

89 Palacios, J.M., Receptor autoradiography, 10 years later. J. Receptor Res. *4* (1984) 633–644.

90 Palacios, J.M., and Maurer, R., Autoradiographic localization of drug and neurotransmitter receptors: focus on the opiate receptor. Acta histochem. Suppl. *29* (1984) 41–50.

91 Palacios, J.M., Probst, A., and Cortés, R., Mapping receptors in the human brain. Reg. Peptides *15* (1986) 341–346.

92 Pazos, A., Cortés, R., and Palacios, J.M., Thyrotropin-releasing hormone receptor binding sites: autoradiographic distribution in the rat and guinea-pig brain. J. Neurochem. *45* (1985) 1448–1463.

93 Penney, J.B., Pan, H.S., Young, A.B., Frey, K.A., and Dauth, G.W., Quantitative autoradiography of [^{3}H]muscimol binding in rat brain. Science *214* (1981) 1036–1038.

94 Pert, C.B., Kuhar, M.J., and Snyder, S.H., Opiate receptor: Autoradiographic localization in rat brain. Proc. natn. Acad. Sci. USA *73* (1976) 3729–3733.

95 Pilotte, N.S., Sharif, N.A., and Burt, D.R., Characterization and autoradiographic localization of TRH receptors in sections of rat brain. Brain Res. *293* (1984) 372–376.

96 Quirion, R., Hammer, R.P., Herkenham, M., and Pert, C.B., Phencyclidine (angel dust), 'opiate' receptors: Visualization by tritium-sensitive film. Proc. natn. Acad. Sci. USA *78* (1981) 5881–5885.

97 Quirion, R., Gaudreau, P., St. Pierre, S., Rioux, F., and Pert, C.B., Autoradiographic distribution of [^{3}H]neurotensin receptors in rat brain: visualization by tritium-sensitive film. Peptides *3* (1982) 757–763.

98 Quirion, R., Shults, C.W., Moody, T.W., Pert, C.B., Chase, T.N., and O'Donohue, T.L., Autoradiographic distribution of substance P receptors in rat central nervous system. Nature *303* (1983) 714–716.

99 Quirion, R., Dalpe, M., DeLean, A., Gutkowska J., Cantin, M., and Genest, J., Atrial natriuretic factor (ANF) binding sites in brain and related structures. Peptides *5* (1984) 1167–1172.

100 Quirion, R., and Dam, T.-V., Multiple tachykinin receptors in guinea-pig brain. High densities of substance K (neurokinin A) binding sites in the substantia nigra. Neuropeptides *6* (1985) 191–204.

101 Quirion, R., Multiple tachykinin receptors. Trends Neurosci. *8* (1985) 183–185.

102 Rainbow, T.C., Bleisch, W., Biegon, A., and McEwen, B.S., Quantitative densitometry of neurotransmitter receptors. J. Neurosci. Meth. *5* (1982) 127–138.

103 Reubi, J.C., and Maurer, R., Visualization of LHRH receptors in the rat brain. Eur. J. Pharmac. *106* (1985) 453–454.

104 Reubi, J.C., and Maurer, R., Autoradiographic mapping of somatostatin receptors in the rat central nervous system and pituitary. Neuroscience *15* (1985) 1183–1193.

105 Reubi, J.C., Cortés, R., Maurer, R., Probst, A., and Palacios, J.M., Distribution of somatostatin receptors in the human brain: an autoradiographic study. Neuroscience *18* (1986) 329–346.

106 Reubi, J.C., Palacios, J.M., and Maurer, R., Specific LHRH receptors in the rat hippocampus and pituitary: An autoradiographical study. Neuroscience (1987) in press.

107 Ritter, J.K., Gehlert, D.R., Ribb, J.W., Wamsley, J.K., and Han-

son, G.R., Neuronal localization of substance P receptors in rat neostriatum. Eur. J. Pharmac. *109* (1985) 431–432.
108 Rothman, R.B., Herkenham, M., Pert, C.B., Liang, T., and Cascieri, M.A., Visualization of rat brain receptors for the neuropeptide, substance P. Brain Res. *309* (1984) 47–54.
109 Rothman, R.B., Danks, J.A., Herkenham, M., Cascieri, M.A., Chicchi, G.G., Liang, T., and Pert, C.B., Autoradiographic localization of a novel peptide binding site in rat brain using the substance P analog, eledoisin. Neuropeptides *4* (1984) 343–349.
110 Sarrieau, A., Javoy-Agid, F., Kitabgi, P., Dussaillant, M., Vial, M., Vincent, J.P., Agid, Y., and Rostène, W.H., Characterization and autoradiographic distribution of neurotensin binding sites in the human brain. Brain Res. *348* (1985) 375–380.
111 Schultzberg, M., and Hökfelt, T., The mismatch problem in receptor autoradiography and the coexistence of multiple messengers. Trends Neurosci. *9* (1986) 109–110.
112 Seifert, H., Chesnut, J., De Souza, E., Rivier, J., and Vale, W., Binding sites for calcitonin gene-related peptide in distinct areas of rat brain. Brain Res. *346* (1985) 195–198.
113 Simantov, R., and Snyder, S.H., Morphine-like factors in mammalian brain: Structure-elucidation and interactions with the opiate receptor. Proc. natn. Acad. Sci. USA *73* (1976) 2515–2519.
114 Snyder, S.H., The opiate receptor in normal and drug altered brain function. Nature *257* (1975) 185–189.
115 Snyder, S.H., and Bennett, J.P. Jr, Neurotransmitter receptors in the brain: Biochemical identification. A. Rev. Physiol. *38* (1976) 153–175.
116 Snyder, S.H., and Innis, R.B., Peptide neurotransmitters. A. Rev. Biochem. *48* (1979) 755–782.
117 Snyder, S.H., Brain peptides and neurotransmitters. Science *209* (1980) 967–983.
118 Snyder, S.H., and Goodman, R.R., Multiple neurotransmitter receptors. J. Neurochem. *35* (1980) 5–15.
119 Speth, R.C., Wamsley, J.K., Gehlert, D.R., Chernicky, C.L., Barnes, K.L., and Ferrario, C.M., Angiotensin II receptor localization in the canine CNS. Brain Res. *326* (1985) 137–143.
120 Uhl, G.R., and Kuhar, M.J., Chronic neuroleptic treatment enhances neurotensin receptor binding in human and rat substantia nigra. Nature *309* (1984) 350.
121 Uhl, G.R., Whitehouse, P.J., Price, D.L., Tourtelotte, W.W., and Kuhar, M.J., Parkinson's disease: Depletion to substantia nigra neurotensin receptors. Brain Res. *308* (1984) 186–190.
122 Uhl, G.R., Tran, V., Snyder, S.H., and Martin, J.B., Somatostatin receptors: Distribution in rat central nervous system and human frontal cortex. J. comp. Neurol. *240* (1985) 288–304.
123 Undén, A., Peterson, L.-L., and Bartfai, T., Somatostatin, substance P, vasoactive intestinal polypeptide, and neuropeptide Y receptors: Critical assessment of biochemical methodology and results, in: International Review of Neurobiology, vol. 27. Eds J.R. Smythies and R.J. Bradley. Academic Press, Inc., Orlando, New York 1985.
124 Unnerstall, J.R., Kuhar, M.J., Niehoff, D.L., and Palacios, J.M., Benzodiazepine receptors are coupled to a subpopulation of aminobutyric acid (GABA) receptors: Evidence from a quantitative autoradiographic study. J. Pharmac. exp. Ther. *218* (1981) 797–804.
125 Unnerstall, J.R., Niehoff, D.L., Kuhar, M.J., and Palacios, J.M., Quantitative receptor autoradiography using [^{3}H]Ultrofilm: Application to multiple benzodiazepine receptors. J. Neurosci. Meth. *6* (1982) 59–73.
126 Van Dijk, A., Richards, J.G., Trzeciak, A., Gillessen, D., and Möhler, H., Cholecystokinin receptors: biochemical demonstration and autoradiographical localization in rat brain and pancreas using [^{3}H]cholecystokinin as radioligand. J. Neurosci. *4* (1984) 1021–1033.
127 Van Leeuwen, F.W., and Wolters, P., Light microscopic localization of [^{3}H]arginine-vasopressin binding sites in the rat brain and kidney. Neurosci. Lett. *41* (1983) 61–66.
128 Van Leeuwen, F.W., Van Heerikhuize, J., Van Der Meulen, G., and Wolters, P., Light microscopic autoradiographic localization of [^{3}H]oxytocin binding sites in the rat brain, pituitary and mammary gland. Brain Res. *359* (1985) 320–325.
129 Von Schroeder, H.P., Nishimura, E., McIntosh, C.H.S., Buchan, A.M.J., Wilson, N., and Ledsome, J.R., Autoradiographic localization of binding sites for atrial natriuretic factor. Can. J. Physiol. Pharmac. *63* (1985) 1373–1377.
130 Wamsley, J.K., Zarbin, M.A., Young, W.S. III, and Kuhar, M.J., Distribution of opiate receptors in the monkey brain: an autoradiography study. Neuroscience *7* (1982) 595–613.
131 Whitford, C.A., Candy, J.M., Bloxham, C.A., Oakley, A.E., and Snell, C.R., Human cerebellar cortex possesses high affinity binding sites for [^{3}H]somatostatin. Eur. J. Pharmac. *113* (1985) 129–132.
132 Wolf, S.S., Moody, T.W., O'Donohue, T.L., Zarbin, M.A., and Kuhar, M.J., Autoradiographic visualization of rat brain binding sites for bombesin-like peptides. Eur. J. Pharmac. *87* (1983) 163–164.
133 Wolf, S.S., Moody, T.W., Quirion, R., Shults, C.W., Chase, T.N., and O'Donohue, T.L., Autoradiographic visualization of substance P receptors in rat brain. Eur. J. Pharmac. *91* (1983) 157–158.
134 Young, W.S. III, and Kuhar, M.J., A new method for receptor autoradiography: [^{3}H]opioid receptors in rat brain. Brain Res. *179* (1979) 255–270.
135 Young, W.S. III, Kuhar, M.J., Roth, J., and Brownstein, M.J., Radiohistochemical localization of insulin receptors in the adult and developing rat brain. Neuropeptides *1* (1980) 15–22.
136 Young, W.S. III, and Kuhar, M.J., Neurotensin receptor localization by light microscopic autoradiography in rat brain. Brain Res. *206* (1981) 273–285.
137 Zarbin, M.A., Innis, R.B., Wamsley, J.K., Snyder, S.H., and Kuhar, M.J., Autoradiographic localization of cholecystokinin receptors in rodent brain. J. Neurosci. *3* (1983) 877–906.

0014-4754/87/070750-12$1.50 + 0.20/0

Combined axonal transport tracing and immunocytochemistry for mapping pathways of peptide-containing nerves in the peripheral nervous system

by H.C. Su and J.M. Polak

Department of Histochemistry, Royal Postgraduate Medical School, Hammersmith Hospital, London W12 OHS (England)

Summary. The various combinations of axonal transport tracing and immunocytochemistry used for mapping pathways of peptide-containing nerves, and in particular those of the peripheral nervous system, are reviewed. The advantages and disadvantages of these methods are discussed. The applications and results presented illustrate the future potential value of this approach.
Key words. Anterograde tracing; axonal transport; immunocytochemistry; neuropathways; peptide-containing nerve; retrograde tracing.

Introduction

In the beginning of the 1970s neuroanatomy experienced a methodological revolution with the development of a new technique for tracing neural pathways based on the axonal transport of compounds such as horseradish peroxidase (HRP)[50, 55, 56, 100], radiolabelled amino acids[14, 20, 22] and the fluorescent dye Evans blue (bound to albumin)[49]. New tracers, both ingenious and powerful, are still rapidly being added to the tools of the neuroanatomist[1, 2, 23, 28, 31, 44, 48, 52–