Chemical Neuroanatomy

Editor

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Preface

In recent years, there has been a substantial increase in the number of techniques available for neuroanatomical studies. Some of these have represented improvements for mapping CNS pathways using methods for anterograde or retrograde labeling of neurons or their processes. However, along with these improved pathway-tracing techniques, methods have been developed to localize putative neurotransmitters (or their synthetic enzymes) to particular neurons. This type of study was pioneered by the development of catecholamine histofluorescence methods by Bengt Falck and Nils-Åke Hillarp and by the use of acetylcholinesterase staining (as a potential marker for cholinergic neurons) by Charles Shute and Peter Lewis.

Since these initial studies, the number of putative transmitter substances in the nervous system has increased dramatically with discovery of the neuronal localization of a number of neuroactive peptides including the enkephalins and substance P. In the case of the neuronally localized peptides, it is not at all clear if these compounds are neurotransmitters; however, the development of immunohistochemical techniques for peptide localization has provided neuroanatomists with a number of additional markers to characterize individual neurons and neuronal systems. A further complication that was not anticipated was that many of these neuronally localized peptides coexist in neurons known to contain better-established transmitter candidates, such as dopamine, γ-aminobutyric acid, acetylcholine, and 5-hydroxytryptamine. In nearly all of these cases, researchers have little idea of the physiological roles of the neuroactive peptides, although clues are gradually emerging.

This book attempts to superimpose what is currently known about the chemistry and distribution of putative transmitters and their receptors onto the basic organization of the mammalian nervous system. Apart from the intrinsic interest generated by this type of study, it is hoped that these efforts may encourage both the neurochemist to delve further into the organization of the mammalian brain (rather than a homogenate) and the neuroanatomist to use the antibodies and ligands developed by the neurochemist.

P. C. Emson
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Contents

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The Peripheral Nervous System

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The idea that acetylcholine (ACh) and norepinephrine (NE) are the transmitters in the peripheral nervous system is well established and has been reviewed in detail (56,93,140,151,241). However, pharmacological and morphological studies have provided evidence for peripheral neurotransmitters other than ACh and NE. The evidence for peripheral noncholinergic and nonadrenergic transmitters has in the past been critically evaluated by Burnstock (50) and others (57), and the idea that there might be an extensive system of peripheral neurons using a purine nucleotide as transmitter has been proposed (50,54).

Furthermore, in the last 10 years, the possibility that small biologically active peptides might function as transmitters in both the central and peripheral nervous system has received considerable attention. Some of the peptides now known to occur in peripheral neurons were originally discovered in gastrointestinal endocrine cells and were therefore first thought of as gut hormones. Conversely, other peripheral neuropeptides initially discovered in the brain have since been shown to occur also in gut endocrine cells. The identification, characterization, and localization of these substances have been greatly facilitated by the development of immunochemical methods of analysis, notably radioimmunoassay (RIA), for the measurement of small quantities of peptides in tissue extracts and immunohistochemical techniques for the localization of peptides in tissue sections. There have been a number of excellent reviews of adrenergic, cholinergic, purinergic, and 5-hydroxytryptaminergic neurons at the periphery (49,57,59,78,140,151, 224,225,341,342). This chapter therefore considers in detail the evidence for the identity and distribution of other putative peripheral transmitters, notably neuropeptides. Particular attention is given to the gastrointestinal tract, sympathetic ganglia (especially the prevertebral ganglia), and the adrenal gland, since these systems have been intensively studied and are known to have extensive peptidergic innervation.

GENERAL ORGANIZATION OF THE PERIPHERAL NERVOUS SYSTEM

Modern views on the organization of the peripheral nervous system are largely derived from Langley's classification of somatic and autonomic divisions (263). In reviewing earlier work on the peripheral nervous system, he further divided the autonomic nervous system into sympathetic and parasympathetic parts (157,263). The basic pattern of organization of sympathetic and parasympathetic divisions is well established. Preganglionic sympathetic neurons are located in the lateral horn of the spinal cord at the thoracolumbar level (T1–L3). The majority of these neurons make connections in the paravertebral ganglia of the sympathetic chain or in prevertebral ganglia. The postganglionic neurons, in turn, project to their target tissues.

Preganglionic parasympathetic nerves have either a cranial or a sacral origin. Thus, the cranial part consists of preganglionic neurons in the midbrain and hindbrain, which terminate in ganglia close to or within the target organs in the head and neck region and in the thoracic viscera, as well as in the gastrointestinal tract. The sacral part consists of preganglionic neurons in the lateral horn of the sacral spinal cord (S2–S4), which terminate in the large intestine and the urogenital tract, where the postganglionic neurons are located.

The innervation of the gastrointestinal tract can therefore be said to consist of (a) extrinsic nerves from sympathetic, mainly prevertebral, ganglia, (b) parasympathetic nerves of both cranial and sacral origin, and (c) sensory neurons terminating in the gastrointestinal tract and running in sympa-

thetic and parasympathetic trunks. However, many aspects of gut function continue virtually unimpaired when the extrinsic nerve supply has been interrupted (26). On the basis of this evidence, Langley (263) suggested that the intrinsic gut neurons were best considered as a separate system—the enteric nervous system. Classically, ACh is regarded as the transmitter in the postganglionic parasympathetic neurons of the gut as well as other peripheral organs, whereas the postganglionic sympathetic neurons utilize NE. The identity of the transmitter(s) in sensory neurons has long remained unknown, although lately a peptide, substance P, has been suggested as a possible candidate.

Several lines of evidence have suggested that there are many other types of neurons in the gut and other parts of the peripheral nervous system in addition to the cholinergic and noradrenergic neurons. Pharmacological evidence indicates the existence of neurons that on stimulation cause atropine-resistant contractions of intestinal smooth muscle (15–17,39) and therefore presumably contain an excitatory transmitter other than ACh. There is also clear evidence for noncholinergic, nonadrenergic inhibitory neurons (29,30,52,53, 311). In the cat stomach, stimulation of the vagus in the presence of atropine caused inhibitory responses that were not blocked by adrenergic blocking agents (311). In the guinea pig tenia coli, inhibitory junction potentials were produced by stimulation of intramural nerves. Neither atropine nor guanethidine blocked these inhibitory responses (52). There are several physiological events in which the enteric inhibitory neurons have been demonstrated to play a role. Generally, these neurons are involved in inhibitory reflexes, such as the facilitation of passage of material through the alimentary canal. For instance, receptive relaxation of the stomach (1–4,22,61,347,348), the descending inhibition reflex in the intestine (26,55,139,141,198), and the reflex relaxation of the internal anal sphincter (76,156) are likely to be mediated by enteric inhibitory neurons outside of the gastrointestinal tract.

Nonadrenergic inhibitory neurons have been thought to mediate such functions as bronchodilatation (443) and relaxation of lung musculature (366) and vasodilatation in several tissues (34,51,178,218,346).

Ultrastructural studies have suggested the existence of several types of noncholinergic, nonadrenergic neurons. For instance, Baumgarten and coworkers (25) observed three different types of nerves in the gastrointestinal tract; besides cholinergic and noradrenergic nerve profiles, they found nerve terminals containing large vesicles. These fibers were named "p-type fibers" because of their similarity to the peptide-containing neurons in the hypothalamus (110). The work of Gabella (150) and Cook and Burnstock (71) revealed an even more complex picture; at least eight different types of nerve terminal profiles could be differentiated (see Fig. 1).

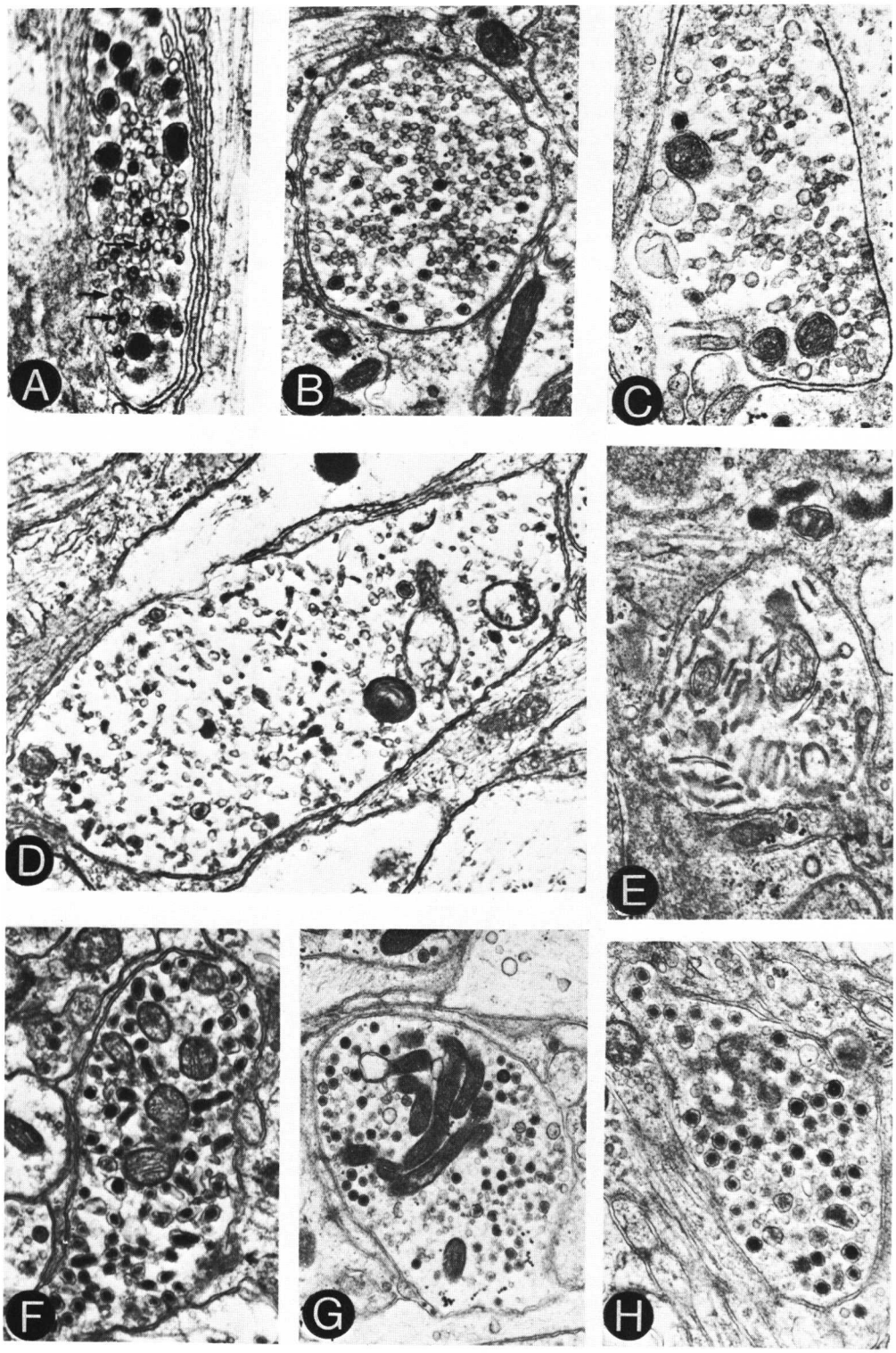
In addition, extracts of gut wall have been shown to possess numerous biological properties that are not attributable to ACh or NE and that could well be caused by other neuroregulatory substances. An early example is substance P, which was originally described as an active factor in extracts of equine intestine and brain that caused atropine-resistant contractions of the rabbit ileum (439).

In the last few years, numerous other examples of putative transmitters have come to light. An account of the evidence for these substances as putative transmitters as well as their histochemical distribution with correlation to functional aspects is given below. (For reviews on the general organization of the peripheral nervous system, see refs. 56,59,60,140,151,152,157,192,261,263,319,384.)

METHODOLOGY

Pharmacological and physiological studies of the nervous system have been greatly advanced by the development of histochemical techniques that made it possible to identify the cellular origin of biologically active substances that seemed likely to function as neurotransmitters. Thus, the Falck–Hillarp fluorescence technique (131,132) proved to

FIG. 1. Electron micrographs of nerve endings in the guinea pig intestine. **A**: Axon profile containing small granular vesicles in which the electron-dense material is located peripherally. ×44,500. **B**: Axon profile containing mostly small agranular vesicles and some larger granular vesicles. ×22,800. **C**: Axon profile containing small round and flattened agranular vesicles and a distinct, large granular vesicle. ×37,600. **D**: Axon profile containing round and flattened agranular vesicles and some elongated vesicles. ×17,400. **E**: Axon profile containing numerous elongate vesicles. × 27,300. **F**: Axon profile containing irregularly shaped dense-cored granular vesicles up to 95 nm in diameter. ×24,300. **G**: Axon profile containing homogeneous round dense-cored vesicles, 50 to 95 nm in diameter. ×18,600. **H**: Axon profile containing large vesicles, up to 115 nm in diameter, with granular cores of variable density. ×28,800. (From Cook and Burnstock, ref. 71, with permission.)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be an invaluable tool both for mapping catecholamine-containing neurons and for functional studies of these neurons. The technique involves the use of formaldehyde, which induces conversion of catecholamines into compounds that fluoresce on illumination with ultraviolet light. Thus, the demonstration of noradrenergic nerve terminals in the enteric plexuses (224,341) (Fig. 2) led to the view that the inhibitory action of NE could be indirect, i.e., mediated by enteric neurons.

The localization of ACh has proved to be more difficult, but a histochemical method that localizes the ACh-degrading enzyme acetylcholinesterase (AChE) can be used, provided the conditions are carefully controlled. There are several reasons for interpreting the results obtained by this method with caution (395). First, AChE activity has a widespread distribution. Although there seems to be a good correlation between ACh content, choline acetyltransferase activity, and AChE content (49,167,182,281), there are several examples of the localization of AChE where there is no evi-

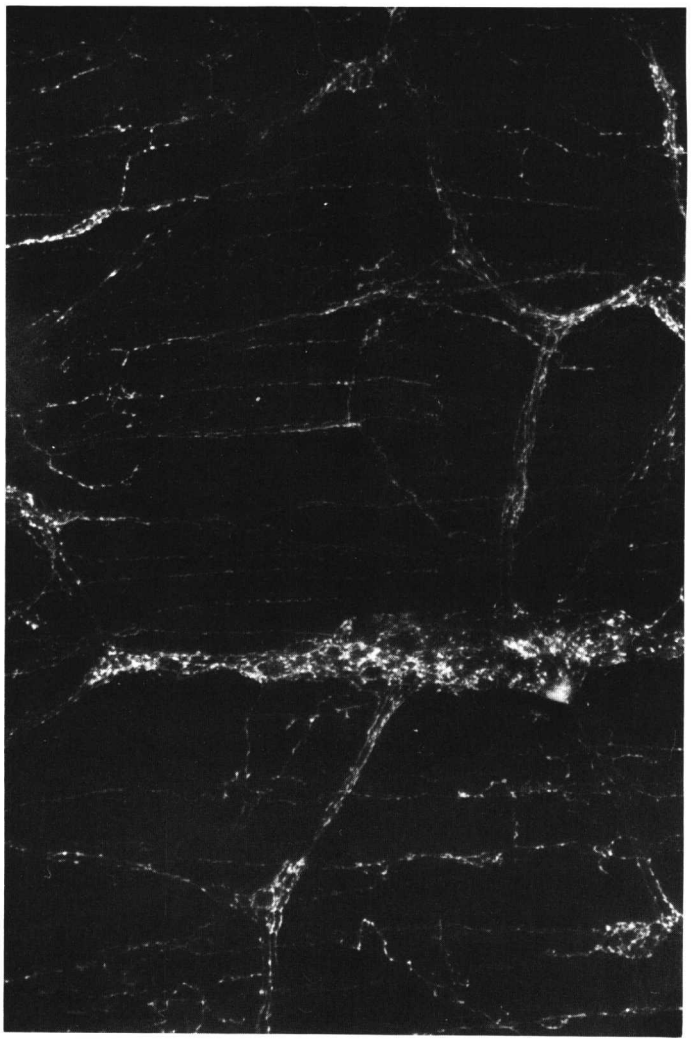

FIG. 2. Fluorescence micrograph of whole-mount preparation of longitudinal muscle and myenteric plexus from rat small intestine processed for catecholamines by the Falck–Hillarp technique. A dense network of varicose noradrenergic nerve fibers can be seen in the myenteric plexus. Fluorescent fibers also run in the interconnecting nerve strands. ×160 (Courtesy of Dr. Lars Olson, Dept. of Histology, Karolinska Institutet, Stockholm.)

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dence for cholinergic nerves; for instance, AChE staining has been observed in adrenergic neurons in the rat (119,226) and in sensory neurons of rat and guinea pig (181,383). Second, there exist other cholinesterases that are unrelated to cholinergic neurons, such as butyrylcholinesterase. However, this problem can be overcome by using inhibitors for nonspecific cholinesterases. Recently, antibodies to choline acetyltransferase have been used in immunohistochemical studies, providing a more specific technique for identification of cholinergic neurons (87).

In some cases, the localization of substances in neuronal structures can be achieved by autoradiographic methods (5–7,296,310,451,452). These methods have commonly been used for the localization of 5-hydroxytryptamine (5-HT) in enteric neurons (109,162,163). The technique obviously demonstrates the capacity of a neuron to take up radiolabeled substance but does not prove that the neuron normally possesses the enzymes for its synthesis (see below). The details of the above-mentioned techniques have been extensively reviewed elsewhere (see, e.g., 59,131,132,395) and are not further discussed here.

In the last 20 years, immunochemical methods have come into wide usage and have been of special importance in localizing small peptides. Antibodies may be readily obtained by immunization of rabbits, sheep, goats, guinea pigs, or mice with peptides coupled to larger proteins, such as bovine albumin (BSA) (170). The antisera may be used in radioimmunoassay (RIA), for quantitative measurements in tissue extracts (453), and in immunohistochemistry (73,333,403). These methods offer many advantages, notably, sensitivity in detecting small amounts of the peptide; however, they are not without problems. The antigen–antibody reaction is generally highly specific, but particular care in interpretation needs to be applied, and it is seldom possible to conclude that the substance present in the tissue and reacting with the antibody actually corresponds to the antigen without careful controls.

In the case of peptides, a sequence of four to seven amino acids generally makes up the antigenic determinant. Therefore, peptides with related sequences may cross react with the same antibodies. For example, gastrin and cholecystokinin (CCK), which have a common COOH-terminal pentapeptide, both react with antibodies that are directed to the COOH terminus (102,362). It is possible to differentiate between the two peptides by using region-specific antibodies, i.e., antibodies that are raised to different parts of the molecules (102,362). However, it is not possible to exclude the existence in the tissue of unknown molecules that may give rise to an immunoreaction. In addition, biologically active peptides are generally synthesized from larger precursors, which may be processed to several different peptides having different biological properties. Thus, caution is needed when dealing with different forms or fragments of a substance. In view of the uncertainty as to the exact nature of the substance responsible for the immunoreaction, such terms as substance P-like immunoreactivity, substance P immunoreactive, and so on, are used. Moreover, it is usually desirable to use different antisera to correlate RIA and immunohistochemistry as far as possible.

In immunohistochemistry, so-called control sera are used to check that the immunoreaction is specific for the antigen. A control serum consists of the specific antiserum, which has been preabsorbed with the antigen in order to block the specific immunoreaction. Any immunoreaction that is observed with a control serum is therefore regarded as nonspecific staining. Preabsorption of the specific antiserum with other substances, preferably in different concentrations, is a common way to investigate whether or not the antiserum cross reacts with substances other than the antigen. Desirably, decrease or inhibition of the immunoreaction is only obtained with the specific antigen.

Another problem concerns negative findings. Thus, lack of immunoreactivity may of course be due to absence of the antigen in the tissue or loss of the antigen from the tissue during the processing of the tissue; poor fixation may result in diffusion of the antigen. However, negative finding may also result from loss of immunoreactivity of the antigen, possibly caused by the fixation procedure. Finally, the levels of antigen in the tissue may be too low for detection, as is often the case for neuropeptides in axons and nerve cell bodies. This problem may be overcome by different experimental procedures. Thus, application of an inhibitor of axonal transport, such as colchicine, has been used to increase the cell body levels of amines (91,92,200,257) and can also be used to increase levels of peptides in nerve cell bodies and axons (24,207). In addition, ligation of nerves results in accumulation of axonally transported amines and peptides at the ligation site (43,90,154,303).

TRANSMITTERS

The history and properties of ACh and NE as neurotransmitters in the peripheral nervous system have been extensively reviewed in the past (see,

e.g., 56,59,60,140,151,224,225,319,343). Therefore, the following section concentrates on the newcomers in this area, i.e., small biologically active peptides in particular, and briefly considers other substances such as 5-HT, dopamine, ATP, and GABA.

Substance P

Substance P was discovered in 1931 by von Euler and Gaddum as they were studying the distribution of ACh (439). Their control was addition of atropine, and they found that ethanol extracts of equine intestine and brain caused contractions, which were atropine resistant, of smooth muscle from the isolated rabbit jejunum. It was not until 1970 that substance P was obtained in pure form and characterized as an undecapeptide (from bovine hypothalamus) (66), although its peptide nature had been suspected earlier (438,439). Chang et al. (67) determined the amino acid sequence of substance P and synthesized the peptide. Studer et al. (405) isolated substance P from equine intestine. Substance P is structurally related to a variety of peptides that have been isolated from frog skin and other tissues (122). The best known of the substance P-related peptides are physalemin (120) and eledoisin (121), isolated from octopus salivary gland. Both peptides share the COOH-terminal sequence Gly-Leu-Met-NH_2 which determines the biological activity of substance P. The COOH-terminal octapeptide is about half as active as the undecapeptide in contracting guinea pig ileum (454). Physalemin and eledoisin have similar biological properties to substance P, e.g., vasodilatation and contraction of intestinal smooth muscle (120–122). Among another group of peptides isolated from amphibian skin, bombesin (124) has the same COOH-terminal dipeptide as substance P but shows rather different biological activities (see section on bombesin below).

The distribution of substance P was first studied by Pernow (354) who used a bioassay on guinea pig ileum or rabbit jejunum. At present, the distribution of substance P is studied to a large extent by RIA and immunohistochemistry. Most substance P antibodies are directed to the COOH terminus, and cross reactivity to physalemin varies from less than 0.1% to 5%, and that to eledoisin between 0.01% and 0.03% (338). Lee et al. (277) have developed an NH_2-terminal-directed antiserum that cross reacts less than 0.01% with physalemin and eledoisin. Cuello et al. (89) have produced a monoclonal antibody to substance P that cross reacts less than 0.01% with eledoisin.

The initial study of the distribution of substance P showed that, in addition to the high concentrations in intestine and brain, the dorsal horn of the spinal cord contained large quantities, whereas only small amounts were found in the ventral horn (338). The same results have since been obtained by RIA (413), and immunohistochemical studies have shown that substance P-like material is present in numerous nerve terminals in the outer layers of the dorsal horn as well as in small-diameter cell bodies in spinal ganglia (209,210). Dorsal rhizotomy results in decreased concentrations of substance P in the dorsal spinal cord as determined biochemically (413), and immunohistochemical studies demonstrate a marked decrease of the number of substance P-immunoreactive nerve terminals in the dorsal horn (209).

Both sets of findings indicate that the substance P-immunoreactive nerve terminals in the dorsal horn originate in cell bodies in the spinal ganglia. Lembeck (279) suggested that substance P may be an excitatory transmitter in primary sensory neurons. Both electrophysiological (250) and biochemical (179,412) data, in addition to the morphological data, support the view that substance P plays a role in the transmission of sensory impulses.

Vasoactive Intestinal Polypeptide

Vasoactive intestinal polypeptide (VIP) was isolated from porcine duodenum by Said and Mutt (376,377) and was characterized as a linear peptide of 28 amino acid residues (331). It is structurally related to secretin, glucagon, gastric inhibitory peptide (GIP), and a newly isolated peptide from porcine intestine, PHI (414,415). As the name implies, VIP is a potent vasodilator (376), but it also has a wide variety of other properties, e.g., relaxation of smooth muscle in gut and respiratory tract, and stimulation of bicarbonate and water secretion from the exocrine pancreas (39,127,376). The latter property is shared with secretin, which is more potent than VIP in the human (107) but less potent in the turkey (97); PHI has been shown to have similar potency to VIP in the turkey pancreas (99).

Vasoactive intestinal polypeptide has been measured in tissue extracts by radioreceptor assay (33) as well as by RIA (96,153,378; see 127). Although the peptides mentioned above have biological actions similar to VIP and are structurally related, they have quite different immunochemical properties. In RIA, the cross reactivity of these peptides with some VIP antibodies is less than 0.005% (100). The nature of VIP in different species seems to vary slightly; chicken VIP (337) differs from porcine VIP in four amino acid residues. Studies with region-specific antibodies have shown that the

human and rat intestines contain several different molecular forms of VIP, of which one corresponds to porcine VIP and the others are less basic molecules (96,98,100). At least in the rat, these different forms are found only in the muscular coat, presumably in the nerves (98,100). However, in human colon, a single form corresponding to authentic VIP is present in the muscle layers, including the myenteric plexus (96).

Vasoactive intestinal polypeptide meets many of the criteria for being a neurotransmitter (127,129,374). Thus, it has been localized in neurons (47,149; see 127,374) and shown to be present in the synaptic vesicle fraction of nerve terminals (165). Several studies have shown the release of VIP on nerve stimulation, and the effect of exogenously applied peptide mimics the response of nerve stimulation (128,129,380). It has been suggested that VIP may be the transmitter in noncholinergic, nonadrenergic inhibitory neurons in the gut (129,171; see also section on distribution below).

Enkephalins

The first isolation of an endogenous ligand for the opiate receptor (417,418; see 255) was made by Hughes and collaborators (217). They isolated from porcine brain two pentapeptides with opioid activity, i.e., analgesic activity and inhibition of electrically induced contractions of guinea pig ileum and mouse vas deferens in a naloxone-reversible fashion. The amino acid sequence of the two peptides differs only in the COOH-terminal amino acid, which is either methionine (met) or leucine (leu), hence the names met- and leu-enkephalin (216,217). It is possible to distinguish between met- and leu-enkephalin in bioassay by the oxidative destruction of met-enkephalin by cyanogen bromide (158,397) or by using region-specific antibodies in RIA (396).

Most of the enkephalin antibodies currently used in immunohistochemical studies, which may have been raised to met- or leu-enkephalin, give a positive immunoreaction with both peptides. Even when the cross reactivity is less than 1% as measured by RIA, the immunoreaction to nerve fibers with a met-enkephalin antiserum may be partially blocked by preabsorption of the antiserum with leu-enkephalin (390). Larsson et al. (265) have employed an elegant technique to overcome this problem. They examined the immunochemical properties of met- and leu-enkephalin antisera by incubating them with Sepharose beads that were previously coated with met- and leu-enkephalin, respectively. They found that their met-enkephalin antisera contained two populations of antibodies, one specific for met-enkephalin and one that reacted with both met- and leu-enkephalin. Preabsorption of these antisera with leu-enkephalin abolished staining of leu-enkephalin-coated beads, and the antisera could therefore be used for staining met-enkephalin-containing structures. Pretreatment with cyanogen bromide or an oxidizing agent, such as acidic potassium permanganate, abolished the staining of met-enkephalin-coated beads. Therefore, a leu-enkephalin antiserum that cross reacts with met-enkephalin can be used for specific staining of leu-enkephalin provided the tissue is pretreated so that the met-enkephalin is oxidized. It was in this way possible to demonstrate separate met- and leu-enkephalin neurons both in brain and gut (265).

Met-enkephalin corresponds to NH_2-terminal pentapeptide of β-endorphin, another peptide with opioid activity, originally isolated from porcine, camel, and human pituitary in 1976 (40,287,288). Beta-endorphin in itself is the 31-amino-acid C-terminal fragment of β-lipotrophin (β-LPH), a 91-amino-acid peptide that was isolated in 1965 by Li and co-workers (285). The function of β-LPH was unknown at the time, but it now seems probable that it is the biosynthetic precursor of β-endorphin and β-melanocyte-stimulating hormone (β-MSH).

A large 31,000-dalton glycoprotein (pro-opiomelanocortin) gives rise to β-LPH and adrenocorticotrophic hormone (ACTH) (306,365). However, immunohistochemical studies with specific antibodies show that β-endorphin- and met-enkephalin-containing neurons have a quite different distribution (35), so that β-endorphin is unlikely to be a biosynthetic precursor for met-enkephalin. Recently, an increasingly impressive body of evidence for other enkephalin precursor molecules has emerged. In large part, this evidence has been obtained from peptides isolated from adrenal glands, where enkephalin-like immunoreactivity was first detected in immunohistochemical studies by Schultzberg et al. (387,391). In addition to met- and leu-enkephalin (95), large amounts of hexapeptides, heptapeptides, and larger forms of 14,000 and 21,000 daltons have since been isolated from the adrenal gland (Table 1). It is likely that opiate peptide variants also occur in the brain. Thus, an octapeptide containing leu-enkephalin with a COOH-terminal extension has been isolated from porcine hypothalamus (318). The sequence is contained within the N terminus of dynorphin, which has been isolated from pituitary and partially sequenced (168). Another leu-enkephalin-related peptide, also isolated from porcine hypothalamus (238), is α-neoendorphin, which is a pentadecapep-

TABLE 1. *Naturally occurring enkephalins (ENK) and related peptides that have been isolated and characterized*

Peptide	Source	Reference
Tyr-Gly-Gly-Phe-Met (met-ENK)	Porcine brain	217
Tyr-Gly-Gly-Phe-Met (0)-Arg	Porcine hypothalamus	215
Tyr-Gly-Gly-Phe-Met-Arg-Phe	Bovine adrenal medulla, striatum	402
Tyr-Gly-Gly-Phe-Met-Lys	Bovine adrenal medulla	282
Tyr-Gly-Gly-Phe-Met-Arg-Arg	Bovine adrenal medulla	282
Tyr-Gly-Gly-Phe-Met-Arg-Arg-Val-Gly-Arg-Pro-Glu	Bovine adrenal medulla	320
8,000-Dalton peptide with COOH-terminal met-ENK	Bovine adrenal medulla	283
14,000-Dalton peptide containing 3 met-ENK	Bovine adrenal medulla	283
34-Amino-acid peptide containing 2 met-ENK	Bovine adrenal medulla	236,244
39-Amino-acid peptide containing 1 met-ENK and 1 leu-ENK	Bovine adrenal medulla	236,244
50,000-Dalton peptide containing 6 or 7 met-ENK and 1 leu-ENK	Bovine adrenal medulla	282
Tyr-Gly-Gly-Phe-Leu (leu-ENK)	Porcine brain	217
Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-OH [Dynorphin (1–13)][a]	Porcine pituitary	168
Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Arg-Pro-($Gly_1Tyr_2Lys_2Arg_1$) (α-neoendorphin)	Porcine hypothalamus	238
Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile	Porcine hypothalamus	318

[a]Recent work using cloning and sequence analysis of cDNA has shown that the dynorphin/α-neoendorphin precursor contains dynorphin 1–17 (Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys_5-Trp-Asp-Asn-Gln), rimorphin/dynorphin B (Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Gln-Phe-Lys-Val-Val-Thr) as well as a α-neoendorphin (237a).

tide. From bovine adrenal medulla, two larger peptides of 34 and 39 amino acids, respectively, have been purified and sequenced (236,244). The triakontatetrapeptide contains two met-enkephalins, one at each end. The larger peptide has a met-enkephalin sequence in the middle and a leu-enkephalin sequence at the COOH terminus. More recently, a dodecapeptide which is contained within the latter peptide was isolated from bovine adrenal medulla (320). The occurrence of a large 50,000-dalton protein in bovine adrenal medulla has been demonstrated by Lewis et al. (282). Trypsinization and treatment with carboxypeptidase B provided evidence for the occurrence of six or seven met-enkephalins and one leu-enkephalin within the protein. Most likely, the large forms are biosynthetic precursors for enkephalins and enkephalin-related peptides, which are produced by the action of processing enzymes (373). Possibly a similar arrangement is true for the central nervous system. The functional significance of a precursor with a repetitive sequence is unclear, but this explains the ratio of met- and leu-enkephalin measured previously (172,186).

Somatostatin

Somatostatin was isolated and purified from bovine hypothalamus by Brazeau et al. (42). Sequence analysis revealed a tetradecapeptide with a disulfide bond between the cysteines in positions 3 and 14. Pradayrol et al. (358) isolated from porcine duodenum a peptide (somatostatin-28) that includes the somatostatin sequence in the COOH terminus. A peptide with the same sequence was recently isolated from ovine hypothalamus, in addition to a smaller peptide that comprised 25 of the amino acid residues in somatostatin-28 (41). Somatostatin occurs in large amounts in the brain and in the gastrointestinal tract, including the pancreas (21a,41,42,201,202). A significant proportion of somatostatin in the gut is present in endocrine cells (201; see 36), especially in the upper part of the tract. These cells have been identified as D cells (357); D cells in the pancreas also contain somatostatin (357). Somatostatin is known to have a number of inhibiting actions. It was originally isolated by Krulich et al. (258) as a factor inhibiting the release of growth hormone, but it is also a potent inhibitor of the release of insulin (8) and glucagon (159) from the pancreas and of gastrin and gastric acid secretion from the stomach (23,169), suggesting that it plays an important part in the regulation of these hormones.

Cholecystokinin

In 1928, a hormonal mechanism for intestinal control of gallbladder contraction was demonstrated by Ivy and Goldberg (223). They named the hormone cholecystokinin (CCK). From hog duodenum they prepared a secretin-free extract

that caused contraction of the gall bladder. Fifteen years later, Harper and Raper (180) demonstrated an intestinal extract of duodenal mucosa that stimulated pancreatic enzyme secretion; they named the active principle pancreozymin. Not until 1966 was the identity of these two substances proven, when Mutt and Jorpes determined the sequence of a 33-residue peptide with both properties isolated from porcine duodenal mucosa (329). It is therefore known as cholecystokinin–pancreozymin, although the latter part of the name is now usually omitted. A 39-residue form of CCK ("CCK variant") consisting of CCK-33 and an NH_2-terminal extension has also been isolated from the same source and sequenced. Other smaller forms have also been isolated. Thus, a CCK octapeptide (CCK-8) and a closely related, slightly less acidic form have been isolated and sequenced from sheep brain (103). In addition, results from RIA measurements suggest that CCK-8 also predominates in the smooth muscle–myenteric plexus preparation of guinea pig intestine (220). Rehfeld and coworkers have suggested that the COOH-terminal tetrapeptide occurs in large amounts in the brain and gut (272,363); however, this matter is under debate, as others have failed to identify this molecular form.

The COOH-terminal pentapeptide of CCK is shared with gastrin—another classical gut hormone. Gastrin was isolated from porcine antrum by Gregory and Tracy (174,175). Including the tetrapeptide, there are at least five different forms of gastrin (270,271,361,363,365), most of which probably mainly occur in endocrine cells in the gut. It has also been reported that gastrin-like immunoreactivity occurs in the vagus nerve of dogs and cats (429). Dockray et al. (104) showed in a study of a large number of dogs and cats that G-17 occurred in the vagus nerve of relatively few animals, whereas CCK-8 appeared to be the main representative of this group of peptides. By ligation and nerve section, it was possible to establish that in the cat, CCK-8 was produced in cell bodies of nodose ganglion and transported toward the gut in afferent fibers (104). The functional significance of this remains to be shown.

Neurotensin

Carraway and Leeman first discovered neurotensin as a factor in hypothalamic extracts that caused vasodilatation and increased vascular permeability followed by cyanosis (63). It has been isolated and sequenced from bovine hypothalamus and small intestine (63–65). The biologically active region of neurotensin is localized in the COOH terminus (45). A peptide extracted from frog skin, xenopsin (see 45), has a similar COOH-terminal sequence and has been shown to have similar biological actions. Immunochemical measurements of neurotensin show that about 10 times more activity is found in the gastrointestinal tract than in the brain. A large proportion of the neurotensin in the gut is localized in endocrine cells (48,136,184, 407), which are most numerous in the ileal mucosa. Ultrastructural studies suggest that neurotensin occurs in a distinct cell type (48). Neurotensin has a number of actions in the alimentary canal, such as inhibiting gastric acid secretion (21) and increasing plasma levels of glucagon and glucose (46,65,332). However, it has also been shown to induce contraction of intestinal smooth muscle (63).

Bombesin

Many of the small biologically active peptides that occur in the mammalian nervous and endocrine systems have counterparts that are found in high concentrations in amphibian skin. Erspamer and his co-workers have isolated and characterized numerous amphibian skin peptides (124,126). Among these are the ceruleins, which are closely related to mammalian cholecystokinins, and physalemin, related to substance P. In addition, it is now clear that the bombesin family of peptides from amphibian skin also has mammalian counterparts. The amphibian peptide bombesin is a molecule of 14 amino acid residues (19); its biological activity seems to reside in the COOH-terminal octapeptide, which is also common to several other amphibian peptides, e.g., litorin, ranatensin, and alytesin (19,20,44,315). Recently, McDonald et al. (313) isolated from porcine nonantral stomach a peptide of 27 amino acid residues that shows an identical sequence in nine of the 10 COOH-terminal amino acids. The mammalian bombesin-like peptide also has similar biological properties to the amphibian molecule. In extracts of guinea pig stomach and intestines, bombesin-like immunoreactivity seems to occur in two molecular forms, one that might represent the heptacosapeptide isolated from pig (313) and one smaller form (220). The two COOH-terminal amino acids of bombesin (14 and 27) are identical with the COOH-terminal dipeptide of substance P. Many antibodies to substance P and bombesin are directed to the COOH terminus of these peptides and may show cross reactivity.

Bombesin has a wide range of biological actions. It causes an increase in blood pressure; stimulates smooth muscle contraction; stimulates secretion of gastric acid, gastrin, and CCK; stimulates pan-

creatic secretion; and lowers body temperature (31,32,46,62,123,316).

Other Peptides

In addition to the peptides already discussed, there may be other as yet undiscovered peptides occurring in the peripheral nervous system. Furthermore, there are some peptides occurring in the endocrine cells of gut and pancreas, such as insulin, glucagon, bradykinin, and secretin, which have been demonstrated by immunohistochemistry to occur in the brain (74,181,295,330,411) and which may well occur in peripheral nerves too, but in very small amounts, since they have not been detected so far. There is, however, suggestive evidence for a few additional peptides in the peripheral nervous system. Angiotensin II-like immunoreactivity occurs in both central and peripheral neurons (148,155). One of the most recent peptides to be found in peripheral neurons appears to be a form of pancreatic polypeptide (PP). Kimmel and coworkers (242,243) isolated and sequenced avian pancreatic polypeptide (APP) from chicken pancreas. Human (HPP) and bovine (BPP) homologs have also been isolated (288), as well as the ovine (OPP), porcine (PPP), and canine (CPP) peptides. There are only minor differences in the sequence of the mammalian peptides, but the avian peptide differs in more than half ot the 36 amino acid residues.

Although APP does not cross react in a RIA using an antibody raised to HPP or BPP, antibodies to APP give a positive immunoreaction with neuronal and endocrine structures in mammalian species (see below). The identity of the peptide that is recognized by the APP antibodies has recently been clarified and it is now believed to be neuropeptide Y (413a).

Luteinizing hormone-releasing hormone (LHRH)-like immunoreactivity has until recently only been found in central neurons but has recently also been shown by immunohistochemistry to occur in sympathetic ganglia in the frog (228,229). The occurrence of another central neuropeptide, thyrotropin-releasing hormone (TRH), in tissue extracts of different parts of the gastrointestinal tract has been shown in RIA (280,328).

Other Putative Transmitters

Apart from neuropeptides, other types of substances have been localized in peripheral neurons. Thus, ATP or a related nucleotide has been proposed as a transmitter in the noncholinergic, nonadrenergic inhibitory neurons (50). The morphological evidence is based on the uptake of radiolabeled ATP or adenosine, which is converted to labeled ATP and was localized in nerves (50). A fluorescence histochemical method using quinacrine was developed by Olson and co-workers (14,349). Quinacrine has been shown to bind ATP (222). However, the relationship between quinacrine-binding structures and ATP is still not fully elucidated. Stimulation of the vagus nerve results in a release of adenosine and inosine, which are interpreted as the breakdown products of ATP. Both ATP and ADP cause a rapid hyperpolarization of smooth muscle that is unaffected by tetrodotoxin, suggesting a direct effect on the muscle cells (50,54).

Serotonin is known to be a transmitter in central neurons, and there is also considerable evidence for transmitter function in the gut (57,108,161,162; see also 78). Autoradiographic and immunohistochemical data show uptake of radiolabeled 5-HT into enteric neurons (109,160,163,368) and the occurrence of tryptophan hydroxylase in submucous and myenteric neurons (161). Serotonin has an excitatory action on gastrointestinal smooth muscle (449,450), and it is believed that 5-HT gives rise to slow excitatory postsynaptic potentials (449). However, it is difficult positively to identify 5-HT-containing neurons in the normal gut. Recently, the development of antibodies to 5-HT (401; and Steinbusch and Nieuwenhuys, *this volume*) has made possible a more direct localization of 5-HT in neurons.

GABA is also known to be a transmitter in the central nervous system. Recent autoradiographic studies have shown the uptake of radiolabeled GABA into gut neurons (231). The significance of this finding is still unclear but is compatible with a population of GABA-containing peripheral neurons.

Intrinsic amine-handling neurons are characterized by their content of aromatic amino acid decarboxylase and monoamine oxidase and their ability to take up and retain catecholamines and indoleamines (137,142). It seems, however, as though these neurons do not in fact synthesize catecholamines, since tyrosine hydroxylase (TH) and dopamine β-hydroxylase (DBH) cannot be detected in extrinsically denervated intestine (146). Enteric noradrenergic cell bodies have been described to occur almost exclusively in guinea pig colon (138). Some cell bodies reacting with DBH antiserum were, however, observed in guinea pig stomach and rat esophagus and colon (388). It is, however, possible that the DBH antiserum cross

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