Coexistence of peptides with classical neurotransmitters

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Summary. In the present article the fact is emphasized that neuropeptides often are located in the same neurons as classical transmitters such as acetylcholine, 5-hydroxy-tryptamine, catecholamines, γ-aminobutyric acid (GABA) etc. This raises the possibility that neurons produce, store and release more than one messenger molecule. The exact functional role of such coexisting peptides is often difficult to evaluate, especially in the central nervous system. In the periphery some studies indicate apparently meaningful interactions of different types with the classical transmitter, but other types of actions including trophic effects have been observed. More recently it has been shown that some neurons contain more than one classical transmitter, e.g. 5-HT plus GABA, further underlining the view that transfer of information across synapses may be more complex than perhaps hitherto assumed.

Key words. Chemical transmission; multiple messengers; synapse; neuropeptides; immunohistochemistry; 5-HT; catecholamines; GABA; somatostatin; enkephalin; NPY; CCK; CGRP.

Biochemical and modern molecular biological techniques have defined a large number of bioactive substances in the central and peripheral nervous system (CNS and PNS). In addition to earlier described low molecular weight compounds such as acetylcholine (ACh), catecholamines and certain amino acids (γ-aminobutyric acid (GABA), glycine), which are considered to act as neurotransmitters, an increasing number of peptides ranging in size from a few up to 40 amino acids and more have been identified in neurons[135]. The biochemical[79, 121] and immunohistochemical[12] demonstration of peptides in well-defined neuronal systems in widespread areas of the nervous system, taken together with physiological investigations, indicate that some peptides, at least in some systems, may have a transmitter role[116, 135]. For example, several lines of evidence suggest a transmitter function for substance P (SP) in primary sensory neurons[117, 124]. Important clues for alternate roles for peptides have also been presented[146]. Subsequent analyses have indicated that peptides exert a wide range of effects.

When the interest in neuronal peptides became manifest 15 or so years ago, and their presence in distinct subsets of peripheral and central neuron populations had been demonstrated, it seemed possible to assume that they might have a transmitter role. For instance, classical transmitters identified at that time, such as ACh, catecholamines and 5-hydroxytryptamine (5-HT) had been found to be present in only a small population of neurons in the central nervous system[34, 71, 120]. The addition of numerous peptides thus appeared to represent a meaningful way to 'fill up' neuronal systems, i.e. those cells that did not contain a classical transmitter produced a peptide. However, immunohistochemical analysis of the distribution of various peptides in comparison to, for example, catecholamine and 5-HT systems revealed that in many cases peptides could be observed in the same neuron that also contained a classical transmitter. This has been documented in many articles and reviews[5, 18, 55, 56, 62, 66, 86, 109, 113, 134] as well as discussed at meetings[21, 30, 65, 114]. The co-localization of classical neurotransmitter and peptide in the same neuron represents a logical continuation of earlier demonstrations showing the presence of biogenic amines and peptide hormones in the same endocrine cells[119, 123] and possible cellular coexistence of transmitters in invertebrate neurons (e.g. Brownstein et al.[16], for discussion, see Osborne[113]). These findings, in a general sense, could be interpreted to mean that neurons either contain more than one transmitter substance (classical transmitter+peptide) or that the peptide in these neurons may be responsible for other types of functions, for example, they could exert long-term trophic effects.

Many examples of neurons that contain more than one peptide but no classical transmitter have been reported. However, it is unclear whether these neurons really lack a classical transmitter or whether the proper marker for a classical transmitter in these neurons is simply 'missing'. There is also increasing evidence that neurons may contain more than one classical transmitter. For example, 5-HT and GABA appear to coexist in the same cells of both pontine and medullary raphe nuclei as first reported by Pujol and collaborators[8, 9, 103]. It does not seem unlikely that these neurons in addition contain one or more peptides. Finally, increasing evidence suggests that adenosine nucleotides may participate as co-messengers in neurotransmission, as early advanced by Burnstock[18].

It is important to note that coexistence of several types of compounds with possible messenger function still largely represents a histochemical concept based on immunohistochemical demonstration of these substances, using antisera raised against various transmitters, transmitter synthesizing enzymes and peptides. Apart from problems concerning the specificity and sensitivity of these techniques, the most important questions are, of course, to what extent are these compounds actually released from the nerve endings and how do they participate in the transmission process? This article represents an initial account of an emerging view that transmission of messages across synapses is a more complicated event than perhaps previously assumed.

How to define coexistence

The nervous system is an extremely heterogeneous tissue, and it is therefore not possible to study coexistence with biochemical techniques with the present status of sensitivity. An exception may be some invertebrate neurons, which are so large that they can be isolated individually and that their content of neuroactive compounds possibly can be determined biochemically[113]. Biochemistry can be used however to demonstrate coexistence indirectly. For example, there are 'specific' neurotoxins such as 6-hydroxydopamine[142] and 5,6-dihydroxytryptamine[6] which destroy catecholamine and 5-HT neurons, respectively. With the latter compound, a concomitant depletion of 5-HT, SP and TRH has been shown and was interpreted to indicate coexistence of these compounds in single neurons[48].

Because neurons can be visualized individually in the microscope and because antibodies can identify substances within a single cell or a slice of a cell, immunohistochemistry[27, 31, 105, 126, 138] offers the most accurate method for determining coexistence. Various immunohistochemical approaches can be used to study multiple antigens in a neuron. They

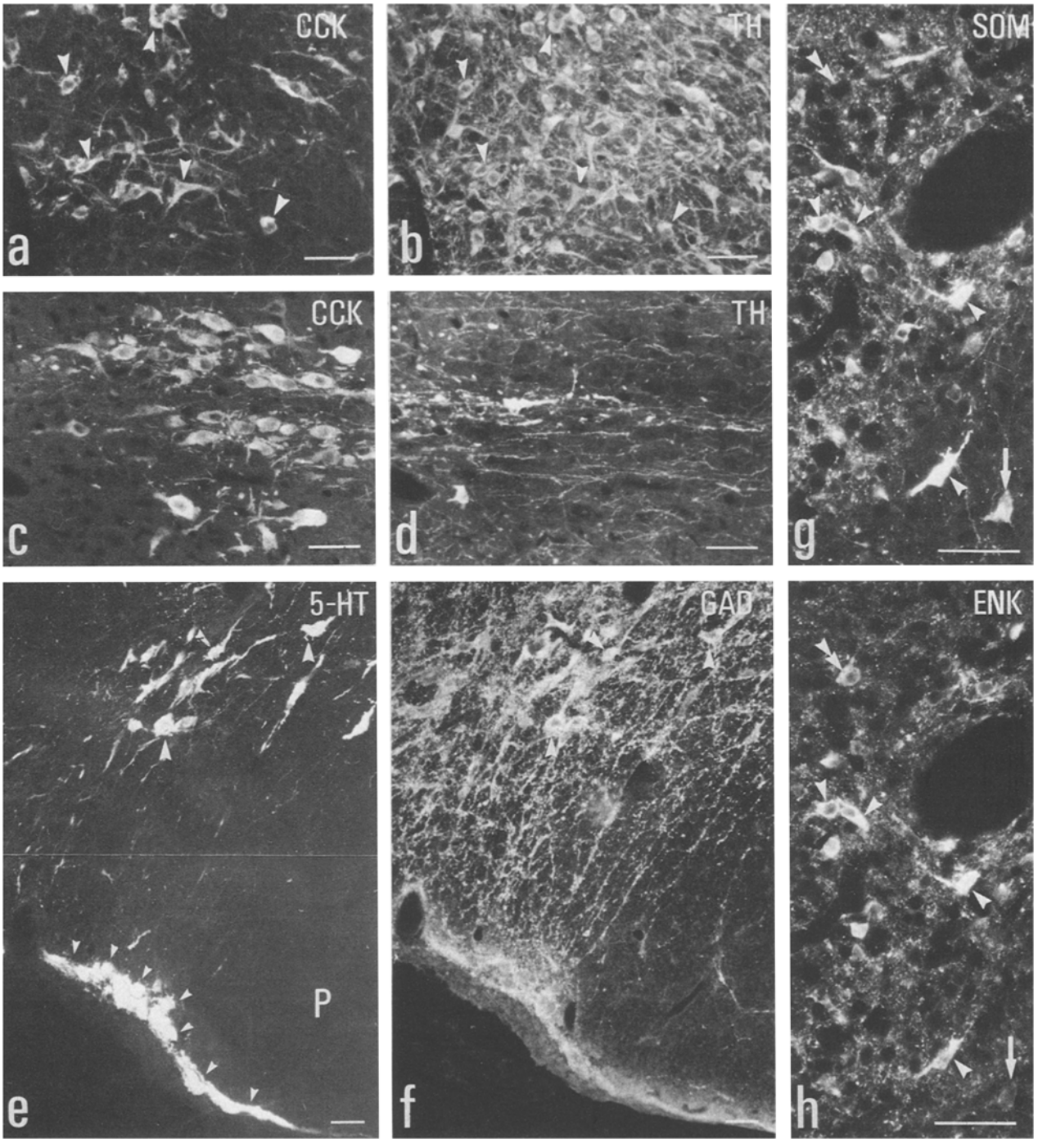


Figure 1. Immunofluorescence micrographs of the ventral tegmental area (VTA) *(a, b)*, the periaqueductal central grey (PAG) *(c, d)*, the ventral medulla oblongata *(f, g)* and the nucleus tractus solitarii (NTS) *(g, h)*, after incubation with antibodies to cholecystokinin (CCK) *(a, c)* tyrosine hydroxylase (TH), a marker for dopamine (DA) neurons *(b, d)*, 5-hydroytryptamine (5-HT) *(e)*, glutamic acid decarboxylase (GAD), a marker for GABA neurons *(f)*, somatostatin (SOM) *(g)*, and methionine-enkephalin (ENK) *(h)*. *a* and *b*, *c* and *d*, *e* and *f* as well as *g* and *h* show, respectively, the same sections which have been processed according to double staining technique using primary antisera raised in different species and secondary antibodies labelled with green fluorescent FITC and red fluorescent TRITC, respectively. This series of micrographs are meant to illustrate coexistence of classical transmitter and peptide (DA plus CCK) *(a, b)*, two classical transmitters (5-HT plus GABA) and two peptides (SOM plus ENK). *a–d* Numerous cell bodies (arrow heads) in the VTA contain both CCK- and TH-like immunoreactivity (LI), whereas in the PAG no double-labelled cells can be seen. *e, f* In the area lateral to the pyramidal tract (P) numerous cell bodies (big arrow heads) contain both 5-HT- and GAD-LI. Note numerous 5-HT cells (small arrow heads) along the ventral surface of the brain which seem to lack GAD-LI. *g, h* In the NTS numerous cell bodies (arrow heads) contain both peptides, but there are also cells containing only one of the peptides (double arrow heads point to SOM-negative, ENK-positive cell and arrows point to SOM-positive, ENK-negative cell). Bars indicate 50 μm. (From references 103, 104)

include the *'adjacent section method'*, where consecutive sections are incubated with different primary antisera. No cross-reaction between antisera can occur and consequently there are no problems of specificity due to interference between antibodies. Only large objects such as cell bodies can be studied but with sufficiently thin sections a cell body can often be identified in two or even more consecutive sections. When epoxy resin-embedded material is used, sections can be cut at 1 μm or thinner, and then numerous sections through a single cell body can be analyzed[10]. *'Elution-restaining methods'*[105,143] have been extensively utilized. After photography of the first staining pattern, the antibodies are eluted with acid solutions, and the sections are then reincubated with a new antiserum, and the new staining patterns are compared with the previously taken photographs. This method can, in our experience, not be used with all antisera, since the elution procedure seems to damage some antigens. The third approach is *'direct double-staining'*, which is based on availability of antisera raised in different species (fig. 1, a–h). Secondary antibodies labelled with different chromogens (e.g. green fluorescent fluorescein isothiocyananate, FITC, and red fluorescent tetramethyl rhodamine isothiocyanate, TRITC) and directed against IgG from the two respective species then allow visualization of the two antigens in the same section by switching between appropriate filter combinations (fig. 1, a–h; see reference 105). In fact, it has recently been shown that three antigens can be visualized in a single section using a third, blue fluorescent dye conjugated to an appropriate secondary antibody[137]. By combining this triple staining technique with elution-restaining, it should be possible to visualize four or even more antigens in a section. The final analysis of coexistence will, however, include electron microscopic studies. It has, for example, been shown that 5-HT and SP are stored in the same vesicles in some nerve endings in the spinal cord[122], and also at the ultrastructural level there are now methods to demonstrate three antigens in one section[37].

Immunohistochemical methods should, in spite of their power and usefulness, be considered with some caution both with regard to specificity and their sensitivity. Thus, it cannot be excluded that the antisera cross-react with compounds which are structurally similar to the immunogens. Recently evidence has been presented that one single amidated amino acid in the C-terminal position may be sufficient to cause cross-reactivity[11,74]. Therefore, expressions such as 'somatostatin(SOM)-like immunoreactivity', 'SOM-immunoreactive', etc. should be used.

It is also important to emphasize the sensitivity problem and that negative results should be interpreted with great caution. It has been demonstrated repeatedly that improvement of the fixation technique and/or production of antibodies with a higher affinity and/or higher avidity reveal a certain antigen in places where it had not been demonstrated earlier. Also, peptide levels in cell bodies are often too low to be visualized in central neurons, but can be increased by pretreatment of experimental animals with a mitosis inhibitor, colchicine[33], and in this way visualized.

Table 1. Coexistence of classical transmitters and peptides in the mammalian CNS[a] (selected cases)

Classical transmitter	Peptide[b]	Brain region (species)	References
Dopamine	CCK	Ventral mesencephalon (rat, cat, mouse, monkey, man?)	57, 58, 64, 66
	Neurotensin	Ventral mesencephalon (rat)	59
		Hypothalamic arcuate nucleus (rat)	59, 70
Norepinephrine	Enkephalin	Locus coeruleus (cat)	24, 80
	NPY	Medulla oblongata (man, rat)	40, 60, 130
		Locus coeruleus (rat)	40
	Vasopressin	Locus coeruleus (rat)	19
Epinephrine	Neurotensin	Medulla oblongata (rat)	59
	NPY	Medulla oblongata (rat)	40, 130
	Substance P	Medulla oblongata (rat)	84
	Neurotensin	Solitary tract nucleus (rat)	59
5-HT	Substance P	Medulla oblongata (rat, cat)	20, 22, 54, 73, 85
	TRH	Medulla oblongata (rat)	56, 73
	Substance P+TRH	Medulla oblongata (rat)	73
	CCK	Medulla oblongata (rat)	94
	Enkephalin	Medulla oblongata, pons (cat)	49, 68
		Area postrema (rat)	4
ACh	Enkephalin	Superior olive (guinea pig)	2
		Spinal cord (rat)	76
	Substance P	Pons (rat)	145
	VIP	Cortex (rat)	38
	Galanin	Basal forebrain (rat, monkey)	100, 101
	CGRP	Medullary motor nuclei (rat)	140
GABA	Motilin (?)	Cerebellum (rat)	23
	Somatostatin	Thalamus (cat)	111
		Cortex, hippocampus (rat, cat, monkey)	52, 72, 131, 136
	CCK	Cortex, hippocampal formation (cat, monkey, rat)	52, 78, 136
	NPY	Cortex (cat, monkey)	52
	Enkephalin	Retina (chicken)	147
		Ventral pallidum, hypothalamus (rat)	75, 150
	Opioid peptide	Basal ganglia (rat)	110
	Galanin	Hypothalamus (rat)	102
	Substance P	Hypothalamus (rat)	75
	VIP	Hippocampal formation (rat)	78
Glycine	Neurotensin	Retina (turtle)	148

[a] The coexistence situations have been defined mainly by immunohistochemistry. Only papers published 1985 or earlier are included. [b] This column contains the peptide against which the antiserum used for immunohistochemistry was raised. The exact structure of the peptide coexisting with the classical transmitter has for the most part not been defined.

Table 2. Coexistence of two classical transmitters in the CNS[a]

Classical transmitter 1	Classical transmitter 2	Brain region (species)	References
GABA	5-HT	Nucleus raphe dorsalis (rat)	8, 9
		Medullary raphe nuclei and adjacent areas (rat)	8, 9, 103
		Retina (rabbit)	115
GABA	DA	Arcuate nucleus (rat)	41
		Olfactory bulb (rat)	46, 77
GABA	Histamine	Hypothalamus (rat)	133
GABA	ACh	Medial septum/diagonal band (rat)	15
GABA	Glycine	Cerebellum (rat)	118

[a] The coexistence situations have been defined mainly by immunohistochemistry using antisera raised against the transmitter itself and/or a transmitter synthesizing enzyme.

Coexistence – overview

During the last years an increasing number of neurons containing coexisting messenger molecules have been described both in the central and peripheral nervous systems. Limited space does not allow a complete account of this work, therefore only selected cases are included. As indicated above, different types of combinations have been encountered: 1) classical transmitter+peptide(s) (fig. 1, a–d), 2) more than one classical transmitter (fig. 1, e, f), and 3) more than one peptide (fig. 1, g, h). In table 1 coexistence of classical transmitters and peptides in the mammalian CNS are listed, which have been described in papers in 1985 and earlier. The purpose of this table is to demonstrate that for each of the classical transmitters there is at least one example of coexistence with one or more peptides. In table 2, examples of the recent evidence that neurons may contain more than one classical transmitter are summarized. The first evidence concerned coexistence of a biogenic amine (5-HT) and an amino acid (GABA)[8, 9], and it seemed possible to argue that 5-HT and GABA indeed belong to different classes of compounds and thus that they might complement each other in some unknown way. More recently, however, there is evidence for occurrence of two inhibitory amino acids (GABA and glycine) in the same Golgi neurons in the rat cerebellum[118]. Also indirect evidence suggests such a coexistence, since GABA nerve endings have been shown to be located opposite to postsynaptic membranes that contain glycine receptors[144].

Coexistence situations in the CNS

As shown in table 1, peptides can be found in virtually all types of classical transmitter neurons in many parts of the central nervous system. It does not seem unlikely that further research will find more and more such examples and that coexistence is a rule rather than an exception. It is of particular interest that many GABA neurons in cortical areas including hippocampus contain one or more peptides[52, 78, 131, 136]. It should be noted that many types of coexistence combinations seem to occur in an unpredictable way and that often only subpopulations of neurons seem to contain a certain peptide. For example, 5-HT neurons in the lower medulla oblongata contain a SP-[22, 54] and also a thyrotropin-releasing hormone(TRH)-like peptide[48, 56, 73], whereas so far no such coexistence has been reported in pontine and mesencephalic 5-HT cells. Moreover, the proportion of 5-HT neurons that contain the two peptides vary within the medullary raphe nuclei[73]. Differential coexistence is also observed in the catecholamine neurons. Thus of the multiple groups originally described and defined by Dahlström and Fuxe[34] (A1–A12; see references 61 and 63), the A1 and A6 noradrenergic and the C1–C3 adrenergic neurons contain a neuropeptide Y(NPY)-like peptide[13, 30, 40], the parvocellular C2 adrenaline group a neurotensin[59] – and a cholecystokinin (CCK)[64] – like peptide. Some A1 and most A6 neurons express a galanin-like peptide[102], and many mesencephalic and some hypothalamic dopamine (DA) neurons exhibit neurotensin-like immunoreactivity (LI)[42, 59, 70], whereby the A12 DA neurons contain galanin-, neurotensin- and growth hormone releasing factor(GRF)-LI[97, 98, 112]. Finally, the caudal part of the A13 DA cell group has a SOM-like peptide[99]. The distribution of CCK-LI in the mesencephalic DA neurons is particularly intricate and is illustrated in figure 2 showing e.g. that the A10 cells in the ventral tegmental area (fig. 1, a, b) have an increasing proportion of coexistence in caudal direction, whereas neurons of the pars lateralis have almost 100% coexistence and hardly any cells containing both DA and CCK are found in pars reticulata. A galanin-like peptide has recently been observed in the basal forebrain cholinergic neurons both in rat and monkey; these neurons project to the hippocampal formation[100, 101].

Coexistence in the PNS

In the peripheral nervous system coexistence is frequently encountered; in fact, it can be observed in most systems. Particularly complicated patterns have been observed in the gastrointestinal tract with up to four peptides in presumably cholinergic neurons (see reference 29). The sympathetic and parasympathetic systems are also rich in peptides (see reference 87). Originally SOM-LI was found in a population of sympathetic noradrenergic neurons[53]. Further study has ex-

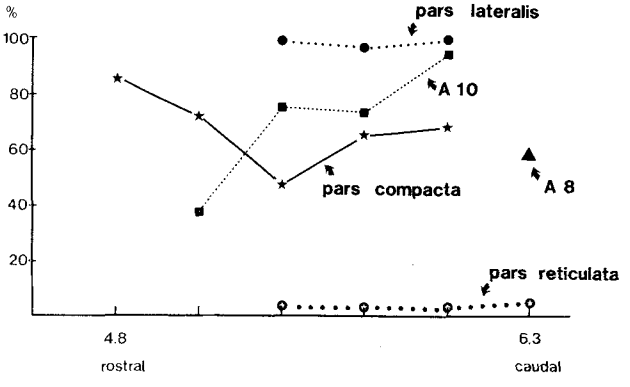


Figure 2. Schematic illustration of the percentage of dopamine (DA) neurons containing cholecystokinin (CCK)-LI in various subregions of the ventral mesencephalon at different rostral-caudal levels. (The most rostral level is approximately 4.8 mm behind the Bregma, the most caudal point 6.3 mm behind the Bregma and sections have been analyzed at 0.3-mm intervals.) Areas analyzed are pars compacta, pars lateralis and pars reticulata of the substantia nigra as well as the ventral tegmental area (A10 DA cell group) and the A8 DA cell group in the mesencephalic reticular formation. Note high proportion of DA/CCK coexistence in pars lateralis and low percentage in pars reticulata. In the ventral tegmental area there is an increasing incidence of coexistence in the caudal direction. (From Staines, Hökfelt, Goldstein et al., in reference 66)

tended these findings. For example, immunohistochemical analysis the coeliac-superior mesenteric ganglion in guinea pig has shown at least three distinct populations of neurons (figs 3, a–c; 4)[82,91,93]:
1) noradrenergic ganglion cells containing an NPY-like peptide (approximately 65% of all neurons), 2) noradrenergic cells containing SOM-LI (25%), and 3) a small population of vasoactive intestinal polypeptide (VIP)/peptide histidine isoleucine(PHI)-positive cell bodies. Some of the latter ones contained NPY-LI and sometimes also noradrenaline (NA). These neurons have specific domains within the ganglion and have been shown to project to different targets in the gastrointestinal wall (fig. 4)[28,45]. Moreover, they seem to be controlled by different afferent inputs (fig. 4). Thus, whereas the afferents from the intestine containing e.g. VIP/PHI and DYN exclusively terminate around the SOM-positive cell bodies (cf. fig. 3, b and c), afferents from the spinal cord and spinal ganglia have a more wide-spread distribution[35,36,82,93,96]. These findings suggest that chemical coding of neurons by a particular peptide in the peripheral nervous system may reflect its participation in a well-defined physiological event.

Are coexistence combinations preserved during phylogeny?

This question has been studied only to a limited extent. However, there are examples both of variation and preservation of certain coexistence situations among different species. For example, NA and SOM coexist in sympathetic neurons in guinea pig and rat but not in cat[90,91]. Coexistence of DA and CCK-LI has been observed in mouse, rat, cat, monkey and probably man, but the proportions and exact distribution of coexistence neurons in the ventral mesencephalon seem to vary among the different species[64]. In contrast, no CCK-LI has so far been observed in mesencephalic DA cell bodies in guinea pig. We have analyzed one of the most primitive vertebrates, the lamprey fish, and although coexistence situations have been encountered, there is so far no evidence for any major coexistence of those compounds which have been described in mammals[17].

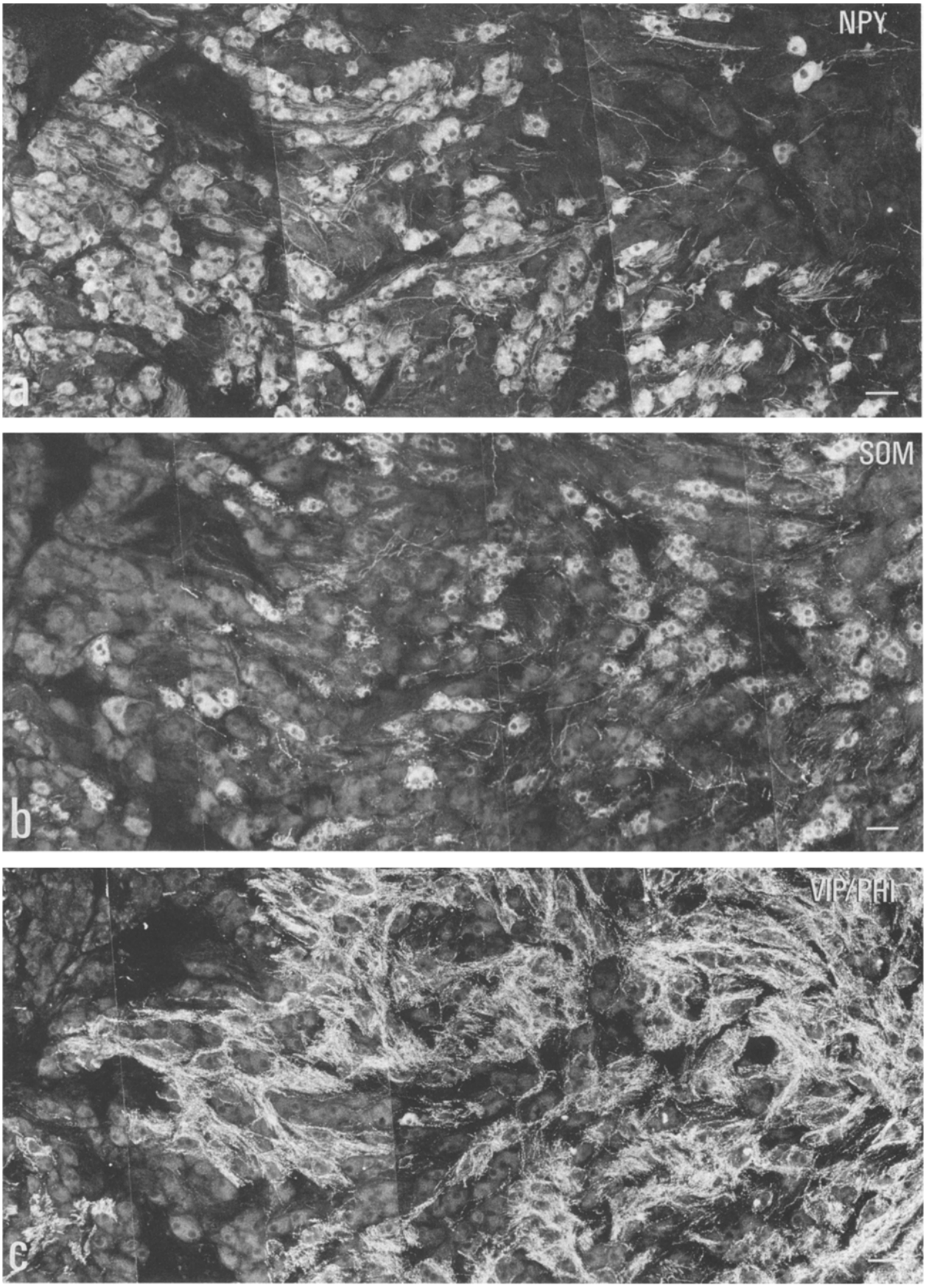
Functional significance of coexistence

The functional significance of coexistence of multiple putative messenger molecules is not very well understood. A key issue is whether or not it is meaningful to have numerous compounds simultaneously conveying messages between neurons or a neuron and an effector cell; i.e. whether these messengers can produce selective and differential responses. There are several models which explain how multiple messengers might work. One shows that the neuron under all conditions releases all types of messenger molecules at the same time, and that the distribution and type of receptors provide selectivity and specificity, i.e. post-synaptic selectivity. An alternative model would be the ability to release the messenger differentially i.e., presynaptic selectivity. Both, of course, may operate together and other types of mechanisms should also be considered. In the following we shall present some morphological evidence for the view that differential release can be obtained and that this is related to differential storage of the transmitter substance.
In general, nerve endings contain at least two types of vesicles, the synaptic vesicle (diameter about 500 Å) and a large type of vesicle (diameter about 1000 Å), often containing an electron-dense core and termed 'large dense-core' or 'granular' vesicle. Immunohistochemical studies at the ultrastructual level have revealed that peptides seem to be located in the large dense-core vesicles. For example, Pelletier et al.[122] demonstrated that SP is present in large dense-core vesicles in nerve endings in the ventral horn of the spinal cord. This general idea is supported by subcellular fractionation studies demonstrating that VIP in the cat salivary gland[89] and NPY in rat vas deferens[44] seem to appear exclusively in a heavy fraction characterized by presence of large dense-core vesicles. These fractions also contained overlapping peaks with the coexisting classical transmitters, i.e. ACh and NA, respectively. In contrast, the lighter fractions, presumably characterized by content of small synaptic vesicles, only contain classical transmitters. These findings suggest that peptides at least in some tissues are stored exclusively in large vesicles, whereas classical transmitters are found in both dense-core and synaptic vesicles. Thus, if a mechanism would exist allowing selective activation and release from the two types of vesicles, it should be possible to obtain differential release of transmitter substance from the nerve terminal. There is evidence that the classical transmitter and peptide can be released differentially and that this release is dependent on the frequency of action potentials[39,90]. According to this hypothesis, a low impulse frequency selectively activates small vesicles resulting in the release of the classical transmitter, whereas at higher frequencies or by bursts of impulses the large vesicles also release their content in addition. In this way the classical transmitter is released selectively or in combination with a peptide(s)[86].

Interaction of coexisting messengers

Some experimental models have yielded interesting and perhaps meaningful results concerning possible interaction among transmitter substances. For example, the cat salivary gland receives a parasympathetic innervation containing ACh together with VIP and PHI and noradrenergic sympathetic, perivascular fibers containing NPY[90]. ACh induces both secretion and an increase in blood flow and these effects are both atropine sensitive[90]. VIP alone has no apparent effect on secretion but causes increased blood flow, thus co-operating with ACh in the regulation of blood flow[90]. Moreover, VIP potentiates ACh-induced secretion, and additive effects on blood flow are seen when ACh and VIP are infused together[90]. With regard to the sympathetic control of blood flow, NA and NPY cooperate in causing vasoconstriction, whereby NPY alone exhibits a slowly developing, long lasting effect[88,90]. A different type of interaction has been observed in rat vas deferens which is innervated by noradrenergic fibers containing NPY[1,92,139], since here the peptide inhibits release of NA. Thus, the peptide seems to exert an antagonistic action at the presynaptic level.
A second model was tested in the autonomic nervous system of the bullfrog by Jan and collaborators[14]. The frog sympathetic ganglion allows a thorough analysis of the coexistence concept, since it is possible to define the roles of the coexistence messengers also with electrophysiological techniques. Neurons in some ganglia of the lumbar chain contain ACh and a luteinizing hormone-releasing hormone (LHRH)-like peptide, and physiological experiments indicate that the preganglionic C-fibers release both ACh and the peptide[14]. However, the targets for the two compounds are not identical. Acetylcholine exerts its actions only on the so-called C-cells, which are in synaptic contact with the preganglionic

Figure 3. Immunofluorescence micrographs (montages) of the coeliac-superior mesenteric ganglion of guinea pig after incubation with antiserum to neuropeptide Y (NPY) (a), somatostatin (SOM) (b) and peptide histidine isoleucine (PHI) (c). The montages show semiadjacent sections in the border zone between the NPY and SOM domaines. On the left hand side the NPY-positive cell bodies dominate, whereas SOM cell bodies are seen mainly to the right, but in this particular area a considerable intermingling takes place. Note that PHI-positive fibers originating in the gastro-intestinal wall preferentially innervate SOM-positive cell bodies. Bar indicates 50 μm. (From reference 82)


NPY
a
SOM
b
VIP/PHI
c

C-fiber from which ACh is released. The LHRH-like peptide causes responses only in some C-cells but does in addition activate B-cells which are many µm apart and thus not in synaptic contact with the preganglionic C-fibers[14]. The physiological analysis reveals that the LHRH-peptide causes slow excitatory postsynaptic potentials (EPSP), whereas it is known that ACh causes a fast EPSP[14]. In conclusion, in this particular model the two compounds both induce excitatory postsynaptic potentials but, whereas ACh induces a fast potential, the LHRH-like peptide is responsible for the slow EPSP. Moreover, whereas ACh acts synaptically on C-cells, the LHRH-like peptide activates only a proportion of these cells but can in addition induce slow EPSP in B-cells located up to 10 µm away. As pointed out by Branton et al.[14], the distribution of receptors and the ability of the messenger molecule to 'survive' long diffusion distances represent important factors for deciding upon what effects are evoked. These and the previous examples indicate that coexistence of messenger molecules is not a 'homogenous' phenomenon and that different types of interaction may take place.

Are extracellular enzymes targets for neuropeptides?

Finally, in the discussion of functional significance of multiple messengers, we would like to focus on a recently discovered peptide, calcitonin gene-related peptide (CGRP)[3, 127]. Using antibodies raised against this peptide, immunohistochemical studies have revealed characteristic and unique distribution patterns within the nervous system, including its presence in primary sensory neurons[127]. In fact, these CGRP-positive primary sensory neurons seem in part to be identical to previously described SP-immunoreactive neurons[47, 149]. It therefore seems likely that CGRP and SP are released from the same nerve endings both in peripheral tissues as well as in the superficial layers of the dorsal horn of the spinal cord[95, 129]. Possible interactions between CGRP and SP have been studied in the spinal cord after intrathecal administration of the two peptides, separately or in combination[149]. After intrathecal injection of SP at the lumbar level, rats exhibit a characteristic behavior with caudally-directed biting and scratching[69, 125], and this could be confirmed in our study on rats, exhibiting a fairly short-lasting behavior (2–4 min)[149]. CGRP alone in doses up to 20 µg did not cause any observable effects. However, if SP and CGRP were injected together, a marked increase in the duration of this behavior was seen, lasting for 30 min or more[149]. A partial explanation for this prolongation of SP-induced behavior by CGRP has been forwarded by Terenius and collaborators[81]. They observed that CGRP is a potent inhibitor of a SP endopeptidase isolated from human CSF[81], suggesting that CGRP may prolong transmission at SP 'synapses' by inhibiting a degrading enzyme. This may represent a new type of interaction of two compounds released from the same nerve endings and raise some general questions concerning chemical transmission, indicating that messenger molecules may not always interact with membrane-bound receptors but perhaps, as in this case, with an enzyme located in the extracellular space.

It has been suggested[67] that such a hypothetical action of a messenger molecule on an extracellular enzyme may be a more general principle. For example, in the substantia nigra it has been reported that nerve cells can secrete acetylcholinesterase[25, 51] (see Greenfield[50]), and it is known that this enzyme can hydrolyze SP[26], which is present in very dense fiber networks in the zone reticulata of the substantia nigra[32, 83]. In spite of this, several groups have failed to demonstrate binding sites for tachykinins in the rat substantia nigra with receptor autoradiography[7, 128, 132]. One hypothetical explanation could thus be that SP released from nerve endings in the zona reticulata primarily interacts with an extracellular enzyme[67].

Studies on CGRP have also suggested another role for a coexisting peptide. It was early observed that CGRP-LI in the spinal cord is present not only in central branches of primary sensory neurons, but also in motoneurons and it therefore seems likely that CGRP coexists with ACh[47, 127, 140, 141]. Recently evidence has been obtained by two groups that CGRP may be involved in regulation of receptor density. Thus, CGRP added to cultured chicken myotubes causes an increase in the number of surface ACh receptors, probably by acting as a long-term anterograde factor in the biogenesis and maturation of the endplate postsynaptic membrane[43, 107]. These findings further underline the view that coexisting messengers may interact and act in a wide variety of ways, characteristic of the particular system in which the coexistence occurs.

Conclusions and speculations

The functional significance of the histochemical demonstration of coexistence of multiple messengers is at present difficult to evaluate, but evidence has been obtained from studies in the PNS that classical transmitters and peptides are co-released and interact in a cooperative way on effector cells. Other types of interaction may also occur, however, since peptides have been shown to inhibit the release of the coexisting classical transmitter. In the CNS, the situation is even less clear but similar mechanisms may also operate. Indirect evidence suggests that peptides may in some cases strengthen transmission at synaptic (or non-synaptic) sites and in other cases inhibit release of the coexisting classical transmitter. Thus, multiple messengers may provide a mechanism for relaying differential responses and for increasing the amount of information transmitted at synapses.

It is emphasized that coexisting messengers may not necessarily be involved directly in the transmission process at synapses but could also exert other types of actions, for example have trophic effects or induce other types of long-term events in neurons and effector cells. For instance, it has been shown that SP exerts growth-stimulatory effects on smooth muscle cells[108], and as discussed above CGRP may be involved in regulation of expression of transmitter receptors[43, 107]. In fact, it may be argued that the coexistence phenomenon as such, i.e. that neurons in addition to classical transmitter(s) contain other compounds, suggests that peptides are involved in other functions, since the neurons already have a classical transmitter at their disposal for accomplishing the task of fast cell-to-cell communication.

Redundancy of neurons and neuronal systems is an important feature of the central nervous system and one may ask the question, why should it not be sufficient to have one transmitter at each synapse when there are so many nerve cells? An answer could be that redundancy is also present at the level of the individual synapse. A highly differentiated transmission process may also be necessary to achieve the enormous operational capacity of our brain, which also includes transfer of messages for long-term effects.

At this point it is, however, also wise to look upon the coexistence phenomenon with a critical eye, in view of the fact that physiological implications so far are very little elucidated. It cannot be excluded that coexistence of multiple messengers is a paraphenomenon representing a consequence of evolution. It is possible that peptides have been important messengers in lower species, but that they have been replaced by the more efficient, small-molecule transmitters, especially in phylogenetically young areas of the brain such as cortex, and that peptides at least in some places are carried along more or less as 'silent passengers'. It will be an important task to establish in the future whether or not, in fact, peptides and classical transmitters are released from the same nerve endings and under which conditions this occurs.

Furthermore, it will be important to determine the models of action and interaction of the different transmitter substances.

Finally, the question may be raised whether or not the coexistence phenomen is of interest in relation to pathological processes. So far, little evidence for such an involvement has been presented. It is obvious that, for example, the presence of a CCK-like peptide in certain mesencephalic dopamine neurons (see above) could be discussed in relation to schizophrenia, since this disease according to one of the hypotheses is related to hyperactivity of mesolimbic dopamine systems (see book edited by Matthysse and Kety[96a]), but this issue has so far not been sufficiently penetrated.

It may, however, be relevant in this overview to speculate how coexisting messengers in a general way could interact in the development of a pathologic process. As an example we have chosen coexistence of acetylcholine and the newly discovered peptide galanin[141a] in forebrain neurons of rat[100] and monkey[101] projecting to hippocampus[100]. As shown by many groups, these cholinergic neurons may be important for higher brain functions such as memory and learning[104a,131a]. Their cholinergic nature and projections to cortical areas have been established in many studies (see Fibiger[42a] and Wainer et al.[145a] for review), and there is strong evidence that they are degenerated in Alzheimer's disease and senile dementia[148a]. It is therefore not unreasonable to consider if and how a possibly coexisting peptide, galanin, could be involved in the development of this disease.

In the hypothalamus galanin may inhibit the release of dopamine in a system where galanin and dopamine coexist[108a]. If galanin inhibits the release of acetylcholine also in the cholinergic forebrain system, this peptide could be of importance for the development of Alzheimer's disease (fig. 5). Our reasoning is based on the hypothesis described above that a coexisting peptide is stored in the large dense-core vesicles (fig. 5) and is preferentially released when neurons are firing at a high rate or with a certain frequency pattern[39,90]. In the case of galanin, it may have the purpose to prevent excessive release of the coexisting transmitter (fig. 5a–c). A further basis for our discussion is that the degeneration of the cho-

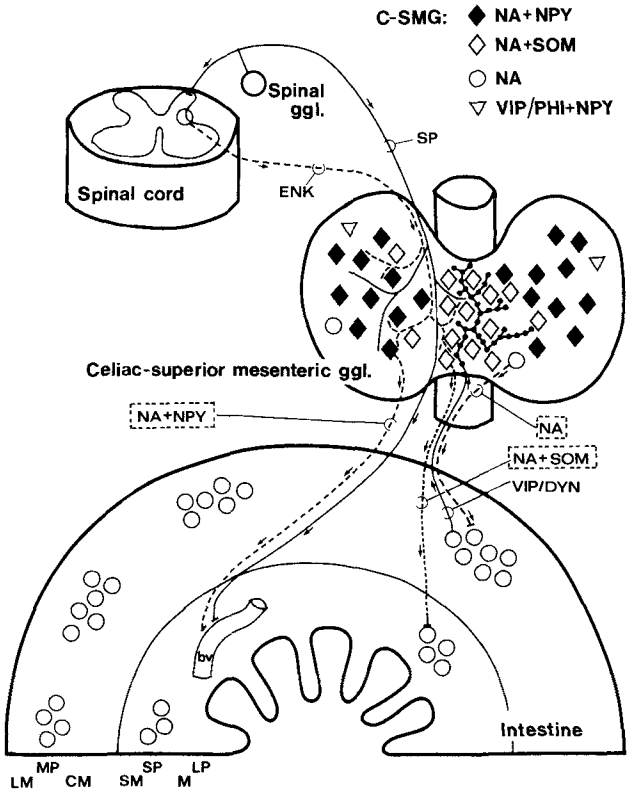


Figure 4. Schematic illustration of the coeliac-superior mesenteric ganglion (C-SMG) and its connection with the spinal cord and intestine with special reference to peptides and coexistence systems. In the C-SMG two main populations of ganglion cells are seen characterized by presence of noradrenaline (NA) plus neuropeptide Y(NPY)-like immunoreactivity (LI) and NA plus somatostatin(SOM)-LI, respectively. The former are located in the lateral parts of the ganglion, whereas the NA+SOM ganglion cells occupy its mid portion. Small population of cells contain vasoactive intestinal peptide (VIP)/peptide histidine isoleucine (PHI) plus NPY, and they are located in the lateral aspects of the 'NPY domaine'. Some NA cells seem to lack a peptide. The NA+SOM neurons project to the submucous ganglion, whereas the NA+NPY neurons innervate blood vessels in the intestinal wall. Cell bodies containing NA alone project to the myenteric ganglia. Projections from the intestine to the C-SMG arise from the myenteric ganglia and contain multiple peptides including VIP, PHI and dynorphin (DYN). They seem to innervate exclusively NA plus SOM cell bodies in the midline areas of the ganglion. The fibers from the spinal cord contain an opioid peptide, possibly an enkephalin(ENK)-like peptide, and they distribute diffusely over the ganglion. To what extent these fibers also contain acetylcholine has not been established. Finally, primary sensory neurons containing i.a. substance P give rise to a diffuse plexus within the ganglion, and these fibers represent collaterals of axons continuing on to the gastrointestinal wall and innervating blood vessels. bv, blood vessel; CM, circular muscle layer; LM, longitudinal muscle layer; LP, lamina propria; M, mucosa; MP, myenteric plexus; SM, submucousa; SP, submucous plexus. (This schematic drawing is based mainly on work in references 28, 35, 36, 45, 82, 91, 93, 96)

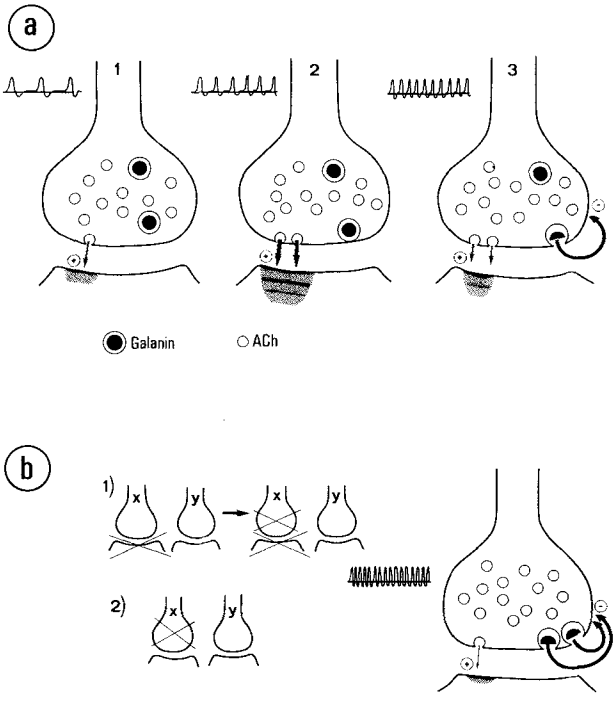


Figure 5a, b. Schematic illustration of a cholinergic nerve ending in the hippocamus containing galanin and originating in the basal forebrain. a) Under normal conditions acetylcholine (ACh) is released in increasing amounts with increasing impulse frequency, causing an increased postsynaptic response (1, 2). However, with very high activity (3), galanin is also released causing inhibition of ACh release. b) If a proportion of the cholinergic forebrain neurons is damaged (x), either as a consequence of degeneration of postsynaptic neurons (1)[135a], or by presynaptic degeneration (2), the remaining neurons (y) may exhibit hyperactivity, leading to increased galanin release, increased inhibition of ACh release and a diminished postsynaptic response. This in turn, via feed-back mechanisms, may further activate the forebrain neurons, leading to a vicious circle causing accelerated cell death.

linergic forebrain neurons is a sequential process and that partial destruction of a system causes a marked hyperactivity in the remaining neurons, as demonstrated experimentally on the nigrostriatal dopamine system by Agid et al.[1]. It may be speculated that such changes occur during progressive degeneration of the cholinergic forebrain system in Alzheimer's disease. As shown in Figure 5b, hyperactivity, i.e. increased firing in the remaining, non-lesioned neurons would lead to a substantial release of galanin and consequently decreased acetylcholine release. If, as one may anticipate, feedback mechanisms operate, low acetylcholine levels in the synaptic space would lead to further increase in impulse activity, further release of galanin and stronger suppression of acetylcholine release. Thus, provided that galanin biosynthesis can be maintained, the more the activity increases, the less acetylcholine is released, taking the system into a vicious circle. Such an increased strain on the neurons could also lead to accelerated degeneration and thus faster development of the disease. It would be interesting to know if one could counteract this process by a compound which blocks galanin binding sites. Such a galanin antagonist could be a potential drug for the treatment of Alzheimer's disease, should one become available in the future.

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Peptides and epithelial growth regulation

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Summary. There is now considerable evidence implicating several peptides in the control of gastrointestinal epithelial cell proliferation and cell renewal. While some of these may act directly, many may be involved in regulating the powerful trophic effects of the intake and digestion of foold on the gut epithelium. – Several peptides have been associated with the regulation of intestinal cell proliferation. There is little doubt that gastrin is trophic to the stomach, but, its role in the rest of the gastrointestinal tract is debatable. Enteroglucagon has often been associated with increased intestinal epithelial proliferation, but at the moment all the evidence for this is circumstantial. The effects of peptide YY and bombesin warrant further study. The availability of recombinant epidermal growth factor (EGF) has recently enabled us to demonstrate a powerful trophic response to infused EGF throughout the gastrointestinal tract. The increasing availability of peptides will eventually allow the rigorous in vivo evaluation of the trophic role of these potentially very important peptides.
Key words. Peptides; gastrointestinal tract; epithelial cell proliferation; gastrin; enteroglucagon; peptide YY; bombesin, epidermal growth factor; cholecystokinin; somatostatin.

Introduction

In many ways the gastrointestinal epithelium is an ideal model for the study and investigation of the control of epithelial cell proliferation, as it is continuously and rapidly renewed with its cell division restricted to an anatomically discrete zone. It is also capable of adapting its rates of proliferation to a wide variety of physiological and other stimuli. The study of epithelial cell renewal is also of considerable importance since most tumours are of epithelial origin[76]. Three main mechanisms are generally considered to be involved in the control of epithelial renewal in the gut namely, a (local?) negative feedback system from the functional (villus) to the reproductive zone (crypt), the direct or indirect effects of food (luminal nutrition and/or intestinal workload) and the effects of humoral factors[103].

Parabiotic studies in which the blood systems of two animals are linked have indicated that a hormonal factor may cross-circulate from a stimulated animal to its partner[52,100]. A similar response has also been noted in less extreme models where isolated loops of small intestine still respond to altered food intake[20], and after intestinal resection [7,37].

The study of cell renewal and epithelial growth control necessitates the use of suitable methods, and unfortunately many studies in this field have been bedeviled by the use of totally inappropriate methods. The problems involved have been spelt out in detail elsewhere[5,19,35,66,102,103], and are as follows; 1) The intestine contains a large proportion of non-epithelial cells (muscle, submucosa lymphoid aggregates); thus any gross measure may give a misleading result. Even the mucosa itself is approximately 20% non epithelial[19]. 2) The choice of a suitable denominator is of vital importance, as many measures, such as labelling index and mitotic index will not detect a general increase in compartment size. These measures also suffer from being 'state' measures, and as such can be misleading if the duration of the DNA synthesis phase or mitosis is altered. 3) Measures based on the gross uptake of tritiated thymidine can be especially misleading, as although usually equated with growth, triatiated thymidine uptake can be affected by a variety of stimuli. Thymidine itself is not a precursor in the de novo synthesis of DNA, but is incorporated by a salvage pathway which depends on the activity of several enzymes and transport mechanisms plus the size of the endogenous thymidine pool. All of these factors can be influenced by hormones or growth factors. Thymidine can also be stored and recycled, and it can also be taken up by bacteria[66].

Most of these pitfalls can be avoided if the accumulation of arrested metaphases in microdissected crypts is determined. This 'rate' measure also avoids the several problems involved in the quantification of sectioned material, and expressing the results on a per crypt basis can account for all the factors that may influence epithelial cell production (cell cycle time, size of the growth fraction and size of the crypt itself)[5,19,35,66,102,103].

Gastrin as a trophic hormone in the gastrointestinal tract

There is a considerable body of evidence for a powerful pharmacological and possibly physiological modulation of cell proliferation by gastrin in the stomach[21,63,99]. There is also evidence, unfortunately mainly based on the gross uptake of tritiated thymidine, that this trophism extends into the small intestine and colon[45,46,48,58]. Claims by Johnson[43,44] for a major trophic role for gastrin were also supported by a study of the effects of gastrin on primary duodenal explants in short-term culture[58]; but this study is especially open to criticism[101].