Ann. Rev. Pharmacol. Toxicol.1988. 28:285–310

REGULATION OF THE RELEASE OF COEXISTING NEUROTRANSMITTERS

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INTRODUCTION

The joy of neurobiologists at the discovery of the coexistence of several neuroactive substances within the same neuron terminals can be compared with the delight of a man who has bought a radio he thought would only receive AM broadcasts, but who then discovers that it also gets all the FM stations. The discovery that the given neuronal network has the possibility of transmitting several different signals at the same time, at many of its synapses, lends new dimensions to the workings of this network. The pace of morphological discovery of ever new examples of the coexistence/colocalization of two or more neuroactive substances in the same neuron is very high, producing several hundred new examples of coexistence phenomena in the periphery and CNS (cf. 1–6). The work on the functional implications of the possibility of multiple signalling through the same synapse—using several coexisting neurotransmitters—has been much slower. In this review, we discuss some important questions concerning the release of coexisting neurotransmitters and through these get some insight into the functional implications of coexistence. These questions are as follows:

(*a*) Will a neuron at every one of its processes (dendrites and axon

0362-1642/88/0415-0285$02.00

terminals) release the same mixture of coexisting neurotransmitters, or can the same action potential mean different chemical neurotransmissions at different synapses of the same neuron?

(*b*) Will the same nerve terminal always release the same mixture of coexisting neurotransmitters? or can the (stoichiometric) ratio of the released neurotransmitters vary, so that in extreme cases the entire chemical nature/composition of the signal may change, i.e., one or another of the coexisting neurotransmitters is not released at all? Can the previously discovered principles of presynaptic regulation of neurotransmitter release apply when more than one neurotransmitter is released?

Furthermore, we describe some of the emerging functional aspects of coexistence both under conditions of acute nerve stimulation and in the presence of chronic drug regimens (on e.g. antidepressant or antipsychotic drugs).

Histochemical Evidence for Coexistence, Use of Lesions, and Releasing Agents

Discovery of the coexistence of more than one neuroactive substance in the same neuron is a result of histological work. Hökfelt and coworkers (7) described the presence of vasoactive intestinal polypeptide (VIP)–like immunoreactivity (LI) in postganglionic neurons which innervate the cat submandibulary gland. These neurons were previously shown to be cholinergic by acetylcholinesterase (AChE) staining and later, more conclusively, with choline acetyltransferase (ChAT) staining.

Further evidence for coexistence of several neuroactive substances in the same neuron stems from other experiments than those in immunohistochemistry. These experiments are: use of lesions (surgical and chemical), leading to simultaneous disappearance of several neurotransmitters in the same area; and use of releasing substances, which cause release of more than one neurotransmitter simultaneously from the same population of nerve terminals. While immunohistochemistry dominated the discovery of cases of coexistence, the other two more functionally oriented approaches are coming into use more and more (discussed below).

One must emphasize that although at least a hundred examples of coexistence of two or more neuroactive substances have been described by immunohistochemical techniques, only in very few instances have the functional implications been studied in as much depth as in the case of the very first example of coexistence. Thus, the coexistence of VIP and acetylcholine (ACh), as studied by Lundberg and coworkers (8–11), also serves as one of the useful examples of the presynaptic and postsynaptic consequences arising from coexistence.

Scope of Coexistence Phenomenon

Because of the histological method by which coexistence is most often discovered, it is impossible to determine how widespread the phenomenon of coexistence is among neurons. Every new neuropeptide mapping shows that this hitherto unknown neurotransmitter coexists at certain locations with previously known neurotransmitters X, Y, and Z. One may, however, safely assert that coexistence of several neurotransmitters is not a marginal phenomenon, since a multitude of examples has been found in less than 10 years time. These examples include neurons of both the peripheral and the central nervous systems. It should also be noted that, in an evolutionary respect, the phenomenon is rather old; examples of coexistence are found, for example, in the Hydra (12) and Aplysia (13).

Types of Coexistence

Several attempts have been made to predict which neurotransmitter will coexist with which other neurotransmitter(s) in a given set of neurons. These attempts have so far been unsuccessful; we do not understand what governs which genes will be expressed in which neurons. It is clear that all of the neurons carry the genes for all of the neuropeptides and for the enzymes that synthesize the classical low molecular weight neurotransmitters. Being unable to predict the coexistence of neurotransmitters in pairs, or triples, one resorts to phenomenological classifications of the known cases of coexistence. A trivial classification is based on the number of coexisting neurotransmitters per neuron: there are cases of two, three, or four coexisting neurotransmitters. This classification, however, offers nothing but a catalog. A classification based on the types of different molecular mechanisms that underlie the phenomenon of coexistence was among others put forth by O'Donohue et al (14). They distinguish four types of coexistence or four types of cotransmitter containing neurons.

(*a*) Multiple neurotransmitters (neuropeptides) are derived from a common gene coding for the prohormone [e.g. neurons with pro-opiomelanocortin derived peptides: α-melanocyte stimulating hormone (α-MSH), β-endorphine (15)].

(*b*) Multiple neurotransmitters (neuropeptides) are the products of distinct genes [e.g. somatostatin and neuropeptide Y (NPY) coexistence (16)].

(*c*) Multiple neurotransmitters include neuropeptide(s) and a classical neurotransmitter(s) [e.g. VIP and ACh (7), noradrenaline (NE) and NPY (17)].

(*d*) Multiple neurotransmitters are all of low molecular weight (classical) neurotransmitter type [serotonin (5-HT), ACh, and octopamine in Aplysia ganglion (13)].

Classes b, c, d, have in common that the synthesis of multiple neuroactive substances involves transcription, translation of more than one gene. Biosynthesis of the classical neurotransmitters often involves several enzymes whose genes all must be transcribed, translated (the gene product post-translationally modified when needed) before the classical neurotransmitter (e.g. NE or 5-HT) is biosynthesized. In addition genes coding for the coexisting neuropeptide must be expressed also.

It should be emphasized that, e.g., coexistence of ACh and VIP in postganglionic neurons of cat submandibular gland has no predictive value with respect to which peptide will occur in other cholinergic neurons. In fact, most cholinergic neurons in the CNS are devoid of VIP-LI [except a small population of bipolar cells in the cerebral cortex (18)]. Similarly, most serotoninergic neurons do not express substance P (SP), neurokinin A [substance K (SK)], or the thyrotropin releasing hormone (TRH), although many of them even have their soma in the raphe nucleus, like those neurons innervating the ventral horn of the spinal cord, where most of SP-LI and TRH-LI coexists with 5HT (19–21).

Some Well-Studied Examples of Coexistence

The pace of histochemical discovery of new cases of coexistence is not matched by functional studies on neurons with coexisting neurotransmitters, due to methodological difficulties. However, a few instances of coexistence do demonstrate corelease, and thus, one may ask questions about the regulation (simultaneous) of the release of more than one neuroactive substance and about their effects on pre- and postsynaptic events. Table 1 lists those arbitrarily chosen examples that we believe permit one to formulate some concepts governing corelease, cotransmission in signalling at synapses with multiple neurotransmitters.

Table 1 Some examples of coexistence where corelease has also been demonstrated

Classical NT	Peptide NT (1)	Peptide NT (2)	Localization	References
ACh	VIP		Parasympathetic nerves in exocrine glands	8–11
	VIP	PHI	cat submandibular salivary gland	49
Catecholamines	Leu-Enk	Met-Enk	cultured adrenal chromaffin cells	41–45
NE	NPY		splenic nerve vas deferens	50, 74, 75
5HT	SP	TRH	ventral spinal cord	27, 51, 84
	SP	CGRP	spinal cord	68
	SP	SK	substantia nigra	52, 53

Some Comments about the Examples

It is easier to establish corelease, synergism of postsynaptic action at an effector organ, etc., in the peripheral nervous system than in the CNS, and such examples dominate in Table 1. Nevertheless, examples of coexistence of several neurotransmitters are very frequent in the CNS (cf 1–6). The studies on ACh/VIP or on SP/5HT/TRH coexistence could have been (and partly have been) extended in width, since additional coexisting neurotransmitters were found in those neurons during the past 5–6 years. The VIP gene is also known to code for peptide PHI or peptide PHM-27 (Figure 1; see also 22, 23) which is a neuroactive peptide. Furthermore, the SP gene codes for another tachykinin, SK (24–26), and these SP/SK/5 HT neurons also contain and release TRH (27). Thus, while the study is in progress, the scope of coexistence for the given set of neurons may gain additional dimensions. It appears both more important and more practical, at this point, to try to formulate concepts based on a few selected examples, than to try to provide an exhaustive description of all cases of coexistence.

BIOSYNTHESIS OF COEXISTING NEUROTRANSMITTERS

Cellular Localization of the Biosynthesis of Classical and Peptidergic Neurotransmitters

The general distribution of the biosynthetic activities that produce neuroactive peptides and neuroactive small molecules (classical neurotransmitters) is uneven. The cytosolic enzymes responsible for biosynthesis of classical neurotransmitters, like ChAT (for ACh), tyrosine hydroxylase [for NE and dopamine (DA)], and tryptophan hydroxylase (for 5HT) occur both in the soma and in the nerve terminals; and their specific activity is highest in the nerve terminals (28). The neuropeptides, on the other hand, are synthesized on ribosomes, attached to the endoplasmic reticulum, mostly in the soma. The presence of synthesized and processed neuropeptides in the nerve terminals is achieved through *axonal transport* of the neuropeptide containing vesicles, which are filled in the soma. One consequence of the anisotrop distribution of biosynthetic activities is that it is possible to deplete the nerve terminal with respect to neuropeptides, while the classical neurotransmitter levels are only slightly or not at all lowered at the same intensive stimulation, because the biosynthetic enzymes, present in the nerve terminal, will keep up with increased release. Indeed, some biosynthetic enzymes, like tyrosine hydroxylase, increase their activity as the impulse flow and rate of release at the nerve terminal increases (29).

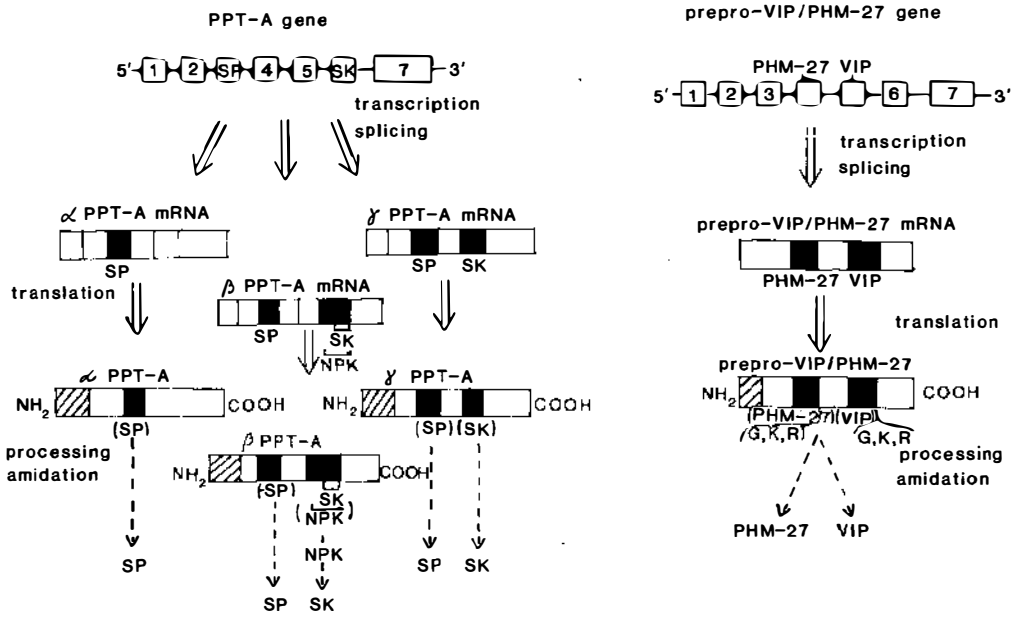


Figure 1 Biosynthetic routes leading to coexistence. Some examples of the biosynthesis of coexisting peptides coded on the same gene. The major steps of rat SP/SK (left) and of human VIP/PHM-27 (right) biosynthesis including transcription, splicing of relevant exons into preprohormone mRNA, translation into the primary translation product preprohormone with the signal peptide and the coexisting neuropeptides which is cleaved at basic residues Lys Arg (or K, R in the one letter code) and finally post-translational processing (in these cases amidation of C-terminal) is shown. These examples were chosen to illustrate coexistence as a consequence of genomic organization, but also to show that alternative splicing may change which neuropeptides are expressed in which tissue (cf NPK and SK). (Data from 22–26).

Biosynthesis of Coexisting Neuropeptides Encoded on the Same Gene

Several examples of coexistence of neuropeptides can be deduced from study of the structure of mRNA and from the study of the gene coding for these neuropeptides. Studies on the sequence of cDNA for preproenkephalin (30–32), prepro VIP (22, 23), and prepro SP or preprotachykinin(s) (PPT) (24–26)—to mention a few examples—show the general feature of multiple neuropeptide carrying preprohormones. These preprohormones have the following general composition:

NH_2-putative signal peptide -X- coding region for peptide 1 -Y- coding region for peptide 2 -Z-COOH

The putative signal peptide (20 amino acids), characteristic for most secretory peptides, is followed by a peptide X separated from the first neuropeptide by an arginine; peptide Y separates the first and second neuropeptides, and peptide Z carries the C terminus end of the preprohormone. The neuropeptides 1 and 2 are cleaved at Lys-Arg residues (Figure 1: VIP/PHM-27). In many cases the same neuropeptide occurs in many copies in the same preprohormone [e.g. the preproenkephalin which carries six Met-enkephaline copies and one Leu-enkephalin copy (30–32)].

It is not a priori known whether peptides X, Y, or Z, liberated during the post-translational processing of the preprohormone, are neuropeptides with synaptic activity or not.

This question led Allen et al (33) to synthesize the peptide representing the C-terminal portion of NPY-preprohormone (CPON) and to test it for synaptic activity. This paradigm may lead to the discovery of previously unknown neuropeptides (coexisting with some already known neuropeptides).

The other approach taken in looking for coexisting neuropeptides uses the known primary sequence of a neuropeptide, synthesizing an oligonucleotide probe to hybridize with mRNA from the tissue and sequencing all mRNA species found. This approach has led to identification of peptide PHI or peptide PHM-27 (22, 23) on the same mRNA that codes for VIP. [It is worth mentioning that PHI was isolated by chemical means in the same laboratory as VIP, by Mutt and his colleagues. Radioimmunological and immunohistochemical approaches just started to provide evidence for a colocalization of PHI with VIP in several tissues and species (34) when the apparent reason for this coexistence, namely, that PHI and VIP are synthesized on the same preprohormone, was elucidated by studies on VIP-mRNA, thus also PHI-mRNA (Figure 1; see also 22, 23)].

Stoichiometry of the relative amounts of coexisting neuropeptides coded on the same gene, transcribed and translated in the same preprohormone, still

varies greatly: Fahrenkrug showed that the VIP/PHM-27 ratio may vary between 0.5 and 8.5 in different human VIP and PHM-27 producing tumor, although there is only one precursor present for VIP/PHM-27 in all of these (35). Thus, differences in post-translational processing (e.g. amidation, proteolysis, etc) must explain these differences.

The expression of coexisting peptides coded on the same gene may be even more complicated when alternative splicing generates several different mRNA species, which code for one or two of the coexisting peptides. This situation is observed with tachykinins, where mRNA coding for SP; SK, and for (SP + SK) have been isolated (Figure 1; see also 24–26). The ratio of different mRNA species generated varies greatly from tissue to tissue (25). [Another example of the role of post-translational modification in changing the coexisting neuropeptide repertoire is the biosynthesis of SK in form of neuropeptide K (NPK) which acts itself as a neuro signal or can be cleaved to yield SK (Figure 1)].

Those arbitrarily chosen examples suggest that coexistence of neuropeptide is more the rule than an exception, since most preprohormones code for more than one neuropeptide—hence, for coexisting neuropeptides.

Coexistence of several neuropeptides coded on different genes and coexistence of classical neurotransmitters and neuropeptides represent similar cases of coexistence, since the enzymes of classical neurotransmitter biosynthesis are coded on different genes than are the coexisting neuropeptide (e.g. NPY/NE coexistence, VIP/PHM-27/ACh coexistence, where enzymes of noradrenalin and of ACh biosynthesis are separated from NPY or from VIP/PHM-27).

Storage and Transport of Coexisting Neurotransmitters

Subcellular fractionation studies of nerve terminals from several tissues with coexisting neurotransmitters show that peptide neurotransmitters are localized in large, dense core vesicles, while low molecular weight classical neurotransmitters (ACh, 5HT, DA, NE) show bimodal distribution as a function of bouyant density (cf 36). These neurotransmitters occur in small synaptic vesicles and also sometimes in large, peptide-containing vesicles. The results of subcellular fractionation are somewhat confounded by the possibility of cross-contamination of the fractions. The advent of immunogold labeling techniques (37, 38) permits electron microscopy level examination of the content of small and large synaptic vesicles (39–41). Such studies have yet been carried out in few cases only and suggest that e.g. 5-HT but not SP occurs in small vesicles, as well as that some large vesicles contain both SP and 5HT (39). Whether or not several coexisting neuropeptides are stored in the same peptidergic vesicle (and if so at what stoichiometric ratio) is not known for most systems containing several neuropeptides. In the sensory ganglion cells of the guinea pig mesenteric artery, however, colocalization of

calcitonin gene–related peptide (CGRP)—and SP-LI in large secretory vesicles—was demonstrated by the double immunogold labeling (40).

The existence of separate vesicle populations, containing only one or the other of the coexisting neurotransmitters, implies that stimulus paradigms can be found (and most likely exist and are used by the organism in vivo), and these paradigms permit selective release of the content of one but not the other vesicle population. Different vesicle populations (with their different neurotransmitters stored) may attach to different releasing sites and may be released at different frequencies of stimulation, yielding different intracellular Ca^{2+} concentrations. The possibility exists of pharmacological manipulation of one vesicle population, without affecting the other vesicle population, by releasing agents such as reserpine and para-chloroamphetamine (PCA). We discuss the use of these agents later in the review, but we want to state that these agents may cause (as does varying the stimulation frequency) selective release of coexisting neurotransmitters, this suggests that not all classical neurotransmitters are localized in vesicles which contain peptides (cf section on releasing agents).

Coexistence of opioid peptides and catecholamines in the adrenal medulla (42) represents an interesting example of coexistence, but also of vesicular colocalization (41) and of corelease (43–45), which can be evoked by nerve stimulation or reserpine (46).

One of the oldest examples of coexistence, albeit in a different meaning than that used in general (and in particular in this article), is the coexistence of adenosine triphosphate (ATP) and ACh (47) or of ATP and NE in the same vesicle (48).

In these cases the two low molecular weight substances (e.g. ATP and ACh) are costored in the same vesicle (in fact loading of the cations ACh or NE requires presence of negatively charged ATP). The consequences of the vesicular costorage is that ATP/ACh and ATP/NE are coreleased at a constant ratio, independent from frequency of stimulation and extracellular Ca^{2+} concentration. The genes coding for the ATP, ACh, and NE synthesizing enzymes are separate genes, and the coexistence, costorage, and corelease of ATP/ACh and ATP/NE are consequences of the bioenergetics of pumping and storing the cationic substances NE and ACh with ATP.

RELEASE OF COEXISTING NEUROTRANSMITTERS

Frequency Dependence of the Release of Coexisting Peptide Neurotransmitters and Classical Neurotransmitters

Few preparations are available in which the electrically evoked release and, more importantly, corelease of coexisting neurotransmitters could be studied directly (Table 1). These include (*a*) postganglionic neuron at the cat submandibular gland which releases the neuropeptides VIP, PHI (49) and the

classical neurotransmitter ACh (9); (*b*) splenic nerve of the pig which releases NE and NPY (50); (*c*) nerve terminals of descending neurons from the raphe nucleus in the ventral spinal cord, which upon field stimulation release 5 HT, TRH, SP [SK release also occurs but has not been measured under the same conditions (51)]; (*d*) nerve terminals in the substantia nigra which contain and corelease SP and SK-like immunoreactivity (52, 53).

The general trend of these systems is that release of classical neurotransmitters (ACh, NE, 5HT) occurs at lower frequencies of stimulation than release of the coexisting peptide neurotransmitters. (It was well established from studies on classical neurotransmitter neurons and on peptidergic neurons that peptide release requires, generally, higher frequencies of stimulation, thus neurons with coexistence keep with this "rule"). In some cases, such as release of NPY from the splenic nerve, a bursting pattern of stimulation is required for the peptide release [this stimulus pattern is not effective in increasing the release of NE from this preparation (50)]. Facilitation of release of classical neurotransmitters occurs in the frequency range 1–10 Hz (54), while peptide release shows facilitation between 5–40 Hz [e.g. SK release (55)]. The structural basis of the differential release of classical neurotransmitters and peptides may be the presence of small synaptic vesicles, containing only the classical transmitter (cf above). It is noteworthy that tissue stores of classical neurotransmitters are often 50–1000 times higher than those of the peptide neurotransmitter, when these stores/pools are measured at the nerve terminal level. Thus, the nerve terminal is equipped for more frequent use of the classical neurotransmitter than that of the peptide.

Chemical Frequency Coding

The above examples indicate that variation of the stimulation frequency may not only alter the amount of neurotransmitter released, i.e. the intensity of the signal, but it may also alter the signal qualitatively. Low frequency stimulation releases the classical neurotransmitter, while high frequency stimulation releases both the classical and peptide neurotransmitters. Thus, stimulation frequency codes for more than just intensity of the chemical signal, when neurons with coexisting neurotransmitters are concerned. The relatively sharp limit of 3–5 Hz, as the lowest frequency of stimulation which has been shown to release detectable amounts of the neuropeptide, indicates that low frequency stimulation (and signalling with classical neurotransmitters) and high frequency stimulation (signalling with peptides and classical transmitters) represent two fundamentally different patterns of communication. The above experiments indicate that, at a given synapse, the chemical nature (i.e. the composition) of released neuroactive substances may vary as a function of stimulation frequency. An additional important question, in this respect, is whether the same neuron releases the same signal substance(s) at all of its

processes, upon a given stimulus. So far few experimental data are available to answer this question, but it is likely that different processes of the neuron, axon terminals, dendrites, terminals in bouton, en passant arrangement can be invaded with different probabilities at different frequencies of stimulation (56). The "previous history" of recent stimulation of one terminal as well as, in particular, its localization as a postsynaptic target of other neurons, may also influence which of the coexisting neurotransmitters will be released by this given terminal. Thus, specific synaptic inhibition or stimulation of a terminal (e.g. in axo-axonal synapse) may decide which neurotransmitter is released at specific sites of the same neuron. This fact, in combination with the anisotrop distribution of the biosynthesis of peptide and classical neurotransmitters, may mean that the same stimulus suffices for peptide release from dendrite but not from the distant axonal nerve ending, which is several branch points away from the axon hillock.

The Effect of Releasing Agents on Coexisting Neurotransmitters

Pharmacological agents known to release monoamines have been frequently used in the study of coexistence of monoamines and peptides. Reserpine, a drug which depletes NE stores by blocking vesicular storage of the catecholamine, (1 mg/kg, i.v.) has been shown to deplete most of NPY in the nerve terminals but not in the soma of NE/NPY nerves innervating the heart and spleen (57). Lundberg et al have suggested that increased activity at terminal level or increased release of the peptide NPY per impulse is responsible for the depletion in the terminal fields and that axonal flow of NPY filled vesicles cannot replenish the terminals sufficiently rapidly in the reserpine treated animals, despite the presence of normal or even elevated NPY-LI in the soma of these neurons (57). Reserpine treatment releases both DA and the coexisting peptide sulphated cholecystokinin octapeptide (CCK-8S) (40% of tissue store) from the posterior part of nucleus accumbens (58), suggesting that the peptide occurs both in DA containing vesicles and in peptidergic vesicles without DA. The known 5HT-releasing substance PCA (2.5 mg/kg, i.p.) can cause release of 50–70% of stored 5HT from peripheral and central serotoninergic neurons (59). This applies also to the descending neurons in the raphe nucleus, which innervates the ventral spinal cord of the rat (19–21). These serotoninergic nerves also contain SP and TRH, although probably in a separate vesicle population. While 5HT [and 5-hydroxyindoleacetic acid (5HIAA)] tissue levels drop within 2–6 hr after PCA treatment, the tissue levels of SP-LI are barely affected at this dose of PCA (6) [it should be noted that at much higher doses of PCA, both 5-HT and SP-LI can be released (60), and that PCA stimulation can ultimately kill these neurons]. However, for our argument, the main point is that there exists at least one such dose of PCA at

which 5HT and SP can separately be released. (Presumably this is because they are also contained in separate vesicle populations. TRH release was not measured in these experiments).

Capsaicin has been shown to release SP-LI from primary sensory nerves (61, 62) and cardiac nerve fibers (63). Thus, the question arose whether it caused release of peptides other than SP, if they coexist in the same terminals as SP. Hua et al (64, 65) showed that the tachykinin SK [which is the product of the same tachykinin gene as SP (24–26) and thus coexists with SP] is released by capsaicin treatment together with SP. Capsaicin also released the peptide CGRP which coexists with SP/SK (66–68) but is the product of another gene (69).

This finding is in line with electronmicroscopic results, indicating colocalization of SP-LI and CGRP-LI in the same peptidergic vesicles (40).

Use of "releasing agents," thus, gives some idea about colocalization of coexisting neurotransmitters in releaseable pools, since the above experiments show two things: (*a*) For both reserpine and PCA one can find doses that affect predominantly the monoamine store (probably that in the small vesicles) and do not cause release of the peptides and of that portion of monoamines which are colocalized in the large synaptic vesicles. (*b*) Conversely, capsaicin, which can release SP, also releases other peptides which coexist with SP, like SK and CGRP (the latter of which is stored in the same vesicles as SP). [The neurotoxins 5,7- and 5,6-dihydroxytryptamine were used to cause specific degeneration of serotoninergic neurons. This treatment caused simultaneous disappearance of 5HT, TRH, and SP in the spinal cord (60), demonstrating that the same nerve terminals contain these signal substances, while leaving the question of vesicular localization open.]

REGULATION OF THE RELEASE OF COEXISTING NEUROTRANSMITTERS VIA PRESYNAPTIC RECEPTORS

The study of the release of classical neurotransmitters ACh, DA, NE, 5HT in the peripheral and the central nervous system has shown that their release is subject to regulation by presynaptic receptors (cf 70–72). Many of the presynaptic receptors are autoreceptors which bind the neurotransmitter released from the nerve terminal, where the receptor itself is localized. The concept of presynaptic receptors and of autoreceptors needs to be reexamined when more than one neurotransmitter can be released from the same nerve terminal. One may ask the following questions: Can a presynaptic receptor which is known to regulate the release of one of the coexisting neurotransmitters, also regulate the release of the other neurotransmitters from the same terminal? Are there autoreceptors for all of the coexisting neurotransmitters? Is there a "cross-

regulation" of the release of coexisting neurotransmitters via their respective autoreceptor?

These questions have been studied in great detail in the NE/NPY containing neurons at rat and mouse vas deferens (73–75) and pig spleen (50); ACh/VIP containing neurons at the cat submandibular gland (8–11) and in bipolar neurons in the rat cerebral cortex (18); in the ventral root of spinal cord, at the terminals of 5HT/TRH/SP containing neurons (51, 110–112); in the posterior nucleus accumbens, which contains terminals with DA and CCK-8S (76); in the terminals of septal cholinergic afferents in the ventral hippocampus, which contains ACh and galanin (77).

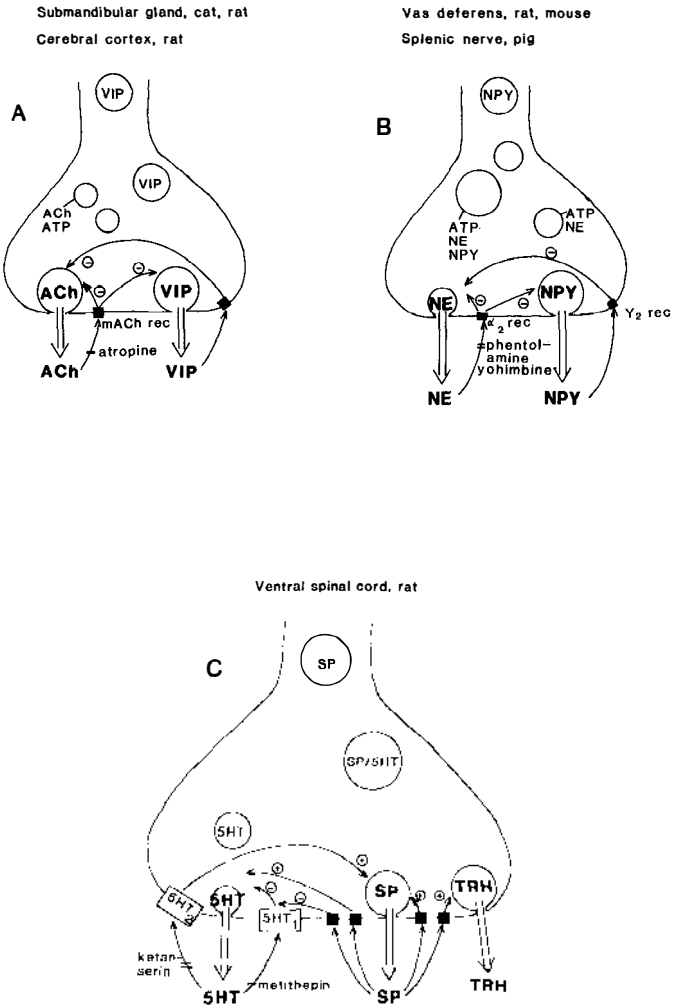
Figure 2 summarizes schematically the results which, in all of these systems, unequivocally indicate that (*a*) a presynaptic receptor that regulates (inhibits or enhances) the release of one of the coexisting neurotransmitters will regulate the release of the other coexisting neurotransmitters in the same direction. In addition to the well-known autoreceptors for classical neurotransmitters, (*b*) there are peptidergic autoreceptors which may regulate the release of the classical neurotransmitter. (*c*) There may be more than one type of autoreceptor present for a given neurotransmitter in the same tissue.

Autoreceptors Regulate the Release of Coexisting Neurotransmitters

It was first demonstrated, in the case of ACh/VIP coexistence in the postganglionic neurons of the cat, that the well-known muscarinic cholinergic autoreceptor, whose agonist occupancy inhibits ACh release (78, 79), also potently inhibits the release of VIP (8–11). Blockade of these cholinergic autoreceptors by atropine, enhanced the per pulse release of both ACh and VIP. (Later, it was shown that the release of one additional coexisting peptide, PHI, is also under the same control.) Similar results were obtained in the cerebral cortex where the degree of coexistence between ACh/VIP is much smaller (18). VIP, on the other hand, enhanced ACh release in both preparations (Figure 2A).

NE inhibits very potently its own release in most preparations via α_2-adrenergic receptors (80). This applies to the vas deferens (81) and the spleen (50), both of which contain NPY in their noradrenergic nerves. The output of NPY (in a way similar to that of NE) at the spleen was substantially (100%) enhanced when phentolamine blocked the α_2-adrenergic autoreceptor (50), indicating presynaptic noradrenergic control of NPY release. NPY itself, in the vas deferens (73–75) and in the right atrium (57), is a potent inhibitor of the electrically evoked release of NE. This NPY-inhibition of NE release is not exerted at the α_2 receptors but rather is additive to the effects exerted at that receptor (75), since the effect exists in the presence of saturating concentrations of yohimbine (Figure 2B).

5HT autoreceptors of two types, 5HT type-1 and type-2, are present in the



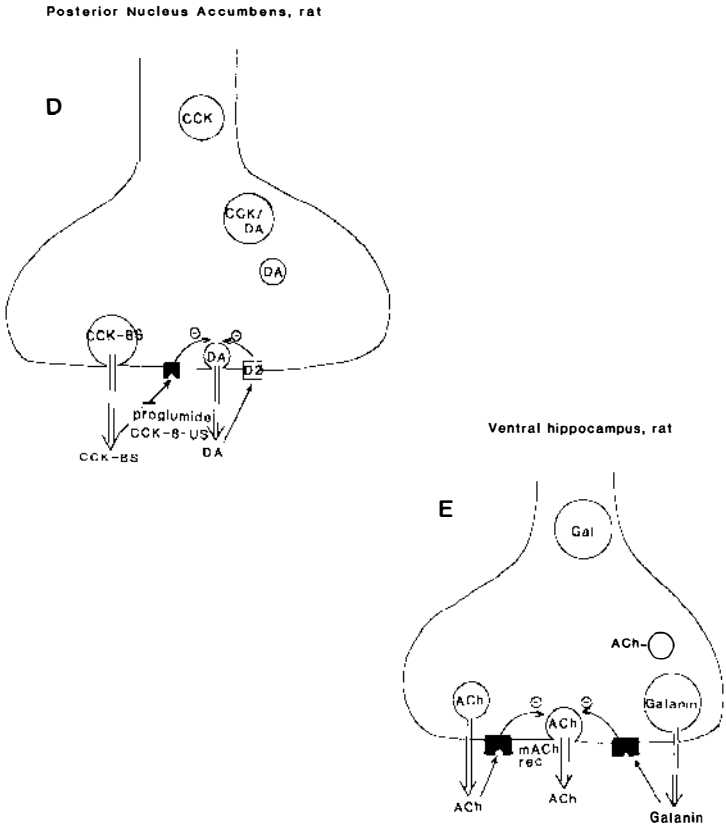


Figure 2 Schematic model indicating cross- and autoregulation of release from neurons where coexistence has been shown. A) In the submandibular gland and in rat cerebral cortex slices, the muscarinic autoreceptor inhibits the release of ACh and of the coexisting VIP. VIP inhibited the release of [^{3}H]-ACh (4, 8–11). [Release of coexisting PHI which is under similar control and frequency dependency as that of VIP (49) is not shown]. B) In the rat , mouse vas deferens, and at the pig spleen, NA inhibits at α_2 receptors both NA and NPY release, while NPY inhibits [probably at NPY_2 (Y_2) receptor (110)] the release of NA (50, 57, 73–75, 101). C) In the ventral spinal cord TRH, SP and 5HT coexist (19–21). 5HT was shown to increase the evoked release of SP-LI from slices of the rat spinal cord via a 5HT type-2 receptor (51). Autoinhibition of 5HT release was also demonstrated in synaptosome preparations probably via a 5HT type-1 receptor (110). The presense of SP could antagonize this autoregulation (111), while others showed an increase in the basal release of [^{3}H], 5HT was also mediated by SP (112). A C-terminal fragment of SP (SP 6–11) potentiates both the release of SP-LI and TRH-LI (84). D) In the posterior part of nucleus accumbens of the rat and in the cat nucleus caudatus-putamen, where DA/CCK-8S coexistence was demonstrated, CCK-8S inhibits the release of DA probably acting on specific autoreceptors (85, 86). DA also regulates its own release via D2 presynaptic receptors. E) In the ventral hippocampus of the rat, where high concentration of galanin specific binding sites was demonstrated on cholinergic nerve terminals (77), galanin inhibits the ACh release (88). In this region ACh release is also modulated by presynaptic muscarinic receptors (89).

ventral spinal cord, the type-1 receptors mediate inhibition of 5HT release (82, 83) while the type-2 receptors seem to mediate enhancement of the release of the coexisting SP (51). Autoreceptors for SP or tachykinin receptors may be present also, since SP fragment 1–6 enhances the release of both TRH and SP-LI (84). Finally autoreceptors for TRH may also participate in the regulation of the release of SP and 5HT in this tissue (Figure 2C).

In vivo experiments and some in vitro experiments with [^{3}H] DA release suggest that DA release is inhibited by CCK-8S in the posterior nucleus accumbens of the rat (Figure 2D; see also 85) and also in the nucleus caudatus-putamen of the cat (86) where DA/CCK-8S coexistence was found (76, 87).

Galanin was shown to coexist with ChAT-LI in septal somata, some of which project to the ventral hippocampus (77). In the ventral hippocampus, but not in the dorsal hippocampus, galanin receptor activation causes a concentration-dependent inhibition of ACh release both in vivo and in vitro (88). ACh release is also under the inhibitory control of muscarinic cholinergic receptors in this structure (Figure 2E; see also 89).

Significance of Cross-Regulation of the Release of Coexisting Neurotransmitters

The presynaptic receptor and especially the autoreceptor mediated control of release, studied in the case of classical neurotransmitters, has shown that these mechanisms play an important role in the number of quanta released for each stimulus or each pulse.

It is not difficult to discern the effects of autoreceptor regulation of the release via several autoreceptors for the coexisting neurotransmitters. Let us examine in detail NE-NPY interactions in regulating the release from a terminal, e.g. at the rat vas deferens. At low frequencies of stimulation (below 5–10 Hz) only NE is released, and the release is regulated by the NE autoreceptors (α_2) which suppress NE release at stray impulses with low frequency. At frequencies higher than 2–4 Hz, release of NE is facilitated (81). The release of NPY is effectively inhibited by the need for higher stimulus frequency and by occupancy of α_2 adrenergic receptor, which is occupied by the released NE and inhibits both NE and NPY release. At even higher frequencies of stimulation, NPY is also released, and it will inhibit the facilitated NE release, putting a ceiling to it (74, 75). The type of stimulation (frequency, pulse pattern) during previous stimulation periods, may alter this picture somewhat, e.g. "burst" type stimulation is more effective to release NPY than NE, depletion of NE by reserpine yields enhanced NPY output (57), the rate of desensitization of NPY and NE receptors may vary, diffusion of NPY (a rather stabile peptide, $t_{1/2}$ = 10–20 min) may affect vicinal varicous terminals. These variables all contribute to the complex signaling at synapses with coexistence.

One may summarize these effects as yielding a high signal-to-noise ratio, where low level release of either NE or of NPY is counteracted by autoinhibition, and real distinct stimuli are required for NE or NE + NPY to appear as signals.

Tissue Levels of Coexisting Neurotransmitters and the Autoreceptors

That autoreceptors for the classical neurotransmitters play a decisive role in determining the tissue levels of classical neurotransmitters in several tissues is well established. For example, the most effective way to lower hippocampal or striatal ACh levels is the use of muscarinic antagonist ligands, which, via blockade of the autoreceptor, will lower the ACh levels by as much as 30% (90). The strong autoreceptor control of release at the level of nerve terminal is of even greater importance with respect to the tissue levels of coexisting peptides. The peptide release, in most studied cases, is under the control of autoreceptors for the coexisting classical neurotransmitter. When this inhibitory control is removed by presynaptic autoreceptor antagonists and release of both classical and peptide neurotransmitter is enhanced, one may rapidly deplete the nerve terminal with respect to the peptide neurotransmitter. The supply of neuropeptide in the nerve terminal is dependent on axonal flow of peptide filled vesicles from the soma, while the classical neurotransmitter is synthesized both in the nerve terminal and in the soma. Thus, at rapid rate of release of both classical neurotransmitters and peptides, the latter store may be depleted. Studies on the effects of chronic drug treatment on tissue levels of coexisting neurotransmitters indicate this.

EFFECTS OF CHRONIC DRUG TREATMENT ON COEXISTING NEUROTRANSMITTERS

Changes in the Tissue Levels of Coexisting Neurotransmitters

Chronic atropine treatment of rats depletes the rat submandibular gland in VIP (91), causing a VIP-receptor supersensitivity reflected by an almost 100% increase in the [^{125}I]VIP binding. ACh levels also fall, but the synthesis of ACh and the uptake of choline are coupled to each other (92), thus ACh stores are never fully depleted. The depletion of VIP may be the consequence of the suspension of muscarinic autoreceptor-mediated control on VIP release, by atropine. In acute experiment, VIP release is increased several hundred percent in the presence of atropine. Under these conditions the replenishment of VIP by axonal transport is too slow. Thus, the classical muscarinic antagonist atropine causes VIP-ergic supersensitivity at sites where VIP and ACh coexist, namely at the submandibular gland and to lesser extent in the cerebral cortex (7, 18).

Chronic treatment of rats with antidepressant drugs (such as zimelidine, a

5HT uptake blocker, and imipramine (93) or amitryptiline (94), monoamine uptake blockers) caused an increase in SP-LI levels (93) and in TRH-LI levels (94) in the spinal cord where 5HT, THR, and SP coexist. It is assumed that a lowered rate of firing, caused by chronic treatment with these drugs, does not affect or slow down the biosynthesis, packaging, and transporting of peptides (SP, TRH) which coexist with 5HT. Thus, there is a build up of SP-LI (93) and of TRH-LI (94) upon chronic treatment with these antidepressant drugs. The same treatment lowers 5HT and 5HIAA levels, probably because the biosynthetic enzymes of 5HT production are sensitive to decreased impulse flow and firing rate (95).

Thus, "monoaminergic drugs," like zimelidine and imipramine, can alter the tissue levels of coexisting peptides (SP, TRH) in a direction opposite to the change caused in 5HT levels. The increased SP levels are reflected in the increased size of releaseable pools and, thus, have a functional synaptic consequence (84).

Chronic treatment with neuroleptic drugs, like haloperidol, chlorpromazine, and clozapine, causes a decrease in DA turnover and was found to cause a build up in CCK-8S levels (96) in the rat midbrain, where DA and CCK-8S coexist (76). These changes in tissue levels of peptides which coexist with the monoamine neurotransmitters may contribute to the therapeutic and side effects of psychoactive drugs designed to affect monoaminergic neurons (Figure 3).

SOME SYNAPTIC INTERACTIONS OF COEXISTING NEUROTRANSMITTERS

It is an important question whether or not a postsynaptic target cell can distinguish between two neurotransmitters which are released from two neurons and two coexisting neurotransmitters released by the same neuron. While we have not been able to address this question directly at a synapse, where the presynaptic element contains coexisting neurotransmitters, it seems clear that postsynaptic elements, in such synapses, can interpret the coexisting neurotransmitters as synergistic signals. Iontophoretic or bath application of the same pair of neurotransmitters in another area, which is innervated by nerves carrying both of them, but not in coexistence, often does not produce any synergism in their effects. Thus, the postsynaptic counterpart of nerves, which release more than one signal-substance, may contain receptors for these, in such special arrangements that synergistic action of the co-released coexisting neurotransmitters is possible.

The first dramatic examples on VIP-potentiation of ACh induced salivation in the cat submandibular gland (9–11) were followed by demonstrations of synergistic effects of VIP and ACh in promoting phosphatidylinositol turn-

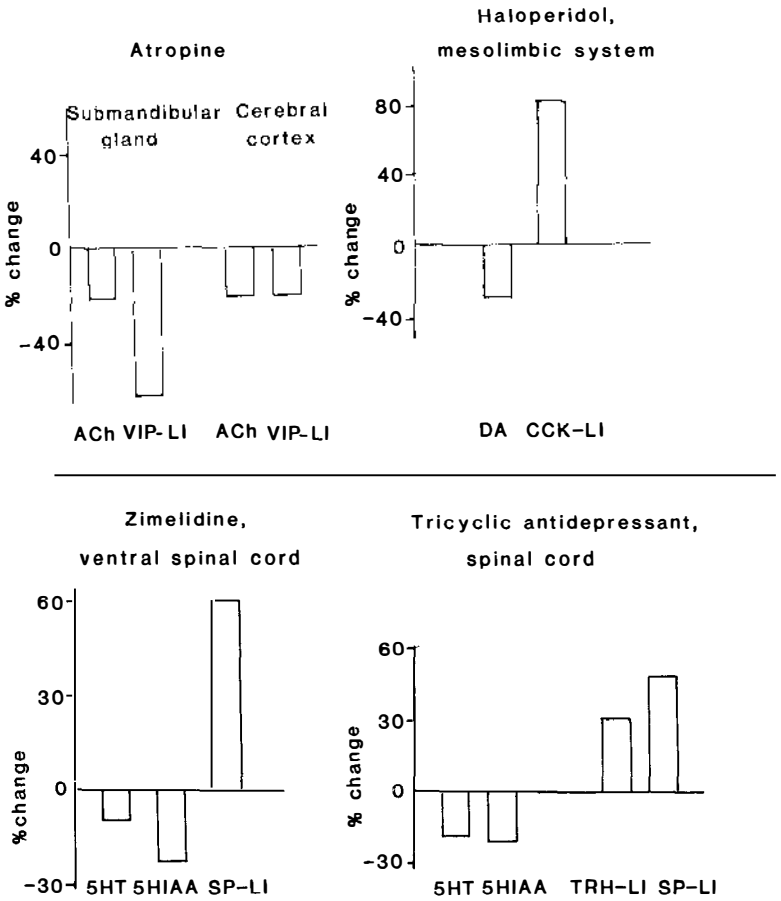


Figure 3 Chronic drug treatment effects on tissue levels of coexisting neurotransmitters. Chronic drug treatment causes differential changes in the tissue levels on coexisting neurotransmitters. Changes in the tissue levels (as compared to saline treated controls) are shown for VIP/ACh as a result of atropine treatment (data from 90, 91); for DA/CCK-8S as a result of haloperidol treatment (data from 96) and for 5HT (and its metabolite 5HIAA)/SP/TRH as a result of zimelidine (data from 93) and tricyclic antidepressant treatment (data from 93, 94).

over in the cerebral cortex (97) (i.e. at sites where ACh and VIP coexist).

CCK-8S was shown to potentiate DA induced hyperlocomotion when the peptide is infused in the posterior nucleus accumbens. The effect of CCK-8S at this site of DA/CCK-8S coexistence could be both pre- and postsynaptic (98, 99).

NPY enhanced the adrenergically mediated and electrically evoked contractile response in the rabbit femoral artery which is innervated by NE/NPY nerves (100, 101).

Synergistic effects of 5HT, TRH, and SP at the spinal cord have also been noted (102, 103).

Mechanisms of Synergistic Effects of Coexisting Neurotransmitters

Some of the synergistic effects stem from the interaction of coexisting neurotransmitters with degrading enzymes, in a way that blocks or slows down degradation of the more efficacious neurotransmitter. This mechanism may explain the CGRP mediated potentiation of some actions of SP, since CGRP acts as an inhibitor of SP-endopeptidase (104). (Other synergistic actions of CGRP-SP in protein extravasation—increase of local blood flow—may involve other postsynaptic events in addition to blockade of SP degradation.)

Receptor-receptor interactions in the postsynaptic membranes have been reported between receptors for coexisting neurotransmitters such as VIP/ACh in the submandibular gland (11), DA/CCK-8S (105), SP/5HT (106), NE/NPY (107). These interactions are demonstrated by changes in the affinity of receptors or in the number of available receptors for the classical neurotransmitter in the presence of neuropeptide. Receptor-receptor interactions may involve other membrane proteins such as G proteins (108) as a common link in the plane of the membrane.

Postsynaptic effects of coexisting neurotransmitters may be complementary in duration, amplitude, or in the second messenger systems utilized by these neurotransmitters.

The coexisting SP and SK for example seem to bind to distinct receptors but both of these tachykinins promote 5HT release, e.g. in the cerebral cortex (M. Solti and T. Bartfai, in preparation). The microscopic distribution of SP and SK receptors and degrading enzymes may be different on the postsynaptic cell, and it is clear that SK has higher stability than SP—so a complementary action in "space and time" may be achieved by corelease of SP and SK.

In some cases the coexisting neurotransmitters represent apparently opposing signals with respect to a given second messenger system. Consider for example NPY and VIP coexistence; VIP activates while NPY inhibits adenylate cyclase activity in the rat cerebral cortex (109).

This apparent contradiction with respect to cAMP synthesis is resolved by taking into account that the inhibition of adenylate cyclase by NPY acting at NPY_1 (Y_1) receptors (109; C. Wahlestedt et al, in preparation) becomes measurable first when the enzyme is activated (e. g. by VIP). Thus, the two coexisting and coreleased neurotransmitters in this case contain both the "on" and "off" signal for cAMP synthesis, producing a sharp, well-defined cAMP signal.

CONCLUSIONS

Coexistence of more than one neurotransmitter in peripheral and central neurons is a widespread phenomenon involving virtually all known neurotransmitter systems. At distinct sites where two or more neurotransmitters occur in the same neuron, they may be released sequentially or simultaneously, depending on frequency of stimulation. The presynaptic receptors and autoreceptors are capable of regulating the release of every coexisting neurotransmitter from the same terminal. There are possibilities for as many types of autoreceptors in a nerve terminal as the terminal contains neurotransmitters. Some of the paradoxical effects of classical pharmacologic agents can be understood within the frame of coexistence (e.g. atropine resistant "cholinergic vasodilatation i.e. VIP"). Synergistic pre- and postsynaptic actions of coexisting neurotransmitters have been noted at sites of coexistence. Chronic drug treatment may affect the tissue levels of coexisting neurotransmitters differentially. Therapeutic and side effects of antidepressant and antipsychotic drugs may involve changes in the neuropeptide signalling involving the neuropeptides which coexist with monoamines in monoaminergic neurons affected by these drugs.

Acknowledgments

The authors are grateful to a number of colleagues for useful discussions and want to express their gratitude to Drs. T. Hökfelt, R. Håkansson, V. Mutt, T. Melander, B. Meister, J. Lundberg, Ö. Nordström, N. Landquist, and L. Diaz Arnesto. This work was supported by grants from the National Institutes of Mental Health, Bethesda, Maryland, and the Swedish Medical Research Council. Dr. P. Serfözö was an exchange visitor, and Dr. G. Fisone is on leave of absence from the Mario Negri Institute, Milan, Italy.

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