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Abstract
Neuropeptides are small protein molecules (composed of 3–100 amino-acid residues) that have been localized to discrete cell populations of central and peripheral neurons. In most instances, they coexist with low-molecular-weight neurotransmitters within the same neurons. At the subcellular level, neuropeptides are selectively stored, singularly or more frequently in combinations, within large granular vesicles. Release occurs through mechanisms different from classical calcium-dependent exocytosis at the synaptic cleft, and thus they account for slow synaptic and/or non-synaptic communication in neurons. Neuropeptide co-storage and coexistence can be observed throughout the central nervous system and are responsible for a series of functional interactions that occur at both pre- and post-synaptic levels. Thus, the subcellular site(s) of storage and sorting mechanisms into different neuronal compartments are crucial to the mode of release and the function of neuropeptides as neuronal messengers.

Keywords Neurotransmission . Synapses . Neuropeptides . Large granular vesicles . Ultrastructure . Colocalization

Abbreviations
5-HT 5-hydroxytryptamine or serotonin
ACTH corticotropin
AGRP agouti gene-related protein
CART cocaine- and amphetamine-regulated transcript
CCK cholecystokinin
CGRP calcitonin gene-related peptide
CNS central nervous system
CRH corticotropin-releasing hormone
DRG dorsal root ganglion
DSIP delta sleep-inducing peptide
GABA γ-amino-butyric acid
GLP-1 glucagon-like peptide 1
GPCR G-protein-coupled receptor
IAPP islet amyloid polypeptide
LGV large granular vesicle
LHRH luteinizing hormone-releasing hormone
α-MSH α-melanocyte-stimulating hormone
NO nitric oxide
NPY neuropeptide tyrosine
PACAP pituitary adenylyl cyclase-activating peptide
PHI peptide histidine isoleucine
PP pancreatic polypeptide
PCR polymerase chain reaction
PNS peripheral nervous system
PYY peptide tyrosine tyrosine
SP substance P

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Introduction

Neurobiologists still face the problems of neuron-to-neuron communication and of the organization of neuronal net-works; this has been further complicated by the recognition of neuron-to-glia signaling. In the 1960s, when the concept of neurochemical transmission became universally accepted, the complex computational operations of the brain were commonly believed ultimately to require simply one excitatory and one inhibitory transmitter in order to take place. However, in the late 1970s and the 1980s, several tens of small peptide molecules, commonly referred to as neuropeptides, were localized by immunocytochemistry to discrete cell populations of the central (CNS) and peripheral (PNS) nervous systems, and the concept of "chemical neuroanatomy", originally developed by Hökfelt and coworkers (1980, 1984) entered the scene of neurobiology. Thus, it became clear that neurons could produce and utilize more than a single molecule to exchange their information, and the concept of the presence of multiple messenger molecules within the nerve cell was fully established (Hökfelt et al. 1987). Initially, difficulties were encountered with respect to receptor identification (Hershey and Krause 1990; Tanaka et al. 1990; Yokota et al. 1989) and the lack of effective pharmacological agonists/antagonists (see Bock et al. 1989; Folkers et al. 1982; Wang and Shoenfeld 1987). More recently, the generation of transgenic models (see De Felipe et al. 1998; Schwartz and Epelbaum 1998; Zimmer et al. 1998; Wang and Dockray 1999; Woolf et al. 1998) and the development of nonpeptide agonist/antagonists (see Calo' et al. 2000; Doods et al. 1996; Folkers et al. 1990; Hill 2000; Snider et al. 1991; Yaksh 1999) have proved to be invaluable tools for improving the dissection of the functional role of neuropeptides in the brain and spinal cord. This review focuses on the anatomical and functional findings related to neuropeptide distribution in mammalian CNS and centers upon their importance in the transfer of neuronal information at synaptic and non-synaptic sites under physiological conditions.

Historical notes

In the 1950s, Guillemin, Schally, Vale, Arimura, and collaborators were the first to demonstrate that most hypothalamic releasing and inhibiting hormones could be chemically identified as small peptides (see Guillemin 2005). Work on hypothalamic hormones led to the development of the concept of neurosecretion, which was, at that time, considered to be a peculiarity of the magnocellular neurons in the supraoptic and paraventricular nuclei. Other peptides were detected in the brain in the 1960s and 1970s, such as substance P (SP) originally discovered by von Euler and Gaddum in 1931 (Nicoll et al. 1980), and the tachykinins/bradykinins extracted from the frog skin by Erspamer and co-workers (Erspamer 1981; Renda et al. 1989), the opioids (Burgen et al. 1980; Hughes et al. 1975), and the gut hormone cholecystokinin (CCK; Mutt 1979, 1980b). Peptide histidine isoleucine (PHI), peptide tyrosine tyrosine (PYY), and neuropeptide tyrosine (NPY; Mutt 1980a; Polak and Bloom 1984; Tatemoto et al. 1982) were discovered in the 1980s, after development of more sensitive procedures of extraction. In parallel, the existence of calcitonin generelated peptide (CGRP) was inferred from studies on alternative mRNA splicing of the calcitonin gene by Rosenfeld, Amara, and co-workers (Amara et al. 1982, 1985). In the 1990s, the identification of several new neuropeptides, such as nocistatin (Okuda-Ashitaka and Ito 2000), nociceptin/orphanin FQ (Darland et al. 1998; Schlicker and Morari 2000), prolactin-releasing peptide (Hinuma et al. 1998, 1999), urotensin II (Coulouarn et al. 1999), and ghrelin (Kojima et al. 2001; Kojima and Kangawa 2005) relied upon the initial discovery of a series of orphan receptors, i.e., receptors for which the endogenous ligand was unknown (Civelli et al. 2001). Another strategy used was that of directional tag polymerase chain reaction (PCR) subtractive hybridization; this led to the identification of the hypocretins/orexins (Mondal et al. 2000).

Basic characteristics of neuropeptides

Neuropeptides are small protein molecules composed of 3–100 amino-acid residues. Most neuropeptides influence membrane excitability, but this should perhaps not be thought of as their main biological action. Peptides have many other effects, such the regulation of gene transcription (Landgraf and Neumann 2004), local blood flow (Cauli et al. 2004), synaptogenesis, and glial cell architecture (Theodosis et al. 1986). With regard to the role of neuropeptides in cell-to-cell communication, the first thing to take into consideration is that they are about 50 times larger than

low-molecular-weight “classical” neurotransmitters. As a consequence, neuropeptides possess several more recognition sites for receptors than smaller neurotransmitter molecules. This is reflected by the higher binding affinity (about 1000×; with values in nmol/l versus μmol/l) and selectivity of neuropeptide molecules for their own receptors compared with those of classical neurotransmitters. Thus, they are capable of eliciting a biological effect when released in lower quantities. Neuropeptide half-lives in the extracellular space are long: for example, the half-lives of oxytocin and vasopressin are about 20 min in the brain compared with just 2 min in the blood (Mens et al. 1983). Large neuropeptide molecules diffuse and bind more slowly, but more tightly, to receptors. These latter are usually seven-transmembrane-region G-protein-coupled receptors (GPCRs). Neuropeptide GPCRs are internalized upon activation as a desensitization mechanism. For example, the neurokinin 1 receptor (the preferred SP receptor) is internalized 5 min after agonist activation and is then recycled and restored to the cell membrane within 30 min (Mantyh et al. 1995). The common existence of a combination of neuropeptides and classical neurotransmitters in neurons (see below) enables fast (2–5 ms) and slow (100–500 ms) synaptic communication to take place.

Distribution of neuropeptides in CNS

A systematic map of the sites of distribution of most neuropeptides in central neurons has been built up over the last few decades. With the continuous addition of new members, attempts have been made to group neuropeptides into families based upon various criteria, such as their anatomical localization, biological function, sequence homology, and derivation from a common precursor (Table 1).

Of relevance here, peptides may be expressed in three main modes in CNS and peripheral tissues (Hökfelt et al. 2000b). In the type 1 mode, substantial levels are synthesized and stored under physiological conditions, such is the case for SP and CGRP in primary sensory neurons (Charnay et al. 1983; Cuello et al. 1976; Dalsgaard et al. 989; Gibson et al. 1984; Hökfelt et al. 1987; Fig. 1a,b), galanin in the hypothalamus (Melander et al. 1986; Rokaeus 1987; Skofitsch and Jacobowitz 1985), VIP (Emson and Lindvall 2001; Giachetti et al. 1977), and NPY (Aoki and Pickel 1990; Gray and Morley 1986; Jones and Hendry 1986) in the cerebral cortex. In the type 2 mode, low levels are found under normal conditions, but peptide expression is up-regulated following appropriate stimuli, e.g., VIP, galanin, or NPY in primary sensory neurons (Hökfelt et al. 1994; Ma and Bisby 1998; Verge et al. 1995). In the type 3 mode, peptides are expressed transiently during development, such as in the case of somatostatin in central neurons (Fitzpatrick-McElligott et al. 1991; Maubert et al. 1994; Yew and Chan 1999), galanin in sensory neurons (Hökfelt et al. 1994; Wynick et al. 1998), CGRP in motorneurons (Gibson and Clowry 1999; Johnson et al. 1992; Monks et al. 1999), SP in the embryonic spinal cord (Charlton and Helke 1986; Delander et al. 1997; Ribeiro-Da-Silva and Hökfelt 2000; Yamashita et al. 1990), and secretin in neurons of the raphe nuclei (Lossi et al. 2004; Fig. 1c,d). The type 2 and 3 modes demonstrate the existence of a plasticity in peptide expression under normal and experimental conditions that is related to their pleiotropic function.

Coexistence of neuropeptides and other neurotransmitters in neurons

Coexistence, the concurrent presence of two or more transmitters in a single neuron, is now regarded as a common feature of central and peripheral neurons (Hökfelt 1991; Lundberg 1996; Merighi 2002). Neuropeptides coexist with other neuropeptides (Table 2), low-molecularweight fast-acting neurotransmitters (Table 3), the gaseous transmitter NO (Dun et al. 1994; Yang et al. 2000; Xu and Hökfelt 1997), and certain neurotrophins (Salio et al. 2005). As a rule, neurons produce a combination of one (or more) low-molecular-weight transmitter(s) and one (or more) high- molecular-weight neuropeptide(s). One remarkable exception seems to be represented by the oxytocin/vasopressin magnocellular neurons that contain a complex cocktail of peptides, but apparently no low-molecular-weight transmitters. When a neuropeptide coexists with a classical transmitter, the latter is generally believed to be the principal messenger. A possible exception is represented by the GABA-synthesizing CRH neurons in the paraventricular hypothalamic nucleus (Meister and Hökfelt 1988), where the amino-acid seems to be a modulator of the CRH action.

Table 1 Mammalian neuropeptides in the central nervous system (CNS; ACTH corticotropin, AGRP agouti gene-related protein, CART cocaine- and amphetamine-regulated transcript, CCK cholecystokinin, DSIP delta sleep-inducing peptide, CGRP calcitonin gene-related peptide, CRH corticotropin-releasing hormone, GHRH growthfactor-releasing hormone, GLP-1 glucagon-like peptide 1, 5-HTmoduline 5-hydroxytryptamine-moduline, IAPP amylin or islet amyloid polypeptide, LHRH luteinizing hormone-releasing hormone, *α*-MSH α-melanocyte-stimulating hormone, NPY neuropeptide tyrosine, PACAP pituitary adenylyl cyclase-activating polypeptide, PHI peptide histidine isoleucine, PP pancreatic polypeptide, PYY peptide tyrosine tyrosine, SP substance P, TRH thyrotropin-releasing hormone, VIP vasoactive intestinal polypeptide)

Classified peptides	**Unclassified peptides**
Hypothalamic peptides	ACTH, AGRP, Apelin, Brain natriuretic peptide, CGRP, CCK, CART/peptide, Corticostatin, DSIP, Endomorphin-1 and -2, Glucagon-like peptide 1, Galanin, 5-HT-moduline, Hypocretins/orexins, IAPP, MCH, Metastin, α-MSH, Neuropeptide B, Neuropeptide FF, Neuropeptide S, Neuropeptide W, Neurotensin, Nociceptin/orphanin FQ, Nocistatin, Parathyroid hormone-related protein, Prolactin-releasing peptide, Secretin, Secretoneurin, Urocortin
CRH, GHRH, Ghrelin, LHRH, Oxytocin, Somatostatin, TRH, Vasopressin	
NPY and related peptides	
NPY, PP, PYY	
Opioid peptides	
Dynorphin, Met-enkephalin, Leu-enkephalin	
Tachykinins	
Neurokinin A, Neurokinin B, Neuropeptide K, SP	
VIP-glucagon family	
GLP-1, PHI, PACAP, VIP	

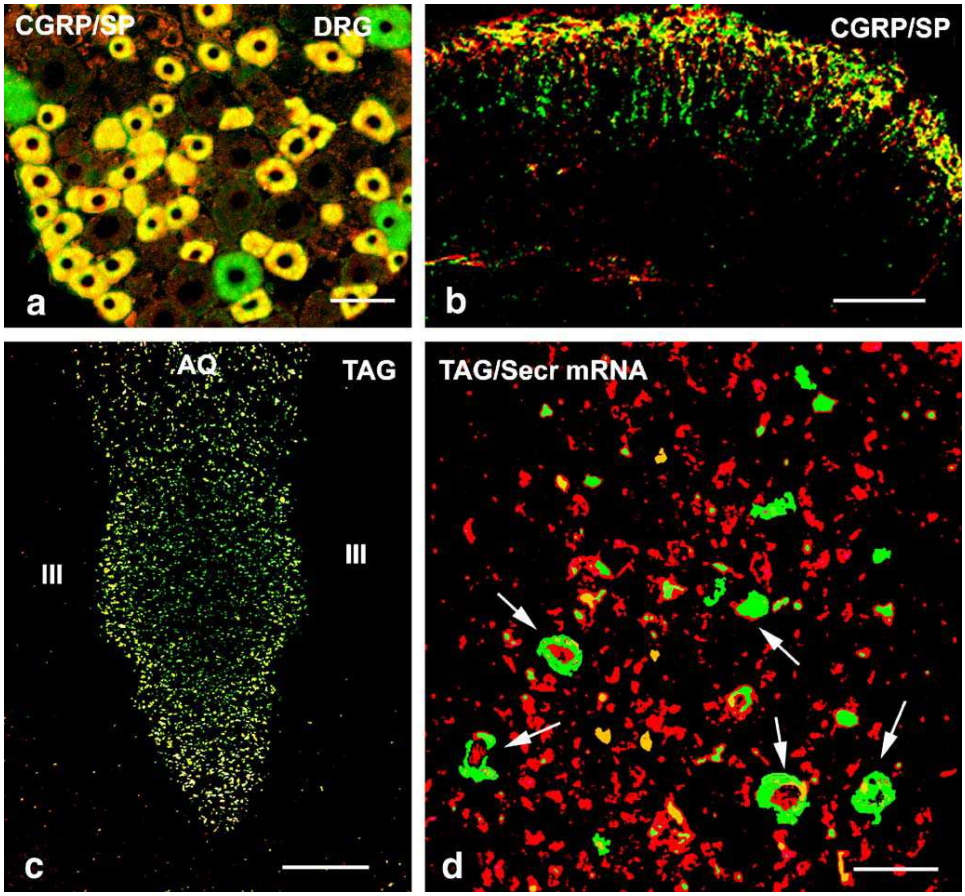

Fig. 1 Examples of two of the three main modes of expression of neuropeptides. a, b Calcitonin gene-related peptide (CGRP, green) and substance P (SP, red) are normally expressed at high levels (type 1 mode) in dorsal root ganglion (DRG) neurons (a) and spinal cord (b). The two peptides coexist in most of the DRG neurons and their fibers within the superficial dorsal horn (yellow). Dual immunofluorescence. c, d Secretin is transiently expressed during development (type 3 mode) in neurons of the raphe nuclei of the mesencephalon. To amplify peptide expression and thus enable its detection, transgenic mice were generated in which the expression of the large T antigen of the SV40 virus (TAG) was driven by the secretin gene promoter. When the gene was turned on during development, the population of secretin-expressing neurons was amplified by the transforming properties of TAG. A well-defined cluster of TAG-immunoreactive neurons (c) is detected below the cerebral aqueduct (AQ) at the midline between the two somatic nuclei of the oculomotor nerve (III). The image in d has been elaborated with Photoshop software to display in pseudocolors the immunocytochemical localization of TAG (red) and secretin mRNA (Secr mRNA, green). A few neurons (arrows) clearly show nuclear TAG immunoreactivity and a positive in situ hybridization signal. For further details, see Lossi et al. (2004). Bars 50 μm (a, d), 200 μm (b), 400 μm (c).

Table 2 Neuropeptide coexistence in CNS (5-HT 5-hydroxytryptamine, CCK cholecystokinin, CGRP calcitonin gene-related peptide, CRH corticotropin-releasing hormone, GABA γ-amino-butyric acid, NPY neuropeptide tyrosine, PACAP pituitary adenylyl cyclase-activating polypeptide, SP substance P, VIP vasoactive intestinal polypeptide)

Neuropeptide + low-molecular-weight transmitter	**CNS area**	**References**
SP+5-HT	Medulla oblongata	Arvidsson et al. 1994; Pelletier et al. 1981
	Dorsal raphe nuclei	Arvidsson et al. 1990a; Henry and Manaker 1998;Marson 1989; Millhorn et al. 1989; Thor and Helke 1989
Enkephalin+5-HT	Dorsal raphe nuclei	Henry and Manaker 1998; Mijnster et al. 1997;

		Millhorn et al. 1989; Ward and Dorsa 1996
Galanin+5-HT	Dorsal raphe nuclei	Hökfelt et al. 2000a
CGRP+5-HT	Medulla oblongata	Arvidsson et al. 1990b
Secretin+5-HT	Dorsal raphe nuclei	Lossi et al. 2004
SP+noradrenaline	Medulla oblongata	Halliday et al. 1988
NPY+noradrenaline	Hypothalamus	Leibowitz 1989
Galanin+noradrenaline	Cerebral cortex, Hippocampus	Hökfelt et al. 2000b
Galanin+acetylcholine	Basal forebrain	Crawley 1993; Miller et al. 1998
	Hippocampus	Chan-Palay 1988; Consolo et al. 1990; Crawley 1990; Miller et al. 1998
Somatostatin+acetylcholine	Hippocampus	van der Zee et al. 1991
CGRP+acetylcholine	Vestibular nuclei	Ohno et al. 1991; Safieddine et al. 1997
SP+aspartate	Spinal cord	Merighi et al. 1991
NPY+aspartate	Arcuate nucleus	Zsarnovszky et al. 2000
Enkephalin+glutamate	Locus coeruleus	van Bockstaele et al. 2000
PACAP+glutamate	Retinal ganglion cells	Hannibal et al. 2000
SP+glutamate	Spinal cord	De Biasi and Rustioni 1988, 1991; Merighi et al. 1991
Enkephalin+GABA	Striatum	Penny et al. 1986; Zahm et al. 1985
	Spinal cord	Laing et al. 1994; van Bockstaele et al. 2000; Zahm et al. 1985
Somatostatin+GABA	Visual cortex	Lin et al. 1986
	Entorhinal cortex	Wouterlood and Pothuizen 2000
	Suprachiasmatic nucleus	Buijs et al. 1995
	Amygdala	Mcdonald and Pearson 1989
CCK-8/VIP+GABA	Hippocampus, dentate gyrus	Kosaka et al. 1985
	Amygdala	Mcdonald and Pearson 1989
	Suprachiasmatic nucleus	Buijs et al. 1995
CGRP+GABA	Cerebellum	Kawai et al. 1987
NPY+GABA	Arcuate nucleus	Horvath et al. 1997
	Amygdala	Mcdonald and Pearson 1989
	Spinal cord	Horvath et al. 1997; Rowan et al. 1993
SP+GABA	Hypothalamus	Rowan et al. 1993; Nitsch and Leranth 1994
	Retinal ganglion cells	Caruso et al. 1990
Galanin+GABA	Spinal cord	Simmons et al. 1995
Somatostatin+GABA	Entorhinal cortex	Wouterlood and Pothuizen 2000
CRH+GABA	Hippocampus	Yan et al. 1998
Vasopressin+GABA	Suprachiasmatic nucleus	Buijs et al. 1995

Table 3 Neuropeptide co-storage in CNS (CCK cholecystokinin, DSIP delta sleep-inducing peptide, CGRP calcitonin gene-related peptide, CRH corticotropin-releasing hormone, LHRH luteinizing hormone-releasing hormone, SP substance P)

Co-stored neuropeptides	**CNS area**	**References**
Tachykinins +CGRP	Spinal cord	Aimar et al. 1998; Merighi et al. 1991, 1992; Ribeiro-Da-Silva 1995
	Central nucleus of amygdala	Salio et al., in preparation
Tachykinins+enkephalin	Spinal cord	Merighi et al. 1989
Galanin+SP	Spinal cord	Hökfelt et al. 1993
Galanin+SP+neurotensin	Spinal cord	Zhang et al. 1993
Oxytocin+vasopressin (only in certain experimental conditions)		
	Supraoptic nucleus of hypothalamus	Glasgow et al. 1999; Jirikowski et al. 1991; Mezey and Kiss 1991
CRH+vasopressin	Paraventricular nucleus of hypothalamus	Whitnall et al. 1985a; Whitnall 1993

	Median eminence of hypothalamus	Hisano et al. 1987; Whitnall et al. 1985b
Oxytocin+enkephalin	Magnocellular neurons of hypothalamus	Hisano et al. 1986; Rossier et al. 1983
Vasopressin+dynorphin	Magnocellular neurons of hypothalamus	Whitnall et al. 1983
Oxytocin+CCK	Hypothalamus	Bondy et al. 1989
Oxytocin+CRH	Hypothalamus	Bondy et al. 1989
CCK+CRH	Median eminence	Juaneda et al. 1999
LHRH+galanin	Hypothalamus	Liposits et al. 1995
LHRH+DSIP	Median eminence of hypothalamus	Vallet et al. 1991

Subcellular segregation of neuropeptides and low-molecular-weight neurotransmitters at synapses

Identification of the site of storage of neuropeptides within the nerve cells remains crucial to the understanding of their mechanism of action at synaptic (or non-synaptic) sites and interaction with other transmitters. From the very beginning, low-molecular-weight classical neurotransmitters, such as acetylcholine, biogenic amines, and amino-acids, have been found to be present together with peptides in several areas of the CNS (Chan-Palay and Palay 1984; Cuello 1982; Hökfelt et al. 1987). From initial studies, the two classes of transmitter molecules were believed to be stored in different subcellular compartments, i.e., the small clear synaptic vesicles (SSVs) for classical transmitters and large granular vesicles (LGVs) for the neuropeptides (Fried 1982; Fried et al. 1985; Thureson-Klein et al. 1988; Zhu et al. 1986). In the past, the debate regarding such a compartmentalization was left open, since it could not be excluded that, at least in some cases, LGVs also contained some more-conventional transmitters, although peptides never seemed to be found in small clear vesicles (Fried 1982; Pelletier et al. 1981). Nowadays, the concept that LGVs are the sole site of neuropeptide storage is widely established. The histological demonstration of differential subcellular sites of storage for neuropeptides and low-molecular-weight neurotransmitters is consistent with the possibility that they are selectively released upon specific stimuli. On the other hand, coexisting peptides, even when synthesized from different mRNAs, are usually stored together in LGVs, at least in the type 1 mode of peptide distribution. This has a series of functional implications, since the differential release of costored peptides is most readily accomplished by regulating their relative proportions of synthesis (Dalkin et al. 1989; Fisher et al. 1988; Kessler 1985). From this perspective, the study of tachykinin and CGRP co-storage (in particular within primary sensory neurons) has been highly informative with regard to the way that multiple neuropeptides are stored in neurons. About 20 years ago, pioneering immunogold labeling methods localized tachykinin and CGRP to individual LGVs in neuronal cell bodies of the dorsal root ganglia (DRGs; Merighi et al. 1988). Two years earlier, SP- and CGRP-containing LGVs had been described in sensory peripheral terminals supplying the blood vessels (Gulbenkian et al. 1986). Later, ultrastructural SP/CGRP co-storage was observed in terminals supplying the carotid body (Heym and Kummer 1989) and in central terminals within the spinal dorsal horn (Merighi et al. 1991, 1992; Ribeiro-Da-Silva 1995). Virtually all LGVs were multiple/ dually labeled in both central and peripheral projections, whereas double (multiple)-labeled LGVs were relatively rare in the cell body. Although the central and peripheral projections of individual DRG neurons could not be followed, these observations showed that: (1) a mixture of neuropeptides was packed within individual LGVs, i.e., when multiple peptides were produced by one neuron, they were not selectively packaged in different LGV subpopulations; (2) LGVs (and therefore the neuropeptides packed therein) were found at both central and peripheral projections of the DRG neurons. If these points can be generalized, there is presumably no selective transport to functionally different neuronal processes (the axons and the dendrites) and/or to different branches of the same process. In neuronal cell body, LGVs containing just one peptide can be regarded as immature vesicles that will probably incorporate the other peptide(s) of the cocktail before being transported to terminals (Merighi 2002). Another question that has been left open until recently concerns the quantitative ratio of peptides packed within individual LGVs. However, we have obtained evidence for a surprisingly constant ratio of 1:1 for co-stored CGRP/SP in various types of central terminals (Salio et al., in preparation). As previously mentioned, this ratio might be regulated at the level of neuropeptide synthesis, before packaging in the trans-Golgi network (Dalkin et al. 1989; Fisher et al. 1988; Kessler 1985).

Neuropeptides and synaptic transmission

Despite the enormous amount of literature on neuropeptide distribution in mammals, much of our knowledge about neuropeptide function comes from studies in invertebrates. Indeed, the demonstration of the presence of multiple messengers in neurons is far easier than to establish their physiological role or even to show that they have any biological activity. Moreover, although we have built up an organic framework to describe the function(s) of individual neuropeptides at synapses and/or non-synaptic sites, we know relatively little about the functional interactions and the control of release from central synapses of co-stored neuropeptides. The existence of synapses can be unequivocally demonstrated only by electron microscopy. Ironically, however, ultrastructural studies have also shown the existence of non-synaptic transmission. The identification of gases as interneuronal signals (Baranano et al. 2001) or the modulation of neuronal function by lipophilic substances (Baulieu et al. 2001) has left no doubts regarding the existence of non-synaptic information transfer in CNS. It has been more difficult to accept that vesicle-stored transmitters can be released following quantal mechanisms and operate at distant non-synaptic sites (Zoli et al. 1999). This notion applies not only to neuropeptides, but also to low molecular-weight classical neurotransmitters (Liu et al. 1996; Rahman et al. 1999; Reimer et al. 1998). Further complexity is added by the existence of leaky synapses, in which transmitters spill out from the pre-synaptic element and act at a distance on neighboring synapses (Isaac et al. 1999; Nicoll and Malenka 1999).

Co-existence of neuropeptides and fast-acting neurotransmitters

Irrespective of the finding that they may be acting at sites other than their own post-synaptic membrane in strictu senso, neurotransmitters are consistently packaged in vesicles. Two morphological types of vesicles are present at synapses (Fig. 2). The most abundant SSVs are known to accumulate fast-acting low-molecular-weight neurotransmitters. The less frequently observed LGVs store neuropeptides. The distribution of SSVs and LGVs in typical CNS axo-dendritic synapses displays several peculiarities. SSVs occupy a variable, but usually large, fraction of the axon terminal, and some are docked at the pre-synaptic grid (Peters et al. 1976). LGVs, on the other hand, are detected away from the pre-synaptic membrane, singularly or in clusters of variable numbers. Moreover, electron microscopy has provided evidence for the release of LGVs at plasma membranes in the absence of synaptic specializations (Buma 1988; De Camilli and Jahn 1990; Karhunen et al. 2001; Zhu et al. 1986). The major functional implication of such an arrangement is that fast- and slow-acting transmitters may be selectively released, upon activation of different cellular pathways. Early evidence was obtained showing that the release of coexisting peptides and classical neurotransmitters could be differential and dependent on the frequency and pattern of firing (Hökfelt 1991; Hökfelt et al. 2000b; Martinez-Rodriguez and Martinez-Murillo 1994). In general terms, neuropeptide release is triggered by a small increase in the intracellular Ca2+ concentration, whereas release of transmitter amino-acids from SSVs requires a rise of intracellular Ca2+ concentration in the proximity of the Ca2+ channels at synapses. The delay in response to fast-acting transmitters (about 1–3 ms) is a close reflection of their mode of discharge from SSVs and offers an explanation of the reason that neuropeptide release can occur independently from synapses, such as is the case for SP at DRG neuronal somata (Huang and Neher 1996). Among the consequence of the existence of selective mechanisms of release for coexisting peptides and classical transmitters is the possibility that long-lasting intracellular Ca2+ elevation may cause the release of neuropeptides to outlast the duration of electrical activity, thus uncoupling release from spiking (Kits et al. 1997). Therefore, in terminals with both types of vesicles, a focal increase in Ca2+ at the synaptic membrane tends to discharge SSVs, whereas a more diffuse elevation of Ca2+ inside the terminal favors the release of LGVs (Verhage et al. 1991). Even upon prolonged stimulation, not all vesicles at synapses can unload their transmitters. A variable fraction of SSVs (Harata et al. 2001) and LGVs (Kits and Mansvelder 2000) is readily releasable, but the remaining vesicles, forming the reserve pool, need further steps to become competent.

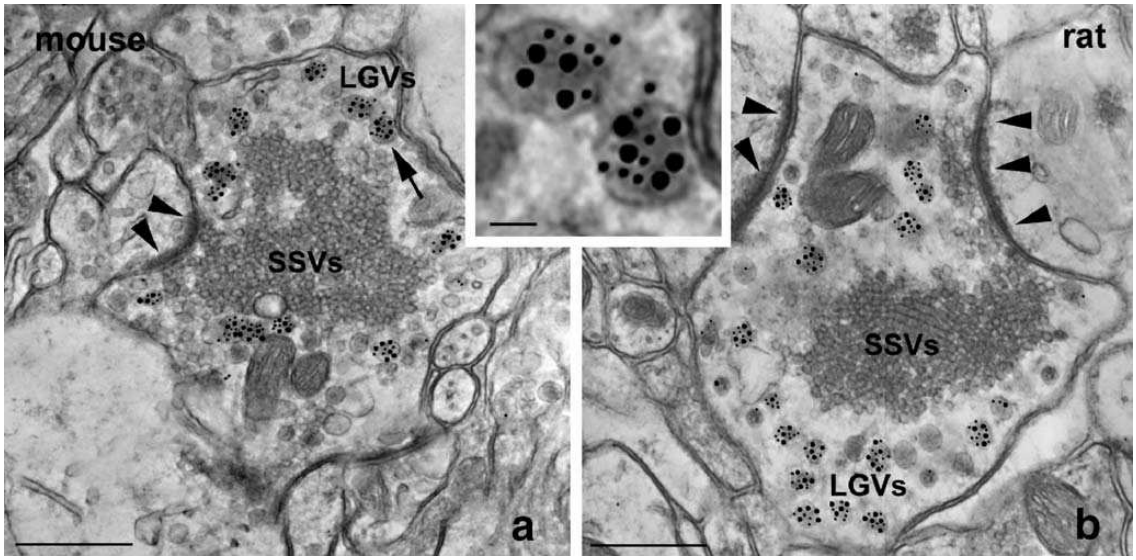

Fig. 2 Subcellular localization of neuropeptides at central synapses. a, b CGRP/SP immunogold labeling of central terminals of DRG neurons in the substantia gelatinosa of the mouse (a) and rat (b) spinal cord. A multisynaptic complex (glomerulus) is shown to contain both small synaptic vesicles (SSVs) and large granular dense-cored vesicles (LGVs). The latter are the sole site of storage of the two neuropeptides, whereas SSVs are consistently unlabeled. Note also that virtually all LGVs are double-labeled and never cluster at the synaptic specialization (arrowheads), unlike SSVs. Insert: Higher magnification of the co-storage of CGRP (large 20-nm gold) and SP (small 10-nm gold) within the two LGVs indicated by the arrow in a. The LGV right is apposed to the axolemma, but there is no evidence of membrane fusion or release of immunoreactive material within the exracellular space. Bars 500 nm (insert 50 nm)

Two different mechanisms of transmitter emptying have been shown to occur in SSVs (Harata et al. 2001) and LGVs (Artalejo et al. 1998). These include the slower classical exocytosis with complete fusion of the vesicle to the plasma membrane or a faster mechanism whereby vesicles come in close proximity to the membrane and, with the formation of a transient pore, release part of their transmitter content by “kiss and run” (Artalejo et al. 1998; Tsuboi and Rutter 2003). The latter mechanism may be particularly important for SSVs in diminutive synapses (Harata et al. 2001). The transient pore mechanism for LGVs (Artalejo et al. 1998; Elhamdani et al. 2001) would allow the quick simultaneous passage of amine transmitters and perhaps other small molecules (present in LGVs in addition to neuropeptides) into the extracellular fluid. On the other hand, macromolecules contained within LGVs (neuropeptides) remain trapped inside the retrievable vesicle. Therefore, release of most neuropeptides from the LGVs is unlikely to operate through kiss and run for several reasons. These include the larger size of the peptides relative to the transient pore (Barg et al. 2002) and the slow emptying of peptide content from LGVs upon exocytosis (Balkowiec and Katz 2000; Lessmann et al. 2003; Brigadski et al. 2005). Moreover, simultaneous capacitance measurements and confocal imaging have shown that peptide release by this mechanism is negligible, whereas complete vesicle fusion is usually required (Barg et al. 2002), through a mechanism involving a priming step (Kits and Mansvelder 2000), followed by retrieval of the vesicle as a coated vesicle (Artalejo et al. 1998; Elhamdani et al. 2001). A divergence in this respect between peptidecontaining LGVs and amine-containing LGVs may merely be additional to the several difference between these two classes of neurotransmitters. Indeed, neuropeptides do not have a known re-uptake mechanism, as opposed to biogenic amines, so that there is no way locally to refill the peptidecontaining LGVs after empting. Moreover, they are synthesized at the rough endoplasmic reticulum in the neuronal perikarya, but not in axon terminals, whereas mines can also be synthesized inside LGVs.

Co-storage of neuropeptides in LGVs

Co-stored neuropeptides are packaged together within LGVs. When a neuron produces more than a single neuropeptide (as appears to be the rule), they can be released all at once at all processes. Alternatively or in addition, individual neuropeptides can be theoretically liberated singularly or in

different combinations at different processes. In Aplysia, the various neuropeptides derived from a common pro-hormone seem to be targeted to different neuronal processes (Klumperman et al. 1996; Sossin and Scheller 1991; Sossin et al. 1990). However, few studies in mammals exist, and the majority of these has been carried out on isolated neurons, with the exception of the anecdotal ultrastructural results on CGRP/SP co-storage in primary sensory neurons. Thus, all co-stored neuropeptides can be reasonably assumed to be released together upon exocytosis (Harling et al. 1991; Holst et al. 1987). For example, the co-release of CGRP and SP (and other tachykinins) has been demonstrated, and this occurs at both central and peripheral endings of the DRG neurons (Arvieu et al. 1996; Collin et al. 1993, 1994; Garry and Hargreaves 1992; Garry et al. 1994; Maggi et al. 1988; Saria et al. 1986; Takano et al. 1993; Vanner 1994). Moreover, if the co-release of co-stored neuropeptides is indeed the rule, then it should occur from any neuronal process containing LGVs, although this latter issue needs further clarification. The major functional implication is that co-released peptides probably act together in determining the response of target cells (Bean et al. 1994). As previously mentioned, neuropeptide release displays some peculiar features with respect to the exocytosis of low-molecular-weight neurotransmitters. After bulk analysis of the LGV compartment in central terminals, we have recently demonstrated that SP and CGRP are stored in a stoichiometric ratio of 1:1 within individual LGVs (Salio et al. in preparation). A differential release of co-stored peptides (if indeed this occurs in vivo) would more likely rely on mechanisms different from those that apply to costored biogenic amines and/or co-existing low-molecularweight neurotransmitters. From this perspective, the relative rate of peptide dissolution from the LGV core might be of primary relevance, since this appears to be the critical determinant of the speed of peptide secretion in vitro (Brigadski et al. 2005).

Significance of neuropeptide co-existence and co-storage at synapses

The advantages of the co-existence of multiple neurotransmitters in neurons have been extensively reviewed (Brezina and Weiss 1997; Hökfelt et al. 2000b; Merighi 2002; Nusbaum et al. 2001).

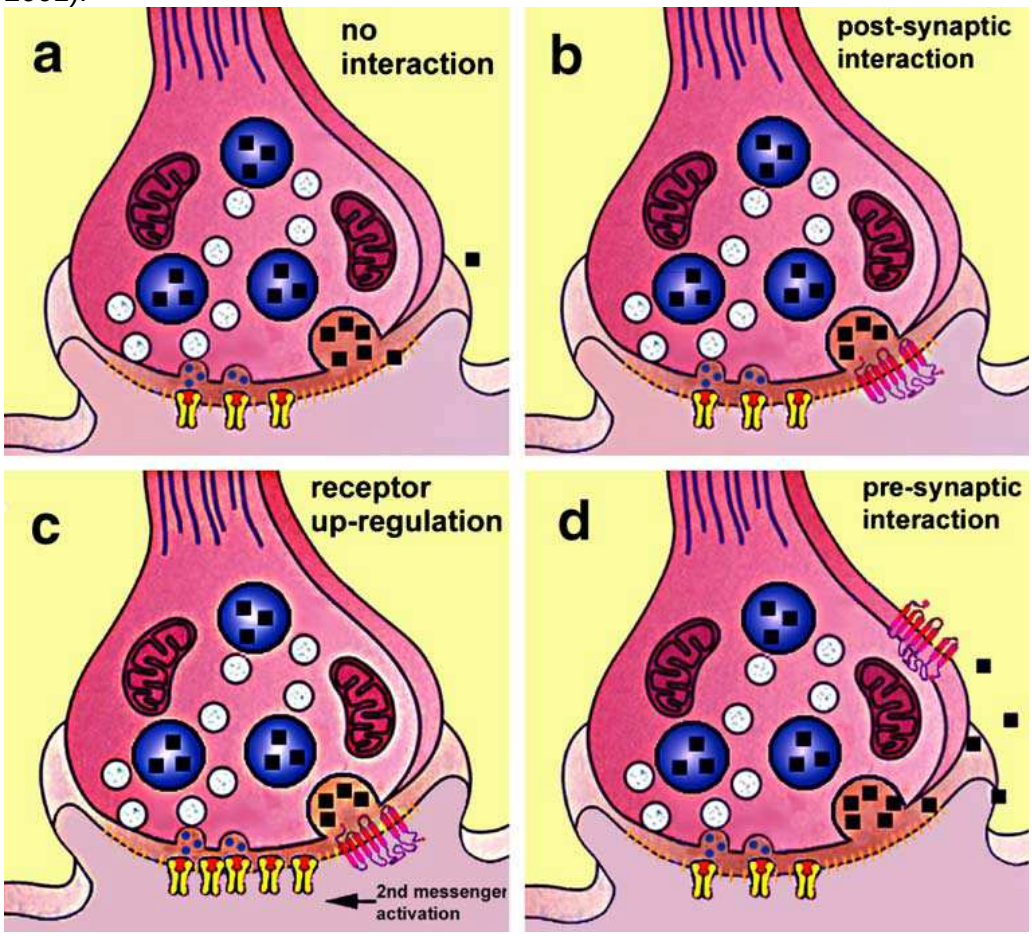

Fig. 3 Representation of possible interactions of low-molecular-weight transmitters and neuropeptides at central synapses. For simplicity, only one neuropeptide is shown (black squares)

within LGVs (blue). The neurotransmitter content of SSVs is represented by small light-blue dots. a The post-synaptic membrane lacks neuropeptide GPCRs, and thus no interaction occurs with the fast neurotransmitter acting on its specific ligand-gated receptors. b Post-synaptic interactions are made possible by the concurrent presence of the receptors for the two co-transmitters. c The neuropeptide binds to its own GPCRs and leads to increased expression of fast transmitter receptors at the post-synaptic membrane. d Activation of presynaptic neuropeptide autoreceptors leads to an increase of the release of one or both co-transmitters

In brief, neuropeptides have a wide diversity of direct or modulatory effects on the electrical responses of target cells, in addition to trophic effects. When they are co-released with other neurotransmitters, the wealth of responses of target neurons increases dramatically (Kupfermann 1991). Neurotransmitters produced and released by a single neuron are often defined as co-transmitters (Fig. 3). However, it is probably unsafe to consider that co-transmitters must display some kind of interaction simply because they are co-released, even though such a co-release occurs under physiological conditions. In the light of the mode of storage and release of neuropeptides at synaptic and non-synaptic sites, fast and slow-acting co-transmitters can act on completely independent targets (Fig. 3a) and do not interact at all (Yang et al. 1996). However, there is a general consensus that, when multiple neurotransmitters are released within the extracellular space, they usually display some type of interactive actions, irrespective of the finding that such a release occurs from the same neuron, i.e., they are true cotransmitters, r from separate sources. The simplest mode of the interaction of two (or more) neurotransmitters occurs when two (or more) distinct receptor complexes are present in the (post-synaptic) membrane of target cells, and a receptor-receptor interaction occurs. When neuropeptides coexist with low-molecular-weight neurotransmitters, the neuropeptide(s) usually act(s) on GPCRs, whereas the low-molecular-weight transmitter generally opens a ligand-gated ion channel (Fig. 3b). The low-molecularweight transmitter is generally the principal messenger, and the neuropeptide interacts with it by altering the ion channel gating properties or its response to further signals. This occurs by direct operation on the receptor complex or by the activation of second messenger systems that, in turn, act on the receptor complex. Hence, one neurotransmitter may alter the number of receptors (Fig. 3c) or the affinity of the receptor to the other(s) simultaneously released. This type of interaction occurs for example between NPY and noradrenaline (Agnati et al. 1983; Illes and Regenold 1990; Martire and Pistritto 1992). Interestingly, receptor recruitment from the interior of the cell to the plasma membrane may be an additional and ubiquitous mechanism of modulation of signal transduction, leading to receptor sensitization (Holtback et al. 1999). The interaction of co-transmitters also occurs through pre-synaptic regulation (Fig. 3d). This implies the existence of pre-synaptic receptors for one or more messengers. In this case, one of the neurotransmitters feeds back on pre-synaptic receptors and thus (upon binding to autoreceptors) affects its own release (Malcangio and Bowery 1999; Salio et al. 2005) or the release of the cotransmitter(s). This latter possibility has been demonstrated, for example, in the striatum in which tachykinins presynaptically stimulate the release of dopamine (Glowinski et al. 1993; Marco et al. 1998) and in the locus coeruleus in which noradrenergic neurons can be activated by the stimulation of neurokinin NK3 receptors (Angulo and McEwen 1994). Finally, unconventional neurotransmitters such as NO can interact with coexisting/co-stored neuropeptides (Aimar et al. 1998). Dendritic localization and release of neuropeptides Magnocellular neurons in the supraoptic nucleus have one to three dendrites with many large swellings displaying strong peptide immunoreactivity. Electron micrographs typically show that these dendrites have a large number of LGVs, similarly to endocrine secretory cells. The first inequivocal evidence of peptide release from these dendrites came from ultrastructural visualization of exocytic profiles (Pow and Morris 1989). Neuropeptides, such as oxytocin and vasopressin, that are released from dendrites function as autocrine or paracrine signals at their site of origin but can also act at distant brain targets to evoke long lasting changes in behavior (Ludwig and Leng 2006). The recently demonstrated ability of neuropeptides to prime LGV stores for activity-dependent release could lead to a temporary functional reorganization of neuronal networks harboring specific peptide receptors, providing a substrate for long-lasting effects. Further investigations will be required to assess whether the dendritic release of neuropeptides is limited to magnocellular hypothalamic neurons or is also used as an autocrine/paracrine signal in other areas of CNS.

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