Chapter 1
Coexistence of Neuromessenger Molecules –A Perspective

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1.1 Chemical Transmission

Chemical transmission is a fundamental process in nervous system function. The chemicals involved were originally termed neurotransmitters, but other names have subsequently also been used: messenger molecule, signaling/transmitter substance, modulator and more – in Sweden we say "a loved child has many names". Early on, with only few substances around, the term "neurotransmitter" appeared distinct and sufficient. However, as more and more categories of molecules appeared to have a signaling function in the nervous system, and sometimes with additional, even not well-defined functions, the name not rarely became on issue of controversy. For example, in the 1960s some eminent neurophysiologists would not accept the monoamines as neurotransmitters. This discussion is today less intense, perhaps because of the insight that the name really is not the critical issue, but rather to understand under what circumstances this spectrum of molecules is produced and released and what their functional significance is.

1.2 Identity and Function of Neurotransmitters

What is clear is that messenger molecules not only are involved in different types of transmission, e.g. slow versus fast signaling (the type of receptor being decisive), but many of them also have other effects, e.g. stimulating growth. And the main function of a messenger may vary during the life of a neuron/the nervous system, e.g. early on exerting a role in developmental processes and later on being a regular transmitter; or being postnatally downregulated and then reactivated under certain conditions, e.g. nerve injury. Thus, our view has advanced from the somewhat stereotype view that the function of a transmitter

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is just to allow axon potential "to jump" from one neuron to another via a chemical message. One could argue, let us only call such a molecule "transmitter" that does exactly that; but in fact there are hardly any messengers with just that function: Even glutamate exerts trophic effects and has both pre- and postsynaptic effects, that is, it also acts as a growth factor and modulator. In summary, molecules released from a nerve ending may have many different functions. If so, we cannot in many casesspeak about co-release of transmitters in a strict sense.

1.3 Neurons Only Produce One Transmitter

My upbringing in the Amine Group – established in 1962 by the late Nils-Åke Hillarp (1916–1965) – in the Department of Histology at Karolinska Institutet, taught me that a neuron only has one neurotransmitter. This view was based on histochemical monoamine research, using the formaldehyde fluorescence (Falck-Hillarp) method developed by Bengt Falck, Nils- Åke Hillarp and collaborators (Falck et al., 1962). With this technique, for the first time, a transmitter could be identified in an individual neuron – if one wants, the first opportunity to approach the coexistence problem. The results clearly showed that dopamine (DA), noradrenaline (NA), 5-hydroxytryptamine (5-HT; serotonin) and (later) adrenaline were synthesized in different systems with their cell bodies distinctly separated along the caudo-cranial axis (Dahlström and Fuxe, 1964; Hökfelt et al., 1974; Hökfelt et al., 1984). Also, early ultrastructural analyses, even when using the highly sensitive potassium permanganate fixation (Richardson, 1966), showed that in the adult animal the peripheral noradrenergic and cholinergic neurons are two separate populations.

Moreover, when it became possible to demonstrate the cellular localization of the large population of inhibitory γ-amino-butyric acid (GABA) neurons, first with ^{3}H-GABA and autoradiography (Hökfelt and Ljungdahl, 1972a, b), and subsequently with immunohistochemistry using antibodies either to the GABA-synthesizing enzyme glutamate decarboxylase (GAD) (Wu et al., 1973; Saito et al., 1974), or to GABA itself (Storm-Mathisen et al., 1983), there was no obvious evidence for overlap and coexistence of GABA with the above-mentioned monoamine neurotransmitter systems (cf. Mugnaini and Oertel, 1985).

1.4 Some Historical Aspects – Dale's Principle

This view was in general agreement with an idea often called the "one neuron-one transmitter" hypothesis. This went back to Sir Henry Dale's statement (Dale, 1935a, b) that a neuron is a metabolic unit and "operates at all its synapses by the same chemical transmission mechanism", one

interpretation being that a neuron releases one and the same messenger from all its branches. The concept was then further modified, saying that each nerve cell makes and releases only one transmitter. In the light of the findings of coexistence of messenger molecules described below, this concept was later discussed in some depth (see e.g. Eccles, 1986; Potter et al., 1986). Nevertheless, the "one neuron-one transmitter" idea was not challenged for several decades. But when multiple messengers were shown in neurons (see below), additional interesting findings with bearing on Dale's principle were reported. Thus, in Aplysia two different messengers could be shown to be directed into different processes of the neuron (Sossin et al., 1990), thus not having the same transmission mechanism, in any case not the same transmitter, at all processes. Another interesting concept has been developed by Ludwig and co-workers, showing that dendrite and nerve endings of a neuron can operate separately and independently in releasing a messenger substance (see Ludwig, 2005; Ludwig and Leng, 2006).

1.5 Early Evidence for One Neuron-Multiple Transmitters

In the mid-1970s, the"one neuron-one transmitter" idea came under serious scrutiny. Thus, studies on isolated (large) invertebrate neurons suggested presence/co-release of more than one putative transmitter from a neuron (Kerkut et al., 1967; Brownstein et al., 1974; Hanley et al., 1974; Cottrell, 1976; Osborne, 1984). Also, Jaim-Etcheverry and Zieher (1973), to my knowledge the first ones using the word "coexistence" in this context, reported presence of NA and 5-HT in the same synaptic vesicles in the pineal gland. In this case serotonin had been taken up from the blood, that is not synthesized in the pineal nerves. Nevertheless, when activated these nerve endings presumably release two transmitters, a topic that will be dealt with in this book.

Elegant experiments, initially carried out in mono-neuron cultures (Furshpan et al., 1976; Landis, 1976), showed that there is a developmental switch in autonomic neurons from a noradrenergic to a cholinergic phenotype and that autonomic neurons for a while can synthesize and release both NA and acetylcholine (ACh). Thus, there is coexistence and co-release of two classic transmitters during development, which also occurs in vivo (Francis and Landis, 1999).

Physiological/pharmacological studies on the peripheral nervous system suggested existence of nerves releasing neither NA nor ACh, and this phenomenon was termed NANC (nonadrenergic, noncholinergic) transmission, which could be shown to exist in many peripheral tissues (see Burnstock, 2007). The prime candidate for this type of transmission was ATP, and Geoffrey Burnstock coined the term purinergic transmission. ATP was also one of the early molecules suggested to be involved in cotransmission, and co-release with NA could be demonstrated (Su et al., 1971; Westfall et al., 1978).

1.6 Coexisting Neuropeptides

Meanwhile, many groups had started to analyze the expression and distribution of a further group of neuronal messengers, the neuropeptides. They have now turned out to represent the largest family of signaling molecules in the nervous system, probably more than hundred members (Burbach, 2008), and with a correspondingly large number of receptors (several hundreds), virtually all of the 7-transmembrane, G-protein-coupled type. Radioimmunoassay and immunohistochemistry, sometimes using the same antibodies, clearly showed a very wide distribution in the brain and in all type of peripheral systems, sensory and autonomic neurons and in the gastro-intestinal tract. Geoffrey Burnstock (1976) wrote an influential review article suggesting reexamination of the "one neuron-one transmitter" concept, pointing in particular to the wide distribution of neuropeptides in the nervous system.

The first direct evidence for presence of a peptide and a classic transmitter in the same neuron was then observed in guinea pig sympathetic ganglia, where somatostatin was found in noradrenergic neurons (Hökfelt et al., 1977). Somatostatin, a tridecapeptide and the principal growth hormone release-inhibiting factor, was discovered by Brazeau et al. (1973), and Renaud et al. (1975) rapidly demonstrated a transmitter function for this peptide. Thus, many sympathetic neurons synthesize two transmitters/messenger molecules, NA and somatostatin. Early on efforts went into establishing that the somatostatin-NA was not a single case, and fairly rapidly more and more examples were found, and early reviews often had tables of varying length showing such examples. However, today they are so abundant that it seems useless to produce such a table. In fact, it is likely that every neuropeptide co-exists with a classic transmitter of some kind, as will be discussed below.

1.7 Neurotransmitter Storage

There are some general points that could be discussed in relation to coexistence. First, it may be said that this primarily is an anatomical term, that is, one has to show that two molecules with transmitter function are synthesized and present (preferably transcript and peptide/protein) in the same neuron. In addition to peptide-monoamine coexistence in the same neuron, it was rapidly shown that the peptides have a special storage site, the large dense core vesicles (LDCVs) (see Pickel, 1985). In fact, it had been recognized in early electron microscopic studies that there are at least two types of storage vesicles in neurons/nerve endings (see Grillo, 1966): (1) synaptic vesicles (diameter around 500 Å); in NA (and other types of monoamine) neurons they can often be shown to have a dense core; and (2) LDCVs (diameter around 1,000 Å); if fixed with

glutaraldehyde they have a dense core and are present in most neurons; if fixed with $KMnO_4$(Richardson, 1966) LDCVs also have a dense core, but only in monoamine neurons (Hökfelt, 1968). So monoamines are stored both in LDCVs and synaptic vesicles.

The evidence for neuropeptide storage in LDCVs was/is based on immuno-histo-chemistry, which showed immunoprecipitate in LDCVs but not in synaptic vesicles (see Pickel, 1985). However, Pelletier et al. (1981) showed with immunohistochemistry that 5-HT is stored in LDCVs, but no precipitate was detected in synaptic vesicles, confirming the old truth that "negative (imuno) histochemistry" is not a final answer. Therefore subcellular fractionation studies were carried out, strongly supporting storage of neuropeptides exclusively in LDCVs (Lundberg et al., 1981; Fried et al., 1985).

An interesting question is whether amino acids are stored not only in synaptic vesicles, but also in LDCVs. Merighi et al. (1991) have triple-stained primary afferent nerve endings in the spinal dorsal horn for glutamate, substance P and calcitonin gene-related peptide (CGRP). Although, often in the same nerve endings, glutamate was never seen in the LDCVs, perhaps one distinct difference between monoamines such as DA, NA and 5-HT on one hand, and aminoacid transmitters on the other hand is that only the former are stored both in synaptic vesicles and in LDCVs.

Taken together, two transmitters present not only in the same neuron but also in the same vesicles suggest co-release, but anatomy does not prove co-release. It is difficult to get direct evidence for co-release, that is, that released molecules indeed are coming from the same neuron/nerve ending(s). This was perhaps first achieved in the mono-neuron cultures discussed above.

1.8 Is the Classic Transmitter Always the Main Messenger?

At one point we considered the interesting question, whether neuropeptides are only present in neurons having a coexisting classic transmitter, and whether the classic transmitter always is the important partner. Here the hypothalamic magno- and parvocellular neurons may provide an answer: Vasopressin, oxytocin and the releasing/inhibitory peptide hormones are of vital importance and, in agreement, these neurons contain, both in cell bodies and nerve endings, large amounts of LDCVs storing the peptides. However, also the earliest electron microscopic studies showed that the nerve endings in the posterior pituitary and the external layer of the median eminence harbor numerous synaptic vesicles in addition, suggesting presence of a classic transmitter. There was early evidence that some CRF neurons produce GABA (Meister et al., 1988), and GHRH-positive neurons have a dopaminergic/GABAergic phenotype (Meister et al., 1986; Meister and Hökfelt, 1988; Hrabovszky et al., 2005a). Moreover, recent studies now

clearly demonstrate presence of one of the recently discovered vesicular glutamate transporters, VGLUT-2, transcript and protein, in parvocellular LHRH- and somatostatin-positive hypothalamic neurons (Hrabovszky et al., 2004; Hrabovszky et al., 2005a; Hrabovszky et al., 2005b), as well as in magnocellular vasopressin and oxytocin neurons in the supraoptic and paraventricular nuclei (Hrabovszky et al., 2006).

Thus, even if the peptide hormone certainly is the main messenger molecule in these systems, classical aminoacid transmitters appear to participate in the modulation of these neurons. So we have a reversed situation as compared to most other systems, that is, the peptide is the main signaling molecule and the aminoacid the auxiliary messenger.

1.9 Also Amino Acid Transmitters Coexist

For a while it seemed as if only monoamines and ACh were involved in coexistence situations, that is, the aminoacid transmitters were "single". However, as mentioned above, GABA and DA coexist in the dorso-medial arcuate neurons (Everitt et al., 1984), but even before that the coexistence of GABA and 5-HT was demonstrated (Belin et al., 1981; Nanopoulos et al., 1981; Belin et al., 1983; Millhorn et al., 1987), and there were indications of glutamate in catecholamine and 5-HT neurons (Kaneko et al., 1990; Nicholas et al., 1990; Minson et al., 1991; Nicholas et al., 1992). Monoamine-glutamate coexistence was also supported by functional studies from single-cell microcultures demonstrating co-release of glutamate and 5-HT (Johnson, 1994; Li and Bayliss, 1998). Evidence for coexistence and co-release of glutamate and dopamine was published by Trudeau and collaborators (Dal Bo et al., 2004), a topic that they will also deal with in this book. Thus, all these studies suggested that neurons can co-release three classes of messengers: Aminoacids, monoamines and neuropeptides. Nevertheless, the glutamatergic nature of these neurons remained somewhat uncertain, because of lack of a truly specific marker. This changed, as indicated above, with the discovery of three vesicular glutamate transporters (see Masson et al., 1999; Fremeau et al., 2004). Thus, using VGLUT3 as a marker final proof was provided for the glutamatergic nature of many serotonin (Gras et al., 2002; Schäfer et al., 2002) and DA (Dal Bo et al., 2004) neurons.

The first example of possible aminoacid–aminoacid coexistence apparent to me was the demonstration by Ottersen et al. (1987) of cerebellar mossy fiber nerve endings characterized by high levels of both GABA and glycine. This type of coexistence, e.g. presence of GABA and glutamate in granule cells/mossy fibers (see Gutierrez, 2003), has during the last years captured increasing interest and is, in fact, the topic of several chapters in this book. It will therefore not be further dealt with here.

1.10 Functional Consequences and Clinical Implications

The insight that neuropeptides coexist with classical transmitters had a major impact, in any case on our own view, on the functional role of this large family of molecules. This indicated that neuropeptides are auxiliary messengers, not the sole messengers responsible for transmission at synaptic and non-synaptic sites in subpopulations of neurons. In fact, many colleagues are unconvinced about a physiological role of neuropeptides, as reflected in a stimulating and thought-provoking article by Bowers (1994).

The functional consequences of coexistence and cotransmission are manifold and have been explored in many experimental models. In the early days various types of interactions between classic transmitters and neuropeptides were considered. A model used here at Karolinska by Lundberg, Änggård and colleagues was the cat salivary gland exploiting interactions between NA and NPY and between ACh and VIP (Lundberg et al., 1980). A particularly convincing and elegant model was the the frog sympathetic ganglion for studies of interaction between ACh and LHRH-like peptides explored by Yuh Nung Jan and Lily Jan working in the legendary Stephen Kuffler's laboratory at Harvard Medical School (Jan and Jan, 1983).

We hypothesize that coexistence and cotransmission also has clinical implications. For example, in the rat the 29-aminoacid peptide galanin (Tatemoto et al., 1983) is expressed both in NA and 5-HT neurons (Melander et al., 1986), and NA-galanin coexistence has also been demonstrated in the human LC (Chan-Palay et al., 1990; Fodor et al., 1992; Kordower et al., 1992). Many NA neurons in the human LC also contain substance P (Baker et al., 1991; Sergeyev et al., 1999). Both NA and 5-HT neurons are targets for so called selective 5-HT and NA uptake inhibitors (SSRIs, SNRIs) for treatment of major (unipolar) depression. And also NK1 (substance P) antagonists have been reported to have antidepressant activity (Kramer et al., 1998; Kramer et al., 2004). This opens up interesting possibilities that coexisting molecules and their receptors can be target for development of novel treatment strategies for various disorders.

1.11 Concluding Remarks

We are witnessing an exciting development in our understanding of chemical transmission in the nervous system, characterized by an amazing complexity, at least as compared to the situation when I started in research some four decades ago. It was difficult enough to explain how a motoneuron in the ventral horn is controlled by some 10.000 boutons releasing one transmitter, but additional messengers in each bouton certainly does not make it easier to understand the functional execution.

Numerous papers have dealt with coexistence: More than 900 hits in PubMed under the terms "neurotransmitter, coexistence", more than 800 on "neuropeptide, coexistence" and more than 100 reviews with the two latter terms in the beginning of February 2008. So much focus has been on the neuropeptides. Early results on coexistence have been summarized in review articles (Hökfelt et al., 1980; Lundberg and Hökfelt, 1983; Furness et al., 1989; Burnstock, 1990; Lundberg, 1996; Merighi, 2002; Burnstock, 2004) and in books (Cuello, 1982; Osborne, 1983; Chan-Palay and Palay, 1984; Hökfelt et al., 1986). There are also important studies on the evertebrate nervous system that lends itself in an ideal way to coexistence/co-release studies, as also mentioned in the Introduction (for review see Osborne, 1984; Kupfermann, 1991; Nusbaum et al., 2001).

In the present book another chapter in the history of transmitter coexistence is written in a series of exciting chapters dealing with topics so far not summarized. They include novel aspects on transmitter combinations, such as NA-ACh, monoamines-glutamate, ACh-glutamate, cross-talk between monoamines, GABA and ATP, and especially various combinations of aminoacid transmitters, actually coexistence of excitatory and inhibitory ones. There are also chapters on synapse formation and invertebrates.

I thank the Editor Dr. Rafael Gutierrez for asking me to write this introduction. Many colleagues I am sure, and I for certain, look forward to read the final "product".

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Chapter 1

Query No.	Line No.	Query
AQ1	04	Please provide a brief abstract.
AQ2	52	The sentence beginning “If so, we can in many then not cases speak. . .sense.” has been changed to “If so, we cannot in many cases speak. . .sense. Is it ok?