

## Chapter 2

# *Ex uno plures: Out of One, Many*

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**Abstract** Ex uno plures, out of one (cell) many (neurotransmitters), seems to be a principle that applies to many, if not all, neuronal types. The co-release of signaling molecules has been long recognized and the terms “classical neurotransmitter” and “neuromodulator” have been used to label the co-released substances, often being the former of low molecular weight and the latter of high molecular weight. Indeed, the use of these terms confers a distinctive function for each substance. However, the co-release of two or more low weight, fast-acting “classical neurotransmitters” is until recently subject of intense investigation. Initially, the co-existence of classical neurotransmitters in a given cell or its terminals was a curious observation, and the possibility of they being released was not directly approached as it contradicted a dogma: “one cell, one neurotransmitter”. Presently, the co-existence and co-release of classical neurotransmitters is known to occur in different animal species and neuronal systems, from invertebrates to human. Moreover, the specification of the neurotransmitter phenotype of neurons has been shown to be plastic. In some cases this plasticity follows a developmental program and, in others, it depends on activity-dependent and even on pathological processes. Therefore, the listener cell should already have the receptors in the postsynaptic site or should actively put them in place to interpret a compound message, carried by two or more neurotransmitters, and integrate it to display a response. The time-locked release and thus, the action of two or more classical neurotransmitter provide the central nervous system with a powerful communication and computational tool.

It is well accepted that neurons can release both a “classical” neurotransmitter together with a modulatory transmitter. However, the recognition that they contain and co-release two or more “classical” neurotransmitters is a new

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avenue in the study of neurotransmission that has started recently to be explored. Colocalization of classical neurotransmitters within single terminals was initially perceived as a curiosity and their potential co-release was viewed with skepticism. The molecular explanation, as well as the physiological and physiopathological relevance of the colocalization and co-release of classical neurotransmitters has recently been the subject of intense research. Indeed, many examples of coexistence and co-release of classical transmitters have been described in invertebrate and vertebrate organisms, and within the mammalian central and peripheral nervous system.

The idea that two or more classic neurotransmitters might coexist in an individual neuron and hence, be co-released by it was not derived from a direct experimental approach. Perhaps with good reason, why would a neuron have and use two chemical neurotransmitters? Indeed, why would it convey more than one message? Unfortunately, the hypothesis of co-release was also often considered as heretical because it contradicted the commonly understood idea behind Dale's postulate that "a single cell releases only one neurotransmitter." Serendipitous observations of neurotransmitter colocalization, as well as of their synthetic enzymes and vesicular transporters, as well as the observation that postsynaptic responses did not match the supposed activity of the fibers stimulated, led researchers to seriously test the co-release hypothesis. Although still questioned in some cases, the overwhelming evidence in favor of this phenomenon has opened new possibilities to understand neural communication and in particular, to address synaptic physiology.

Moreover, it now appears that the neurotransmitter phenotype of neurons may be very plastic. Evidence of activity-dependent plasticity of neurotransmitter phenotype, even after brain insult, has drawn the attention of many investigators to the possibility of finding more cells that express multiple neurotransmitters in response to environmental changes. Specification of neurotransmitter phenotype is a process that initially takes place during development. It is a mechanism by which the genetic program and the environmental signals received by a neuron fine tunes the expression of a series of proteins, to define a given neurotransmitter phenotype. The silencing and the turning on of positive or negative signals are complementary and active processes during development. However, while some genes are definitively turned off by the end of development, others seem to remain latent and can be turned on later in life upon specific demand (e.g., increase or decrease of electrical activity, the action of trophic factors, hormones, etc.). This type of plasticity provides the nervous system with a powerful communicational tool.

## 2.1 What Is a Classical Neurotransmitter?

A "classical neurotransmitter" has been defined as a chemical substance that (1) is synthesized in the cell (indicating that the synthetic machinery has to be present in the cell); (2) is present in the presynaptic terminal and is released

from a specific zone during activity; (3) acts directly on receptors that are present in the postsynaptic cell, altering their activity; (4) if applied exogenously, it mimics the effects of the endogenously released substance by activating the same receptors/channels; and (5) is removed from the extracellular space by defined catalytic or transport mechanisms. Classical neurotransmitter substances are: acetylcholine (ACh); the biogenic amines dopamine (DA), epinephrin (E) and norepinephrine (NE); serotonin (5-HT); histamine (H); and the amino acids glycine (gly),  $\gamma$ -aminobutyric acid (GABA), glutamate (Glu) and aspartate (asp). Some of these classical transmitters activate receptors directly coupled to ion channels, while others activate metabotropic receptors that initiate intracellular signaling cascades that produce the opening or closing of ion channels. Indeed, some transmitters may activate both types of receptors. Accordingly, it is the receptor and not the transmitter that determines whether the action of the released substance will be excitatory or inhibitory.

## 2.2 What Is a Modulatory Transmitter?

In contrast, other substances that are also released from nerve cells, not necessarily from an active zone where the classical transmitters are released, may exert a more diffuse effect and modulate the signal that the primary (“classical”) neurotransmitter conveys. In general, these substances activate intracellular signaling cascades that modify the action of the primary transmitter. Therefore, the usual concept of “co-release” most often implies the simultaneous release of a “classical transmitter” (usually of low molecular weight) and a “modulator” (usually of high molecular weight), which seems to be a general phenomenon in neuronal cells. Among the modulatory substances are peptides, nucleotide (e.g., ATP),  $Zn^{2+}$ , neurotrophic factors, nitric oxide and endogenous cannabinoids. In all these cases the co-released factors are synthesized in the cell (except for  $Zn^{2+}$ ), they are released in a selectively regulated fashion, they act on receptors, and they are removed or inactivated by specific mechanisms. Classical transmitters may also have modulatory roles acting both postsynaptically and/or presynaptically.

## 2.3 Dale’s Principle

Based on the ideas from the studies of Henry Dale on cholinergic and adrenergic neurons in the spinal cord in the early 1930s, John Eccles formulated a functional principle, which was then further transformed to imply that “a single cell releases only one neurotransmitter.”

In fact, what Dale stated was that a neuron functions as a metabolic unity, whereby a single process in a cell can influence all the compartments of the same neuron. From this, Eccles claimed that all the terminals of a given neuron should release the same neurotransmitter. However, in the light of our current

knowledge about the coexistence of transmitter substances, a more correct interpretation of Dale's principle would be: "A neuron *normally* releases the same chemical messengers from all of its synapses." As new evidence for the segregation and compartmentalization in neurotransmission has been disclosed, this can be further adapted to suggest "that the same chemical messengers *could* be released from all the terminals of a given neuron."

After the discovery of the coexistence of classical and modulatory transmitters in single neurons and their co-release, it was thought that co-transmission only permitted this combination of transmitter substances. But what about the coexistence and co-release of two or more classical neurotransmitters?

## 2.4 Coexistence and Co-release of Classical Neurotransmitters

The transformed Dale's principle that "a single cell releases only one neurotransmitter" has influenced many neuroscientists to consider with skepticism the idea that two or more classical neurotransmitters could coexist and hence, be co-released by neurons.

The coexistence and co-release of two or more classical neurotransmitters, each conveying a "principal message," has therefore been studied less extensively than that of classical transmitters and peptide modulators (see Hökfelt in this volume). Indeed, in some ways the initial descriptions of these events still remain a curiosity. However, during recent years, ample evidence has shown that co-release is in fact not that uncommon, and it is now known to occur in a variety of neural systems. In addition, although many populations of adult neurons may not release two classical neurotransmitters under basal conditions, many appear to do so transiently following an established program during early development, or in response to a variety of physiological and pathophysiological stimuli.

A fundamental question in neuroscience is how the remarkable cellular diversity is established during development. The influence of external signals and transcription factors determines when the distinct types of neurons are formed at specific sites. It is equally important to unravel how the specification of their morphological and functional traits takes place, particularly how neurotransmitter phenotypes are specified. The expression of the neurotransmitter phenotype of a given neuron follows a specific program, whereby transcription factors exert a strong regulatory role. However, electrical activity modulated by external signals seems to trigger the expression or repression of a given phenotype by virtue of calcium entering the cell. The only way through which the environmental demands can affect this expression is to activate the existing capability of the neuron to express a given phenotype. Thus, a neuron can possess a latent or potential phenotype, which can be activated during development or in adult life. This also implies that a cell must possess a message that restricts its expression at a given point in time. The effect of its activity and thus, of calcium influx on transmitter selection suggests that the number of

neurons expressing a particular transmitter may be regulated so as to maintain a steady level of excitability in the nervous system (see Borodinsky and Spitzer, this volume; see also Spitzer et al., 2005).

## **2.5 Colocalization of Receptors for Classical Neurotransmitters**

In order for a neuron to respond to a given chemical signal, it must have the appropriate receptors at the correct site. Thus, there must be adequate apposition of the presynaptic and postsynaptic elements. In other words, the neurotransmitter released by the presynaptic terminal must precisely match the postsynaptic receptors for communication to occur across the synapse. This implies that when co-release of classical neurotransmitters takes place, the receptors for the different neurotransmitters must be colocalized in the postsynaptic element, specifically in the subsynaptic zone apposing the site of release. Another possibility is that the receptors for one neurotransmitter are in the subsynaptic zone, while the receptors for the other neurotransmitter are in the perisynaptic or extrajunctional membrane. Such distributions would produce different types of postsynaptic effects. Moreover, the phenotypic plasticity of the presynaptic neuron must be paralleled by plastic changes of the postsynaptic neuron, probably by triggering receptor motility of the matching receptor in the vicinity of the release site. Indeed, receptor selection during development parallels changes in neurotransmitter phenotype (i.e., activity regulates the matching of transmitters and their receptors in the assembly of functional synapses). On the other hand, clusters of different neurotransmitter receptors have been identified, albeit in culture preparations, apposing terminals that apparently release only one type of neurotransmitter. Whether changes in the presynaptic neuron are triggered to make use of these mismatched receptors is still to be determined. Therefore, it would be also important to determine whether a constitutive, redundant co-expression of receptors is a general phenomenon.

Not only do the postsynaptic neurons contain the receptors to the co-released neurotransmitters but also, the terminals or axons that co-release the neurotransmitters can possess receptors that could be readily activated, producing presynaptic auto-modulation or collateral presynaptic actions. This mechanism seems especially relevant for activity-dependent plasticity and can be of marked functional significance in situations of enhanced excitability.

## **2.6 Consequences and Functional Advantages of Classical Neurotransmitter Co-release**

The site of synthesis within the neuron, the rate of synthesis and finally, the type of activity needed to release small-molecule neurotransmitters and peptides differ. While small-molecule neurotransmitters can be synthesized

locally in the nerve terminals where they are to be released, peptides are synthesized in the soma and then transported to the terminals for release. In this regard, the rate at which the chemical substances are produced and prepared for release can produce marked functional differences. Another difference between the release of peptides and “classical” neurotransmitters is that peptides are only released by repetitive action potentials, whereas classical transmitters can be released by single action potentials, providing them with distinct functional characteristics. Thus, most modulators are released in an activity-dependent manner.

This difference underlies the effectiveness of co-released classical neurotransmitters in producing fast signaling and therefore, fast modulatory interactions. In some cases, these interactions are synergistic, for instance, if two inhibitory or two excitatory transmitters are co-released. However, in other cases, two neurotransmitters may exert opposing effects. Of course, the specific receptors have to be in the right postsynaptic site for this interaction of the neurotransmitters to occur. Therefore, because the receptors determine the way in which a cell responds to its inputs, the coexistence of receptors to different neurotransmitters apposed to presynaptic terminals expands the possible responses of the neuron to a variety of signals. In this way fast and efficient modulation of incoming signals can be established at very restricted membrane sites. Therefore, neuronal activity can lead to a high concentration of (classical) neurotransmitters in a very narrow time-window. Accordingly, neurotransmitter-mediated modulation could occur more rapidly than peptide-to-neurotransmitter mediated modulation, and it may take place right at the synapse involved, spatially restricting the modulation. The last chapter of this book will deal with the integration of two or more signals conveyed by classical neurotransmitters: *E pluribus unum*, out of many (signals), one (response).

## 2.7 What Do We Still Need to Know?

The response is simple, more than we already know.

A list of questions might be helpful in illustrating the aspects related to the coexistence and co-release of classical neurotransmitters that need to be investigated. This might include questions such as:

How are two or more neurotransmitters synthesized and packaged within single boutons and in some cases, within single vesicles? For example, glutamic acid is the precursor of GABA and although essentially all GABAergic cells must contain glutamate for its conversion to GABA, they do not release glutamate. If a cell were to have and to release both amino acids, how does it control the passage of glutamate to GABA without running out of glutamate to be used directly? Indeed, if glutamate is used for GABA synthesis, is there a compensatory mechanism to replenish the glutamate needed for release?

This example considers two amino acids that are closely related in metabolic terms. But in neurons containing other transmitters that are not that related, how does the cell cope with the synthetic machinery for two neurotransmitters? Are the vesicles segregated? How is “access” to the releasable and reserve pools organized? How are vesicles distributed and docked in the membrane, and then secreted from the presynaptic terminal? How is the trafficking of vesicles destined to package different neurotransmitters? Are they tagged?

How does the secretory mechanism work when there are two or more neurotransmitters to be released? Are there different release sites for each neurotransmitter? Is the release machinery the same for the different populations of vesicles within single boutons? Is the release machinery (considering the membrane scaffold proteins that anchor the vesicles to the membrane) of one family of vesicles the same as that for a second family? Can the release of either neurotransmitter be differentially controlled? Are the kinetics the same for the release of all the neurotransmitters a neuron contains? What mechanism controls the release of one neurotransmitter at a given time but not at another?

How do the transporters for different neurotransmitters work? How do vesicles “select” the neurotransmitter transporters (or vice versa) that will enable them to capture certain neurotransmitters?

Are all neurotransmitters within a cell released from all terminals? Does segregation occur and if so, how is segregation determined? Is there a postsynaptic retrograde signal (NO, cannabinoids, trophic factors, others) that controls release in a differential manner? Does the postsynaptic target cell have a say in all this? Are the plastic properties the same for all substances released by the neuron?

How is the expression of the neurotransmitter phenotype controlled? Does the same intracellular signal control the expression of a given phenotype and the suppression of the other?

Although some of these questions are currently being addressed by different research groups, the “initial”, more basic questions remain on the table, i.e., which are the neurotransmitters that are co-released? Where from? How? What do their signals do and how are they integrated? etc.

The problem we are dealing with has been clearly presented in this chapter: *Ex uno, plures*, from one (cell) many (neurotransmitters). The chapters that follow will review the specific issues surrounding the coexistence and co-release of these neurotransmitters. Finally, the last chapter will focus on what we are looking for: the meaning of co-expression and co-release of classical neurotransmitters, i.e., *E pluribus unum*, out of many (signals), one (response).

We are sure that this volume will grow rapidly and we will make efforts to update it as new and exciting advances appear.

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