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Review

A review on electron microscopy and neurotransmitter systems

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Abstract

The purpose of this article is to review the contributions of transmission electron microscopy studies to the understanding of brain circuits and neurotransmitter systems. Our views on the microstructure of connections between neurons have gradually changed, and now we recognize that the classical mental image we had on a chemical synapse is no longer applicable to every neuronal connection. We highlight studies that converge to point out that, while the most prevalent fast transmitters in the brain, glutamate and GABA, are stored in small, clear synaptic vesicles (SSV) and released at synapses, neuropeptides are exclusively stored in large dense core vesicles (LDCV) and released extrasynaptically. Amine transmitters are preferentially, but not exclusively, accumulated in LDCV and may be released at synaptic or extrasynaptic sites. We discuss evidence suggesting that axon terminals from pyramidal cortical neurons and dorsal thalamic neurons lack LDCV and therefore could not use neuropeptides as transmitters. This idea fits with the fast, high temporal resolution information processing that characterizes cortical and thalamic function.

Theme: Cellular and molecular biology
Topic: Staining, tracing, and imaging techniques

Keywords: Axon terminal; Electron microscopy; Synaptic vesicles; Transmission; Ultrastructure

Contents

1. Introduction

The use of electron microscope to gain a closer view of brain tissue followed, with a short delay [105], the introduction of this complex instrument into biology. The first years were necessarily committed to learn how best to preserve its morphology and to reappraise the structure of the various cellular and subcellular components of the central and peripheral nervous system [24], previously studied under the light microscope.

This initial approach culminated with the publication in 1970 of "The Fine Structure of the Nervous System" [111], a groundbreaking work that one can nearly read from cover

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to cover, consult as a source of illumination, and use as a field guide. This book provided the general cytology framework for further studies, most of them yet to come, on the ultrastructure of specific brain regions and circuits. Its superb photomicrographs have set high standards that are hard to reach when one uses procedures that degrade the quality of tissue preservation like axonal tracing or immunocytochemical techniques.

In the Preface to the first edition, the authors already advance one point we will try to make in the present review, namely that "no single photomicrograph can be representative of the structure of any cell type" (cell type, that is, from the nervous system). This point is valid not only because single micrographs are two-dimensional samples of three-dimensional structures, but also because of the diversity and complexity that characterize the fine morphology of microcircuits in the brain.

To make sense of the wealth of information provided by electron microscope photomicrographs, it is necessary in most cases to label and identify the presynaptic and the postsynaptic structures. The techniques to identify input–output components of a neural circuit are by no means new, but we have indeed witnessed a gigantic improvement in the existing tools since the publication of "The Fine Structure".

The light microscopy techniques available before the 1970s to trace axonal projections and the Golgi technique to fully stain neurons were adapted to electron microscopy. Tract tracing studies were originally based on the wallerian or anterograde degeneration of axons and terminals after severing them from their cell bodies. Following an empirically determined survival time, the axon terminals examined under the electron microscope showed, in most cases, a dark degeneration that made them stand out against the normal axon terminals from the same region [63,66,160]. The drastic changes in morphology produced by the process of degeneration prevent a detailed description of the axon terminal; however, it is still possible to deduce, to some extent, the original shape and characteristics of the terminal, and to examine the postsynaptic structure.

Understanding brain circuits at an ultrastructural level was successful in the case of studies on structures, like the retina [138] or the cerebellum [106], characterized by a very ordered neuronal organization and by the existence of very distinctive cell types. These early studies did not need to label particular neurons to make functional sense of these structures.

One important role of electron microscopy is the unraveling of neural circuits at the subcellular level. In this regard, the information that is possible to obtain as a consequence of the high resolution of the electron microscope cannot be provided by other morphological techniques, including confocal light microscopy. Other important issues, where ultrastructural studies have made many relevant contributions, will not be addressed in this review. Among them are the key contributions of electron microscopy to the understanding of the morphological basis of synaptic plasticity (see reviews in Refs. [25,47,65]) or the studies analyzing the relationships between the size of axon terminals and their synaptic efficacy [114]. These two areas of research have used serial sections and three-dimensional reconstructions of spines and axon terminals to analyze subcellular structures involved in synaptic plasticity. Our purpose is to review the contribution of electron microscopy to the understanding of brain circuits, with special emphasis on how ultrastructural studies have contributed to a better understanding of transmitter storage and release. The morpho-functional differences between fast and slow transmitter systems will also be discussed.

2. Identification of the postsynaptic neuron

The identification of postsynaptic neuron phenotype initially relied on the Golgi technique [17]. An important improvement was the gold substitution of the silver precipitate [51], resulting in a subtler stain that permitted a better analysis of the ultrastructure. The Golgi-EM technique has produced unique findings, specially in neocortical circuits, [109,110], where the Golgi stain is particularly effective. The postsynaptic neurons may also be identified by retrograde axonal tracing techniques, by cell-specific immunoreactivity or histochemical staining (e.g., orexin immunoreactive for a subpopulation of hypothalamic neurons, or NADPH diaphorase for nitric oxide containing neurons), or by intracellular injection of electron-dense stains [35]. For a review comparing the different labels used for single neuron staining, see Ref. [181]. In general, the Golgi-EM and the intracellular dye injection techniques allow analyzing the inputs to somata and to proximal and distal dendrites. The latter are often not stained by retrograde axonal tracing or by cell-specific immunocytochemistry.

More recently, high-resolution immunogold protocols have been used to define in more detail the spatial distribution of molecular components of the postsynaptic density, including transmitter receptors [104], and associated proteins [167]. Ionotropic and metabotropic glutamate receptors present a considerable structural and functional diversity, resulting from the combination of different subunits [33,70]. This molecular diversity can be studied at the level of identified, individual synapses, by electron microscopy, particularly using immunogold pre- and postembedding protocols that allow a resolution of about 20 nm (for a review, see Ref. [104]).

The functional consequences of this diversity are exemplified in several works. Glutamatergic synapses in the cerebellum or hippocampus [150,151] have both AMPA and NMDA receptors co-localized in the postsynaptic density of particular synapses. In contrast, the faster AMPA receptor is predominant [179] in synapses closer to a peripheral receptor, and probably less plastic, like those involving end bulbs of Held and neurons in the anteroventral cochlear nucleus. In vitro studies on local circuits of

neocortical neurons reviewed by Thomson and Deuchars [155] also suggest the presence of different proportions of NMDA and non-NMDA glutamate receptors in pyramidal neurons and interneurons that are postsynaptic to pyramidal neurons or to thalamic afferents. By including the expression of different subunit isoforms in the analysis, we have a potentially large diversity of postsynaptic responses mediated by different combinations of receptors and receptor subunits. Along this line, results obtained in the lateral amygdala [180] showed that distinct populations of NMDA receptor subtypes, expressed in different synapses made by the same postsynaptic neurons, may account for the differential response to glutamatergic thalamic and cortical inputs of the amygdala neurons.

3. Identification of presynaptic structures, mostly axon terminals

The adaptation of light microscopy tract tracing techniques to electron microscopy has made possible to identify the source of presynaptic axon terminals to a given brain region. Current protocols use the injection of an anterograde axonal tracer [86,120,182] or immunocytochemical labeling of afferents from a single source, like orexin neurons that are exclusively located in the lateral hypothalamic area [41,125,161].

The existence of synaptic connections can be unequivocally demonstrated by electron microscopy, while light microscopic axonal tracing protocols can only suggest such synaptic connections. This distinction is particularly relevant when the afferents to a heterogeneous brain structure, with many cell types, are under close examination. Examples are studies [26,27] on prefrontal cortex terminals projecting to the ventral tegmental area. These cortical terminals synapse selectively on the GABA neurons that project to the nucleus accumbens and on dopamine neurons that project back to the prefrontal cortex.

While electron microscopy has been important to disclose the remarkable spatial precision that is the hallmark of the synaptic connections, it has ironically contributed key evidence to show the existence of a much less precise mode of communication between neurons, namely what is known as volume or nonsynaptic transmission.

4. Volume transmission

It has become increasingly clear that neurotransmission in the CNS can occur without the existence of a synaptic structure. The demonstration of gases as interneuronal signal molecules [8] or the modulation of neuronal function by lipophilic substances [12] left no doubts on the existence of nonsynaptic information transfer in the brain. Perhaps more difficult to accept was the idea that classical transmitters stored in vesicles present in axon terminals or in dendrites, and released following quantal mechanisms, can actually operate on nonsynaptic sites in the target structure [55]. Volume transmission for classical and peptide transmitters in the CNS is now generally accepted, as attested by several reviews [1,2].

The absence of synaptic structures was first demonstrated for the autonomic innervation of peripheral tissue [97], and later on in some sensory receptors like the retina (discussed by Agnati et al. [1]) or the carotid body [157]. Key morphological evidence for the existence of volume transmission in the CNS for classical transmitters has come from ultrastructural studies on serial sections of axon terminals (see review on early EM studies by Descarries et al. [42]) and from high-resolution immunoelectron microscopy studies of transmitter receptor localization (see Ref. [55] for an early review). In some brain regions, amine transmitters are stored in axon terminals that lack in synaptic structure. Documented examples include axons containing dopamine [43]; noradrenaline [14]; acetylcholine [44,95,164], serotonin [19,73,99], and histamine [146].

Two morphological types of vesicles present in axon terminals store neurotransmitters [38,111] (see Fig. 1). The most abundant type, the small, translucent synaptic vesicle (SSV), is known to accumulate fast-acting transmitters. The less frequently observed type of vesicles, larger and with an electron dense core (LDCV), stores peptides. Amine neurotransmitters can be stored in either or in both types of vesicles [38,96]. For example, monoamines in axons and dendrites of the nucleus of the solitary tract may be stored more prominently in LDCV rather than in SSV, since the vesicular monoamine transporter 2 preferentially localizes to LDCV [102]. Serotonin can be stored, within the same terminal, into SSV and in LDCV [22], or exclusively in LDCV [108]. In sympathetic neurons, noradrenaline is stored in LDCV [128], and these vesicles are transported along with SSV in sympathetic postganglionic axons. In these terminals, catecholamines are stored and released from both LDCV and from small dense core vesicles found only in the terminals, and not in the axons. These small vesicles have molecular markers typically found in the sympathetic LDCV, including noradrenaline and the monoamine carrier, indicating that they derive from the LDCV. In contrast, the population of SSV present in sympathetic axons and terminals does not contain catecholamines or the monoamine carrier found in other vesicles and lacks a dense core.

Dense core vesicles typically release their content at nonsynaptic sites of the terminal membrane (see below); therefore, axons with LDCV that contain an amine, even if they also store the amine in small vesicles, may engage in volume transmission. Since many neuronal types in the brain have either an amine transmitter or a neuropeptide, it follows that volume transmission is actually very prominent.

Strong evidence supporting a nonsynaptic mode of transmission comes from light [73] and electron microscopy studies of immunolabeled neurotransmitter receptors. All

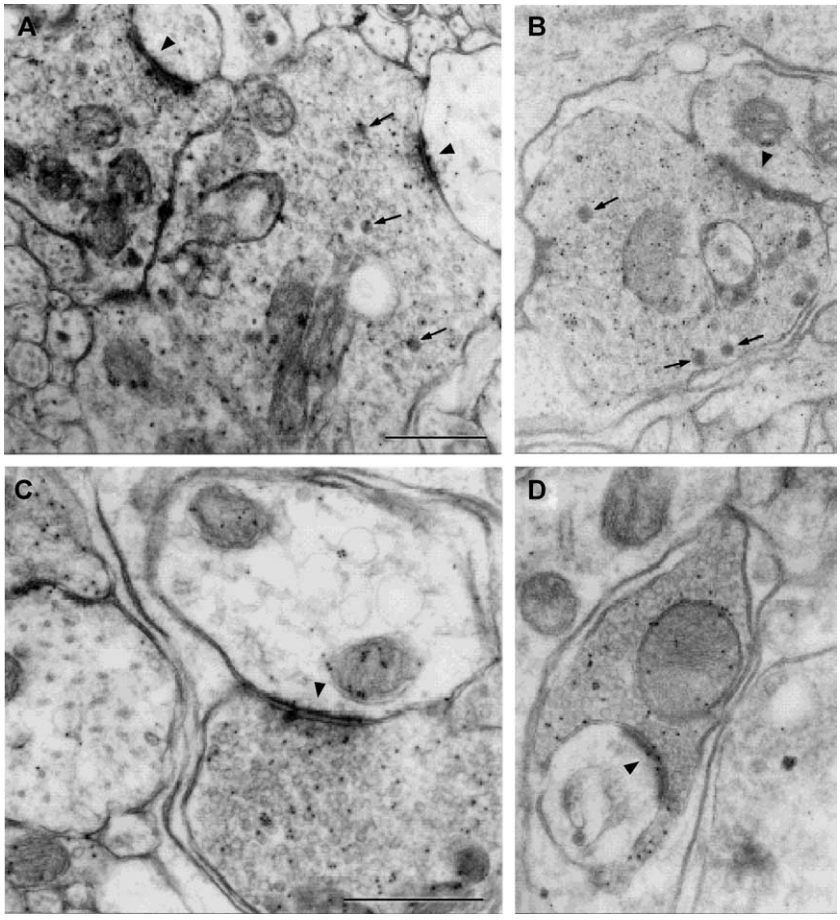

Fig. 1. Asymmetric synapses (arrowheads) in the nucleus tractus solitarius made by glutamatergic axon terminals; postembedding immunogold (10 nm gold particles) to glutamate. A and B show medium-size axon terminals with both types of vesicles: SSV and LDCV (small arrows). This type of synaptic contact is likely the most abundant in the mammalian brain, because the presynaptic structure is a glutamatergic axon terminal with both SSV and LDCV, and the postsynaptic structure is a dendrite. C and D show examples of a less frequent type of glutamatergic synapse, where the presynaptic terminals contain SSV only, indicating that no other neurotransmitters (peptide or amine) coexist with glutamate. Scale bars, 0.5 μm.

studies on the distribution of receptors to neuropeptides, including three-dimensional reconstructions of labeled peptidergic terminals and their target neurons identified by immunolabeled peptide receptors, coincide in showing that these receptors are targeted to membrane sites away from the postsynaptic density [3,10,85,98,113,144,145,170]. Neuropeptide receptors can be preferentially located near synapses, like metabotropic receptors, or distant from synapses, or on presynaptic structures, depending on the receptor. For example, μ-opioid receptor immunoreactivity was localized to extrasynaptic sites along the plasma membranes of dendrites and dendritic spines of nucleus accumbens neurons and, less frequently, to axon terminals [144,145]. The subcellular localization of the μ-opioid receptor on dendrites and on axon terminals within the nucleus accumbens parallels the functional post- and presynaptic modulation, respectively, of μ-opioid agonists. Neuropeptide receptors can also be found on dense core vesicles storing the peptide, a distribution that could allow the selective addition of receptors to presynaptic membranes upon peptide release [28,32].

Classical fast transmitters like glutamate [104] or GABA [58] can have receptors localized to extrasynaptic patches of the plasma membrane of neurons. In the case of glutamate, metabotropic receptors are actually not within the postsynaptic density but, depending on the isoform, they may be in the vicinity of the postsynaptic density or well away from it, or in the presynaptic terminal [104]. Clark and Cull-Candy [29] suggested that rapid changes in the synaptic responses of cerebellar molecular layer interneurons that resulted from increased presynaptic activity of parallel fibers were the consequence of the recruitment of extrasynaptic NMDA receptors by a stronger glutamate release.

Our views on brain function have been strongly influenced by the many studies on the visual system. The visual system function depends on very local, specific, and fast synaptic connections. However, sensory processing is modulated by the large-scale states of brain function (wake, REM, and non-REM sleep) [126]. Amines are the main transmitters of the ascending arousal system that controls this global function. In the case of arousal control, a focal, spatially restricted neurotransmitter action does not seem

essential. Volume transmission may be regarded as modulating the spatially precise synaptic information transfer in nervous tissue.

5. Coexistence of transmitters within the same terminal. A refinement of the classical view of synapses

Initially, co-transmission was perceived as a rarity, an exception to the ruling of fast transmitters. It has become increasingly clear that neuropeptides are present in many neuronal types and that peptides coexist with fast neurotransmitters [69,84] in axonal boutons from many classes of neurons. In fact, it has been suggested [84] that in all neurons, a fast and a peptide transmitter coexist. We will discuss the universality of coexistence later on in this review. Tables 1 and 2 show examples of coexistence of glutamate or GABA, the principal and most abundant excitatory and inhibitory fast neurotransmitters, respectively, with peptide or amine transmitters, as demonstrated by the combined use of immunocytochemistry and electron microscopy.

The widespread use of quantitative immunogold protocols to identify amino acid transmitters in CNS axon terminals has led to a tentative and important conclusion: most axon terminals in the mammalian CNS are either glutamatergic or GABAergic [7,39,49,68,77,87,96,100,117,133,158,159,172,174,176]. In a sense, this idea is related to, and complements, the general proposition that, in many instances [111], asymmetric and symmetric synaptic junctions [60] correspond to excitatory and inhibitory synapses, respectively [163]. This early grand generalization, raised from relating ultrastructural and physiological studies, has guided the interpretation of electron microscopy material for decades.

Large dense core and small, translucent synaptic vesicles have many functional differences (see reviews in Refs. [38,92]), in addition to the obvious dissimilarities in morphology and transmitter content. The LDCVs present in neurons are considered to be equivalent to the secretory granules of endocrine cells because they share a common biogenesis, morphology, and stimulus-secretion coupling [38]. By contrast, SSVs share many features, including size and molecular components, with microvesicles also present in endocrine cell types [38].

Table 1
Neurotransmitters that co-localize with glutamate as determined by high resolution immunocytochemistry

	Location	References
Acetylcholine	Terminals in entopeduncular n.	[30]
CGRP	Mossy fibers, hippocampus	[54]
CGRP	Terminals in dorsal horn	[96]
CRF	Terminals in locus coeruleus	[166]
Dopamine	Ventral tegmental area neurons	[76,142]
Dynorphin	Hippocampus mossy fibers	[93]
Encephalin	Locus coeruleus	[171]
Neurotensin	Terminals in dorsal horn	[156]
Orexin/hypocretin	Terminals in tuberomammillary n.	[161]
Serotonin	Raphe neurons in culture; n. tractus solitarius	[74,75,159]
Somatostatin	Afferents to Mauthner cell	[143]
Somatostatin	Cuneothalamic neurons	[177]
Substance P	Terminals in dorsal horn and n. tractus solitarius.	[96,123]
Substance P	Primary afferents	[37]

Table 2
Neurotransmitters that co-localize with GABA as determined by high resolution immunocytochemistry

		References
Acetylcholine	Cortex, interneurons	[13]
Acetylcholine	Few terminals in entopeduncular n.	[30]
Angiotensin II	Terminals in subfornical organ	[112]
Cholecystokinin	Cortex and hippocampus interneurons	[81,162]
CRF	Cortex interneurons	[183]
CGRP	Terminals in accessory optic n.	[185]
Dopamine	Striatum, intrinsic neurons; nigrostriatal neurons; median eminence	[16,59,127]
Encephalin	Terminals in locus coeruleus or periaqueductal gray	[118,169]
Encephalin	Striato-pallidal terminals	[89]
Galanin	Ventrolateral preoptic nucleus	[132,135]
Histamine	Tuberomammillary nucleus	[129]
NPY	Terminals in medial preoptic area	[71]
NPY	Dorsal horn	[107,116]
Serotonin	No, red nucleus; very few neurons	[4,136]
Somatostatin	Cortex, interneurons	[57,80,81]
Somatostatin	Minority of afferents to Mauthner cell	[143]
Substance P	Boutons in S. nigra	[18]
Substance P	Amacrine cells, retina	[36]
Substance P	Cortex interneurons	[72,78]
VIP	Cortex, interneurons	[57,80]

5.1. LDCV and SSV share most molecular components

Small synaptic vesicles and LDCV share many similar proteins that form part of the core secretory complex involved in Ca^{+2}-dependent membrane fusion. These proteins include, among many others, the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex proteins; synaptobrevin (VAMP) at

the vesicle membrane, SNAP-25 (25 kDa synaptosomal associated protein), and syntaxin, at the plasma membrane [91], as well as synaptotagmin I, a membrane vesicle protein that binds phospholipids and SNARE proteins, proposed as a major Ca^{+2} sensor for both SSV and LDCV [56].

Undoubtedly, the transporters of known amino acid transmitters are found in the SSVs that specifically store them and in a sense define the nature of the terminal. GABA-containing SSVs specifically express the vesicular GABA transporter [148] while [67,147,149] glutamatergic neurons express either of two vesicular-bound glutamate transporter, VGLUT1 or VGLUT2 [149], or, less often, both types of vesicular transporter [67,124]. Biogenic amines are transported by VMAT1, expressed only in LDCV, in endocrine and neuroendocrine cells. In neurons, VMAT2 is the only isoform expressed and though preferentially found in LDCV, it can also be found in SSV as mentioned above [50,102]. There are few proteins specifically expressed in one type of vesicle. Synaptophysins and synapsins were suggested to be present exclusively in the SSV [38], but while synaptophysin is considered a marker of SSV [101], more recent work has found synapsin also associated with LDCV [34].

From the large family of synaptotagmins, the synaptotagmin V isoform has been shown to be localized to LDCV, and not in SSV of PC12 cells, by expression of green fluorescence protein-tagged synaptotagmin V and by subcellular fractionation [122]. However, its limited localization in brain, even though restricted to LDCV, suggests cell type-specific expression of the V isoform [122].

Another protein suggested to be specific for LDCV is calcium-dependent activated protein for secretion (CAPS) [175]. Immunogold protocols applied to rat brain synaptosomes showed that CAPS is mainly associated to plasma membrane and to the membrane of LDCV [15]. Mutational analysis of two CAPS domains, a central pleckstrin homology domain and a C-terminal sequence domain mediating CAPS binding to the plasma membrane and to the LDCV membrane, respectively, supported the involvement of CAPS in LDCV exocytosis [61]. In addition, functional studies in permeable synaptosomes indicated that CAPS was required for LDCV exocytosis but not for SSV-mediated glutamate release [152]. Other functional studies conducted in melanotrophs, which present fast and slow components of calcium-dependent exocytosis, revealed that CAPS was necessary for the fast component only [121]. These results were complemented with immunocytochemical experiments showing the presence of CAPS in only a subset of LDCVs.

Proteins from the chromogranin class, like chromogranin A, chromogranin B, and secretogranin II, are found in LDCVs present throughout the neuroendocrine system and in neurons from the central and peripheral nervous system [90]. They are stored and secreted together with a variety of peptide hormones and neuropeptides, and they have not been found associated with SSV. Recent results point to a crucial role for chromogranin A in the regulation of LDCV biogenesis and the ensuing hormone secretion [82]. Some of the evidences include a severe LDCV loss by downregulation of chromogranin A in PC12 cells, and the induction of LDCV in non-endocrine cells by chromogranin A overexpression. Chromogranin B, meanwhile, though very similar to chromogranin A in structure and biochemical properties, does not share its role in LDCV biogenesis regulation [78]. Other proteins closely related to chromogranins have been described, like NESP55 (neuroendocrine secretory protein). However, in the brain, NESP55 is restricted to the hypothalamus and brain stem, where it was found in cell bodies and axons but not in terminals [52].

Interestingly, lithium ions upregulate the expression of proteins associated with LDCV, like secretogranin II, without affecting mRNAs for proteins predominantly associated with SSV, including the vesicular acetylcholine transporter and synaptophysin [34]. The authors postulate that lithium modulates the turnover of LDCV, and this may play a role on the effects of lithium on manic-depressive illness.

The above-discussed evidence indicates that chromogranins A and B, and secretogranin II are useful markers for LDCV, while synaptophysin is appropriate for SSV.

5.2. Release mechanisms for SSV and LDCV

The intraterminal distribution of SSV and LDCV differs markedly. In a typical CNS synapse, the SSVs occupy a variable, usually large, volume of the axon terminal; a small fraction of these SSV is docked to the presynaptic grid [111]. The LDCVs, in turn, are distributed away from the presynaptic membrane [23,161]. For instance, the LDCVs immunoreactive to dynorphin B are primarily distributed along the inner surface of large mossy fiber terminals in the hippocampus. Seizures reoriented this distribution, increasing the proportion of LDCV apposed to spines [115]. These studies suggest that LDCV could be tethered in some manner to specific sites of the presynaptic terminal. Moreover, electron microscopy has provided evidence for an extrasynaptic release of LDCV [23,38,79,186]. The differential distribution of release sites partly relates to the differences in sensitivity to Ca^{+2} for the exocytosis of both types of vesicles [83,173].

Synaptic delay in the CNS, ca. 1–3 ms, is a close reflection of the discharge of fast neurotransmitters from SSV. The transmitter release from SSV is tightly coupled to Ca^{+2} channels present in a high proportion in the presynaptic grid [137]. The Ca^{+2} channels and the release mechanism must be very close, tens of nanometers apart, because very high concentrations of Ca^{+2} appear to be required to activate this release mechanism [137].

In terminals with both types of vesicles, a focal increase in Ca^{+2} at the subsynaptic membrane tends to

discharge SSV, while a more diffuse elevation in the intraterminal Ca^{+2} favors the release of LDCV [173]. Moreover, neuropeptide release from axon terminals of the neurohypophysis showed two Ca^{+2}-sensitive phases. The first depolarizing pulses of a train primed the terminal for exocytosis evoked by the subsequent pulses [131].

Neuroendocrine axon terminals, as opposed to bovine chromaffin cells, do not have clusters of Ca^{2+} channels in the plasma membrane, but these channels are dispersed in the plasma membrane, as well as concentrated in the LDCV [53,184]. These findings and the Na^{+}- and Ca^{+2}-dependence secretion in these terminals [139–141,153,154] raise questions on the mechanism of excitation-secretion coupling in neuropeptidergic terminals.

Not all vesicles in a terminal or in a neuroendocrine cell can unload their transmitters upon prolonged stimulation. There is growing evidence that a variable fraction of SSV [64] and LDCV [83] is readily releasable and that the remaining vesicles, forming the reserve pool, need further steps to become competent. It has been shown that SSV [64] and LDCV [5] release transmitters by either the slower, classical exocytosis with complete fusion of the vesicle to the plasma membrane or by a faster mechanism involving a transient pore formation (“kiss and run”). The latter mechanism may be particularly important in diminutive synapses that are abundant in the CNS. In these synapses, the size of the readily releasable pool of SSV is so small (30–45 vesicles) [64] that the slower release mechanism, where the retrieval by endocytosis takes 30–60 s, cannot sustain activity higher than 1 Hz. In the faster mechanism, the retrieval takes ca. 1 s [64].

The transient pore mechanism for LDCV [5,48] would allow a quick exchange of the amine transmitter and perhaps other small molecules present in LDCV with the extracellular fluid; instead, the macromolecular content of the vesicles would remain within the retrievable vesicle. In contrast, the release mechanism involving a priming step [83] results in the complete fusion of the vesicles with the plasma membrane, followed by retrieval of the vesicle as a coated vesicle [5,48]. Sustained stimulation shifts the mechanism of endocytosis from dynamin-1-dependent rapid endocytosis to clathrin- and dynamin-2-mediated slow endocytosis in chromaffin cells [5,6,48].

The release of most neuropeptides from the LDCV, however, may not operate through the transient pore mechanism, because of the larger size of the peptide relative to the transient pore [9]. Thus, it is possible that the nature of the transmitter determines the mechanisms for the release of the cargo from the LDCV. A divergence in this respect between peptidergic LDCV and aminergic LDCV would add another difference in their properties. The neuropeptides do not have a known mechanism of reuptake, as opposed to the amine transmitters, so that there is no way to locally refill the peptidergic LDCV. Besides, the neuropeptides are synthesized via ribosomes in the neuronal perikarya, but not in axon terminals, while amines are synthesized within the LDCV.

6. To have or not to have LDCV

It is now widely accepted that most, if not all, neuronal types co-store a fast transmitter in SSV and a peptide transmitter in LDCV. The advantages of having more than one neurotransmitter have been analyzed in several reviews [20,21,69,103]. Peptide transmitters have a wide diversity of direct or modulatory effects on the electrical responses of target cells, in addition to trophic effects. When neuropeptides are co-released with other transmitters, the wealth of responses of postsynaptic (target) neuron increases dramatically [84]. If peptides are co-released always with glutamate, GABA, or other fast transmitter, it follows that the physiological effects of a peptide cannot be properly understood if they are applied alone to brain tissue.

However, there are brain circuits that rely on fast and precisely timed synaptic transmission, and it is in these circuits where co-transmission may introduce undesirable nonlinearities and temporal sluggishness. Such brain circuits may be those involving the excitatory axon terminals of pyramidal neurons in the cerebral cortex, and of relay neurons in the dorsal thalamus. Correlated firing between single cortical neurons changes within a fraction of a second and in tight relation to behavior [119,165] or to sensory stimulation [40]. Moreover, the temporal resolution and reliability of spike timing of cortical neurons seem to increase with the fast synaptic noise associated to fast neurotransmitters [45,88].

The axon terminals of cortical pyramidal and thalamic glutamatergic neurons seem not to have LDCV, but only SSV; therefore, they cannot use peptides as co-transmitters. The lack of LDCV in the axon terminals of a neuron seems enough to predict that this neuron will not have a peptide transmitter. It is necessary to consider, however, that the presence of LDCV in an axon terminal does not necessarily imply they store a peptide that will produce electrical changes in the target neuron. Neurotrophin-3 is transported anterogradely in optic axons, within LDCV, and released from the axon terminals by depolarization in a calcium-dependent way [178].

Direct evidence in favor of the idea that glutamatergic terminals of cortical pyramidal neurons and of thalamic neurons lack in LDCV is scant, in spite of the many electron microscopy works describing thalamo-cortical and cortico-fugal axon terminals. In these studies, the labeling of the terminals either by wallerian degeneration or by the use of electron dense and diffuse horseradish peroxidase labeling obscured the appearance of the terminals or prevented detailed examination of the types of vesicles. However, in a few papers, the authors showed that corticofugal axon terminals [130,168] have SSV, but did not mention the presence of LDCV.

There is, in addition, indirect evidence to support our contention. One line is that no peptide transmitters have been described in pyramidal neurons in the cortex or in dorsal thalamic nuclei. Exceptions involve the expression of peptides only after colchicine treatment, like cholecystokinin [134], or some opioid peptides [94], which in normal rats are present only in interneurons, but after colchicine treatment, they were additionally expressed in a small subpopulation of pyramidal neurons from the neocortex. It is not clear whether this expression represents a low but normal peptide production that needs colchicine to be brought up, or if this is some pathological expression resulting from the treatment.

A second line of indirect support to our proposal is that in the neocortex, a majority of axon terminals [60] from thalamic and pyramidal neurons synapse on spines [46], and most terminals presynaptic to spines have SSV only, as can be confirmed by examining published photomicrographs [31]. In the lateral geniculate nucleus of the thalamus, cortical afferents correspond to the RSD type of axon terminal [62], which lack in LDCV. It is possible that other neurons that are presynaptic to spines also lack in LDCV. In the presynaptic terminals of parallel fibers in contact with Purkinje cell spines, only the presence of SSV, but not of LDCV, is mentioned (page 90) in one classical book on cerebellum [106].

A third line of indirect evidence in favor of the idea that cortical pyramidal and dorsal thalamic neurons do not use neuropeptides as transmitters comes from studies on the distribution of markers for LDCV in the brain. The chromogranins, which are markers for LDCV, as described elsewhere in this review, are notoriously absent from cortical pyramidal neurons (but not from cortical interneurons, which co-store GABA and peptides, see Table 2), and from dorsal thalamic neurons [11].

Therefore, there are some evidences supporting the idea that the synaptic output of cortical pyramidal and of thalamic neurons relies only on the fast, highly local, synaptic mode of transmission and does not have peptidergic, nonsynaptic co-transmission. This scheme makes neurons from the dorsal thalamus and pyramidal cortical neurons an exception in the mammalian brain, because most neuronal types, including cortical inhibitory interneurons, have LDCV and peptide transmitters in addition to a fast-acting neurotransmitter. If the presence of LDCV in a neuron is a prerequisite to express a peptidergic phenotype, perhaps during development the precursors of pyramidal cortical and thalamic relay neurons silenced the genes necessary to build LDCV. In this sense, the crucial role for chromogranin A in the regulation of LDCV biogenesis discussed in Section 5.1 may prove interesting to explore in relation to the development of pyramidal and thalamic neurons. Studies on the input–output transformations in circuits involving identified pairs of neurons, like those conducted in the neocortex by Thomson and Deuchars [155], are needed to evaluate the contributions made by co-transmission versus single, fast-acting transmitters in particular central synapses.

7. Conclusions

Electron microscopic studies have provided invaluable clues on brain structure–function relationships, most notably on the complex and dynamic ways neurons interact with each other. The fruitful combination of molecular tagging with transmission electron microscopy should continue providing new information on the molecular architecture and the life cycle of organelles and structures relevant to cell–cell interactions in the brain. In this line, electron microscopic techniques should be crucial in evaluating evolving concepts of synaptic function, such as whether new synaptic proteins are generated locally, or kept in sequestered pools for quick insertion. The need to understand in detail the vast number of microcircuits existing in the brain should be an agenda that will keep electron microscopists busy for a long time. We hope that enough young neuroscientists will be interested in learning electron microscopic skills and keep ultrastructural approaches to brain function in good health.

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