AN ULTRASTRUCTURAL STUDY OF NERVE PROFILES IN THE MYENTERIC PLEXUS OF THE RABBIT COLON

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Abstract—The ultrastructure of the myenteric plexus from the rabbit colon was examined in both conventionally fixed tissue and also material fixed with the chromaffin method. Montages of the ganglia were analysed semi-quantitatively. Six main types of axon profile are described and classified on a morphological consideration of the vesicle population. Most axon types formed synapses with myenteric neurons. Two kinds of chromaffin-positive nerve fibre were seen, one containing a predominance of small granular vesicles, the other containing many flattened vesicles. The difficulties in relating axon profile types to putative transmitters are discussed.

Recent investigations of the enteric plexuses all tend to emphasise the complexity of the neural circuitry involved. Different experimental approaches have been employed. These include electrophysiology,29 pharmacology,2*4*10 immunohistochemistry,6 and morphology.13 The ultrastructural features of different types of nerves remain unclear. For example, even the identification of adrenergic profiles by the presence of nerves remain unclear. For example, even the identification of adrenergic profiles by the presence of small granular vesicles, which had been accepted by many investigators, has been questioned recently by Furness & Costa.10 Particularly in the gut, there is now increasing evidence to suggest the existence of neurons which may use a variety of transmitters, including purine nucleotides, peptides, γ-aminobutyric acid (GABA) and 5-hydroxytryptamine (5-HT) as well as acetylcholine (ACh) and noradrenaline (NA).6

In the present investigation we attempt to identify morphologically distinguishable categories of nerve fibre in the rabbit myenteric plexus. The nerve types proposed for the rabbit myenteric plexus are compared with those described in the guinea-pig enteric nervous system.1,7,2,13 The myenteric plexus of the rabbit colon is particularly suitable for quantitative analysis of the neuropil since it is very dense under the taeniae; the intrinsic and extrinsic innervation of the muscularis has been studied in detail using electrophysiological techniques.9,1,2,18

EXPERIMENTAL PROCEDURES

Adult rabbits were used, weighing 2-3 kg. Under ether anaesthesia, pieces of the proximal colon including the taenia muscle were removed and placed in ice-cold primary fixative for 2 h (3% glutaraldehyde in 0.1 M phosphate buffer pH 7.4). The tissue was then rinsed in the same buffer and placed in 2% osmium tetroxide in ice-cold buffer for 2 h. After rinsing with distilled water and block staining with a saturated aqueous uranyl acetate solution for 2 h, the tissue was dehydrated in ethanol and embedded in Epon. For the chromaffin reaction, the procedure of Tranzer & Richards47 was followed. Tissue from 4 animals was first fixed for 15 min in ice-cold 1% glutaraldehyde + 0.4% paraformaldehyde buffered to pH 7.2 with 0.1 M chromate-dichromate buffer, immersed in 0.2 M chromate-dichromate buffer pH 6.0 overnight and then placed in 2% osmium tetroxide in 0.1 M chromate-dichromate buffer, dehydrated and processed as above.

Thin sections were cut using a Porter-Blum microtome and were stained with uranyl acetate and lead citrate. They were observed in a Philips 301 electron microscope. For purposes of analysing axon profiles, 10 montages of sections through complete ganglia from conventionally fixed tissue were made at initial magnifications of 5000 or 7500.

In order to overcome some of the problems associated with the analysis of thin sections of neuropil (see Discussion) a very large number of axons (more than 10,000) from 10 different ganglia was considered. Axons were counted from montages at a final magnification of 15,000. The proportion of vesicle-containing profiles varied from ganglion to ganglion. In the largest montage, containing both neuronal and interconnecting nerve bundle areas, 3175 axons were counted of which only 189, or 6%, contained vesicles. Each axon profile encountered was classified only if it contained 10 or more vesicles; thus axons containing only a few scattered vesicles were rejected. A total of 1124 terminal varicosities was classified.

The 6 axon types considered were derived on purely morphological criteria described below. A few nerve profiles transitional between these types were noted separately, but for the sake of accuracy they were not placed in any specific category. No attempt was made to ascribe functional interpretations or determine possible relations between axon types until all the observed profiles had been classified as belonging to one of the morphological axon types used. For each axon examined, it was also noted whether it showed any synaptic specialization and approximately where it lay in the plexus (see Table 1).

 Abbreviations: ACh, acetylcholine; AGV, Small agranular vesicles; ER, endoplasmic reticulum; GABA, γ-aminobutyric acid; HG, heterogeneous granular vesicles; 5-HT, 5-hydroxytryptamine; LGV/LOV, large granular/large opaque vesicles; NA, noradrenaline; SFV/SGV, small flattened/small granular vesicles.
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RESULTS

Morphological categories of axons

Many axon profiles show swellings, or varicosities. These can be connected at one or both ends to inter-varicose narrow portions of the axons, containing microtubules, neurofilaments and occasionally smooth endoplasmic reticulum (ER) and mitochondria. The expanded portions of the axons also contain mitochondria and smooth ER and a few lysosomes, but these organelles are not considered to be distinguishing features of axon types and will not be mentioned again except where they are particularly relevant.

The morphological categories of axons observed are:

Type 1 Small granular vesicles

This kind of profile contains small granular vesicles (SGV) (Fig. 1), which are mostly round, 45–70 nm diameter, with an electron-dense core. The density and size of this core is highly variable in conventionally fixed tissue, and may vary from a small dot located either centrally or eccentrically in the vesicle to a very dark core occupying almost the entire vesicle. There are also a few flattened or irregular vesicles present, as well as some large granular vesicles similar in appearance to those of type 2 (see below).

Type 2 Small agranular vesicles

The main distinguishing feature is the predominance of a large number of small agranular vesicles (AGV) (Fig. 2). These are mostly round, 45–60 nm diameter, but a few are flattened or irregular. A few large granular vesicles (LGV) are usually present, but they are always outnumbered by AGV in a ratio of at least 10 to 1. These LGV are 80–120 nm diameter with a core of very high electron-density. There is a clear halo between the core and the limiting membrane which is always complete and trilaminar. This is the commonest profile type encountered in the plexus, accounting for approximately 60% of all classified axons.

Type 3 Small flattened vesicles

This is characterized by a predominance of flattened vesicles (SFV) (30–40 \times 60–80 nm) together with a variable mixture of small agranular vesicles (45–60 nm diameter), very small agranular vesicles (30–40 nm diameter) and a few large granular vesicles (80–120 nm diameter) (Fig. 3).

Type 4 Heterogeneous granular vesicles

These are characterized by granular vesicles (HGV) of a very heterogeneous size and nature (Fig. 4). They range 70–140 nm diameter and have a core of varying density and granularity. The halo around the granule is often obscure and frequently absent altogether. The limiting membrane is trilaminar but frequently incomplete. A variable number of small agranular round vesicles and a few flattened vesicles is also present. Type 4 profiles frequently form synapses (see below) in which the small agranular vesicles are always found interposed between the large granular vesicles and the presynaptic membrane.

Type 5 Large opaque vesicles

The large opaque vesicles (LOV) have a characteristic appearance with dense cores occupying all or almost all of the vesicle (Fig. 5a). If a halo is present at all, it is usually indistinct. The core is less electron-dense than in large granular vesicles and its texture is slightly variable from one vesicle to another, but the vesicles do not present as heterogeneous an appearance as type 4 (HGV). The vesicles range in size 90–140 nm.

This kind of nerve profile varied widely in its appearance ranging from one extreme which contained only large opaque vesicles to the other which contained both large opaque and a considerable number of small agranular vesicles; for examples, see Figs 5a and 5b.

Type 6 Large granular vesicles

These profiles range from those containing only large granular vesicles (LGV), when they form about 10% of the total identifiable axon population (Fig. 6a), to those that also have a variable number of small agranular vesicles, which are mostly round (45–60 nm diameter) (Fig. 6b). This kind forms about 5% of the total axon population. The LGV's are similar to those found in type 2 profiles—they are 80–120 nm diameter and generally show a clear halo around the core. The core is finely granular and varies only a little in density from vesicle to vesicle. Type 6 profiles are clearly distinguishable from type 2 profiles in that the LGV content is always greater than 30% whereas type 2 profiles always contain less than 10%.

Other nerve profiles

Several other types of profiles have been observed, but in too small numbers to justify separate classification.

Profiles containing 'pleomorphic' large granular vesicles were also seen, but only very rarely (Fig. 7). These are round, ovoid or kidney-shaped in appearance and range in size from 70 nm (minimum diameter) to 300 nm (maximum diameter). The core of these vesicles is very dense and finely granular and always surrounded by a narrow halo. A few small agranular vesicles are present. In view of the scarcity of these axons, no firm comment can be made about either their distribution or whether they form synapses.

Processes containing tubular structures filled with a moderately electron-dense material are occasionally found (Fig. 8). The tubular structures are about 40 nm wide and of variable length. These profiles also contain a few round agranular vesicles and large granular vesicles similar to those of type 2 (AGV).
A classification of axons in the rabbit myenteric plexus

Table 1. Nerve profiles in the rabbit myenteric plexus

<table>
<thead>
<tr>
<th>Nerve profile Type</th>
<th>% of total and actual no (%)</th>
<th>% forming synapses and actual no (%)</th>
<th>% lying superficially and actual no (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small granular vesicles (SGV)</td>
<td>6.3 (71)</td>
<td>9.9 (7)</td>
<td>8.5 (6)</td>
</tr>
<tr>
<td>Small agranular vesicles (AGV)</td>
<td>58.6 (659)</td>
<td>13.8 (91)</td>
<td>28.7 (189)</td>
</tr>
<tr>
<td>Small flattened vesicles (SFV)</td>
<td>4.0 (45)</td>
<td>8.9 (4)</td>
<td>33.3 (15)</td>
</tr>
<tr>
<td>Heterogenous granular vesicles (HGV)</td>
<td>3.7 (42)</td>
<td>16.7 (7)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Large opaque vesicles (LOV)</td>
<td>10.8 (121)</td>
<td>0 (0)</td>
<td>16.5 (20)</td>
</tr>
<tr>
<td>Large granular vesicles (LGV)</td>
<td>14.9 (168)</td>
<td>5.4 (9)</td>
<td>3.0 (5)</td>
</tr>
<tr>
<td>'Pleomorphic' vesicles</td>
<td>&lt; 0.4 (4)</td>
<td>0 (0)</td>
<td>75 (3)</td>
</tr>
<tr>
<td>Tubular structures</td>
<td>0.9 (10)</td>
<td>10.0 (1)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Small mitochondria</td>
<td>&lt; 0.4 (4)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Total</td>
<td>100.0 (1124)</td>
<td>10.6 (119)</td>
<td>21.3 (238)</td>
</tr>
</tbody>
</table>

Axon profiles containing tightly-packed clusters of small mitochondria are observed but only rarely (Fig. 13). A few microtubules and neurofilaments may be present and occasionally such profiles also contain many synaptic vesicles. Similar profiles have been seen by Gabella¹² and Cook & Burnstock⁷ in the guinea-pig myenteric plexus.

In this investigation, unlike that of Cook & Burnstock,⁷ profiles containing large numbers of glycogen granules were not observed. However, there are axons with large empty-looking regions in the axoplasm, which once may have been occupied by glycogen, perhaps extracted during the uranyl acetate block-staining procedure.

A few profiles contain lysosomes, multivesicular bodies and small dense lamellar bodies. Also, occasionally, there are a few profiles containing small electron-lucent vesicles set in a homogeneously darker axoplasm. Table 1 summarizes the numbers and proportions of different types of nerve profile and those forming synapses on ganglion cells or lying superficially.
**Chromaffin reaction**

The chromaffin reaction was introduced by Tranzer & Richards,\(^\text{25}\) as an effective way of visualising biogenic amines in vesicle cores. It is important to note that the chromaffin reaction detects noradrenaline, dopamine and 5-HT.\(^\text{23}\)

In the present investigation, two populations of nerve fibre give a positive reaction. One type of axon gives a very dense deposit in some, but not all, of the small round vesicles. In this kind of profile a few flattened vesicles with a diffuse reaction product are also observed (Figs 10, 11).

Another type of profile with small flattened vesicles reacts to give only a small core or a diffuse product of medium electron-density throughout the vesicle (Fig. 11).

In both kinds of profile, the density of the large granular vesicles does not seem to be increased.

Nerve profiles containing small agranular round vesicles which are chromaffin negative can be observed in the same specimens as those containing the two populations of chromaffin-positive nerves.

**Synapses**

Synapses with membrane specializations are formed by many, but not all, of the classes of nerve profiles described above. The synapses are asymmetrical with the postsynaptic density always being more prominent and thicker than the presynaptic density. The synaptic cleft is always fairly evenly spaced at around 20 nm and contains a moderately electron-dense substance (Figs 1, 2, 4, 6b and 9).

As noted by Gabella\(^\text{1}\) there is a great variability in the size and shape of the active zone and degree of clustering of vesicles. In the present study it has proved impossible to find any obvious relationship between any nerve category and the morphology of the synapses it forms.

Synapses are found on cell bodies and directly on dendrites, and also, but much less frequently, on what appear to be dendritic spines. However, the possibility that these latter structures may represent somatic spines sectioned tangentially cannot be excluded. No synapses involving gap junctions have been observed in this tissue. Some neurons may receive synapses from up to three different categories of nerve fibre.

Type 2 (AGV) fibres form about 75% of all synapses observed; most of these are axo-somatic or axo-dendritic (Fig. 2) and a few are on somatic or dendritic spines.

Types 1 (SGV) and 3 (SFV) both form synapses on cell bodies and on dendrites.

Type 4 (HGV) fibres form synapses on cell bodies, dendrites (Fig. 4) and spines.

Type 5 (LOV) nerve fibres containing large opaque vesicles are never seen to form synapses of any sort. It is difficult in a study of this sort, using only thin sections, to rule out the possibility that such synapses do exist, but in view of the appreciable numbers of these profiles observed, synapses are considered to be rare or absent.

Type 6 fibres have been observed to form synapses, but only when the terminals also contain many small agranular vesicles (Fig. 6b). These synapses are both axo-somatic and axo-dendritic, formed either directly or on spines. The small agranular vesicles are always nearer to the active zone than the large granular vesicles.

**Other features of vesiculated nerve profiles**

Vesicle-containing nerve profiles lying on the surface of the plexus just beneath the basal lamina are frequently found. They may be partially or wholly free of glial cell investment and they may show a clustering of vesicles and a membrane specialization associated with electron-dense material reminiscent of a presynaptic thickening (Fig. 12). About 20% of all identifiable varicosities lie superficially, and of these, the vast majority (80%) are type 2 endings, containing mostly agranular vesicles. Other fibres lying superficially contain small flattened vesicles or a mixture of large opaque and small agranular vesicles. When a vesiculated nerve profile lies in such a superficial position it only very rarely forms a synapse on a neural structure. Instead, it may be that the release of transmitter is directed towards the exterior of the plexus.

**DISCUSSION**

A classification of axon profiles is presented in this paper and is compared with previous studies (see Table 2), although the use of different species, regions of the gut and preparative procedures makes this comparison difficult.

Type 1 profiles (SGV) resembled the SGV containing profiles in enteric plexuses reported by Gabella,\(^\text{11,13}\) Wong, Helme & Smith\(^\text{2}\) and Cook & Burnstock,\(^\text{7}\) who suggested that these profiles were adrenergic. The type 1 profiles also resembled those reported in many other peripheral adrennergically-innervated tissues,\(^\text{7}\) in that the vesicles were of a similar size and contained an electron-dense core.

Type 2 fibres contained mainly small agranular vesicles and only a few large granular vesicles. The majority of enteric neurons has been classically considered to be cholinergic.\(^\text{1,7}\) Most stain to some degree for acetylcholinesterase,\(^\text{13}\) and the myenteric plexus contains large amounts of acetylcholine.\(^\text{22}\) The present study, which showed that 60% of the profiles were type 2, is compatible with the observations of the authors mentioned above.

Type 3 terminals which contained a mixture of small flattened and small round vesicles together with a few large granular vesicles, corresponded to the classes described as type 3 by Gabella\(^\text{12}\) and by Cook & Burnstock.\(^\text{7}\) The different appearances of vesicles observed in type 3 profiles in the present study may be due to different planes of sections through randomly orientated but uniformly disk-shaped or cylin-
Fig. 1. Type 1 axon (a) containing small granular vesicles, diameter 45–70 nm. The arrow shows a synaptic membrane specialization. A type 2 ending (b) is also seen. x 50,000.

Fig. 2. Type 2 axon containing mostly round small agranular vesicles, diameter 45–60 nm. A large granular vesicle, diameter 90 nm, with clear halo is seen in the upper lefthand corner of the picture. The arrow shows a synaptic membrane specialization. x 50,000.

Fig. 3. Type 3 axon with a mixture of a fairly large number of flattened vesicles (about 30 × 75 nm) and a few small round agranular vesicles. Some of them appear to have a moderately electron-dense material inside. x 50,000.

Fig. 4. Type 4 axon characterized by heterogeneous granular vesicles, diameter 80–120 nm. These have cores of differing electron-density. Along the synaptic membrane (arrow) small agranular vesicles are observed. x 50,000.

Fig. 5a. Type 5 axon containing almost exclusively large opaque vesicles with a less distinct halo, diameter 90–140 nm, and only a few small agranular vesicles. x 50,000.

Fig. 5b. Type 5 axon dominated by large opaque vesicles with less clear halo, (90–140 nm) and also some small agranular vesicles. x 50,000.

Fig. 6a. Type 6 axon containing almost exclusively large granular vesicles with a clear halo, diameter 80–120 nm. x 50,000.

Fig. 6b. Type 6 axon dominated by large granular vesicles with a clear halo, diameter 80–120 nm. Some agranular vesicles are seen close to the synaptic region (arrow). x 50,000.

Fig. 7. Axon with pleomorphic large granular vesicles (maximum and minimum diameters around 300 and 70 nm respectively). There are also a few small agranular vesicles. x 50,000.

Fig. 8. Nerve process (a) characterized by tubular structures, (diameter about 40 nm), filled with a moderately electron-dense material. Large granular and small agranular vesicles are also seen. A membrane specialization is observed between this nerve process and axon (b) (arrow). Another membrane specialization is also seen, with small agranular vesicles accumulating at the synaptic region in axon (b) (double headed arrow) x 50,000.

Fig. 9. A high power electron micrograph of the active zone of a synapse formed by a type 6 axon. Moderate electron-dense material can be seen between the pre- and post-synaptic membrane. x 100,000.

Fig. 10. Chromaffin-fixed material. Small round vesicles show cores of high electron-density which represent amine reaction product. The cores of large granular vesicles are apparently not enhanced in density, (arrows). x 100,000.

Fig. 11. Chromaffin-fixed material. Weak reaction product is observed inside the flattened vesicles (a). Similar weakly electron-dense cores are seen in a few flattened vesicles of an adjacent axon with mostly small round granular vesicles (b). x 100,000.

Fig. 12. A cluster of type 2 profiles lying in the peripheral region of the ganglion. An accumulation of synaptic vesicles along the surface cell membrane (arrow) is observed in one of these axons. x 40,000.

Fig. 13. Axon profile (a) at the upper left hand corner may be a sensory nerve ending. It contains a tight cluster of small mitochondria. On the other hand, another axon profile (b) contains both synaptic vesicles and small mitochondria in different parts of the axoplasm. x 30,000.
A classification of axons in the rabbit myenteric plexus

Table 2. Comparison of classifications of nerve profiles in the myenteric plexus

<table>
<thead>
<tr>
<th>Present study Rabbit colon</th>
<th>Cook &amp; Burnstock(^7) guinea-pig</th>
<th>Gabella(^{12,13}) guinea-pig</th>
<th>Baumgarten et al.(^1) Man, guinea-pig, monkey</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small granular vesicles (SGV)</td>
<td>(1) SGV</td>
<td>(1) SGV</td>
<td>—</td>
</tr>
<tr>
<td>Small agranular vesicles (AGV)</td>
<td>(2) AGV</td>
<td>(2) AGV</td>
<td>'cholinergic'</td>
</tr>
<tr>
<td>Small flattened vesicles (SFV)</td>
<td>(3) flattened</td>
<td>(3) flattened</td>
<td>—</td>
</tr>
<tr>
<td>Heterogenous granular vesicles (HGV)</td>
<td>(5b)</td>
<td>HGV</td>
<td>'p-type'</td>
</tr>
<tr>
<td>Large opaque vesicles (LOV)</td>
<td>(5c)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Large granular vesicles (LGV)</td>
<td>(5a)</td>
<td>LGV-AGV (Fig. 10b, 1979)</td>
<td>'adrenergic'</td>
</tr>
<tr>
<td>'Pleomorphic' vesicles</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tubular structures</td>
<td>(4) tubular</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Small mitochondria</td>
<td>(6) 'sensory' small mitochondria</td>
<td></td>
<td>—</td>
</tr>
</tbody>
</table>

Several authors\(^{20,21,27}\) have shown that increasing the osmolarity of the primary aldehyde fixative promotes the appearance of flattened vesicles in some, but not all, nerve terminals containing small round vesicles. Thus, it has been argued that even if the flattened appearance is the result of a fixation artefact, the effect is differential between types of nerves, and flattened terminals represent a separate class of nerve.\(^{7,12}\) Fehér, Csányi & Vajda\(^a\) chronically extrinsically denervated segments of cat ileum and demonstrated that in permanganate-fixed material (elective for biogenic amines), the extrinsically denervated intestine still showed 6% small granular vesicles as opposed to 13% in the intact tissue. In the guinea-pig gut, Gordon-Weeks & Hobbs\(^15\) described two separate categories of small granular vesicle-containing nerves differing in their reactivity with false aminergic transmitters. In the present study, two distinct populations of profiles containing vesicles with some electron-dense deposits (one with small and round vesicles and one with small and flattened vesicles) were observed using both chromaffin and conventional fixation techniques; the chromaffin reaction is positive for biogenic amines.\(^23\)

Baumgarten's category 'p-type' and Gabella's HGV profiles contain large vesicles with granular cores.
Baumgarten originally called the profiles 'p-type' because they showed similarities to polypeptide containing neurosecretory cells. However, there is no direct evidence as yet that these vesicles contain polypeptides. These broad categories probably include both our types 4 and 5 (see Table 2). At least some of these profiles have been shown to be intrinsic in the guinea-pig intestine, for they survive extrinsic denervation. Only Cook and Burnstock attempted to distinguish different profiles of this kind, mainly on the basis of the size of the vesicles; their type 5a appears to correspond to our type 6 (LGV), type 5b to our type 4 (HGV) and type 5c to our type 5 (LOV). The variability in the number of small vesicles seen accompanying the large opaque vesicles both contain large granular and small agranular vesicles, but in different proportions. It has been claimed that different types of autonomic nerves can be distinguished by the proportion of vesicles they contain. In the present investigation, type 6 profiles contained 30% or more large granular vesicles.

Type 6 profiles resemble type 2 profiles in that they both contain large granular and small agranular vesicles, but in different proportions. It has been claimed that different types of autonomic nerves can be distinguished by the proportion of vesicles they contain. In the present investigation, type 6 profiles contained 30% or more large granular vesicles.

Very few irregularly-shaped or pleomorphic vesicle-containing terminals (Type 7) were seen. It is possible that these represent nerves affected in different ways by the preparative procedures used. For example, hypotonic media, adrenergic LGV are known to undergo shrinking and become more oblate with a reduced halo and increased core density.

Axons containing tubular structures and some also containing lysosomes, multivesicular bodies and lamellar bodies (Type 8) are not considered to belong to any special nerve class, but instead may represent either nerves in which transport of membranous organelles is taking place, or dendrites. Tsukita & Ishikawa have observed transport of similar membranous structures in the mouse saphenous nerve.

While it is clearly desirable to find an ultrastructural basis for different types of nerves which contain different transmitters, there are several problems associated with this objective: first, the possibility that the same nerve cell may contain and release more than one transmitter; secondly, the possibility that profile appearances are partly due to an uneven distribution of vesicles within the terminal regions of a single axon type (cf. Fig. 19); thirdly, the effects of fixation on vesicle morphology are still poorly understood. The resolution of this question depends on the development of specific cytochemical methods for transmitters and associated enzymes that allow high resolution at the electron microscopic level.

REFERENCES


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