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THE ANATOMICAL SYNAPSE IN MAMMALS BY LIGHT AND ELECTRON MICROSCOPY

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ABSTRACT

The original descriptions of synapses by light microscopy are (examined, end their widths and numbers per neuron soma are compared in light and electron micrographs. Electron micrographs show the synaptic cleft very frequently normal to the plane of section. It is rare to find any connection of the synaptic knobs to any dendrite of another axon, and the granular appearance on the surface of unfixed mammalian neurons can be shown to be due to intracellular particles - probably mitochondria. It is concluded that the anatomical synapse is an artifact. This raises questions about the nature of chemical transmission and the vesicle hypothesis. It may require a reinterpretation of transmission, but does not affect physiological or pharmacological findings about-the effects of transmitters.

INTRODUCTION

Sherrington (1,2) invented the term 'synapse' to explain the properties of reflexes. At about the same time, Held (3) examining rabbit trapezoid nucleus and Auerbach (4) looking at guinea pig acoustic tubercle described 'end-fusse' on and near somas in tissues stained with silver stains (figs 1,2). Elliott (5) was the first to suggest that adrenalin might be able to transmit excitation. From these findings the concept of transmission became elaborated, and -the idea that it occurred at synapses or neuromuscular junctions gradually evolved. Experiments by Katz and his collaborators on the frog neuromuscular junction - generally regarded as a model of interneuronal synapses - led to the vesicle hypothesis (6,7,8).

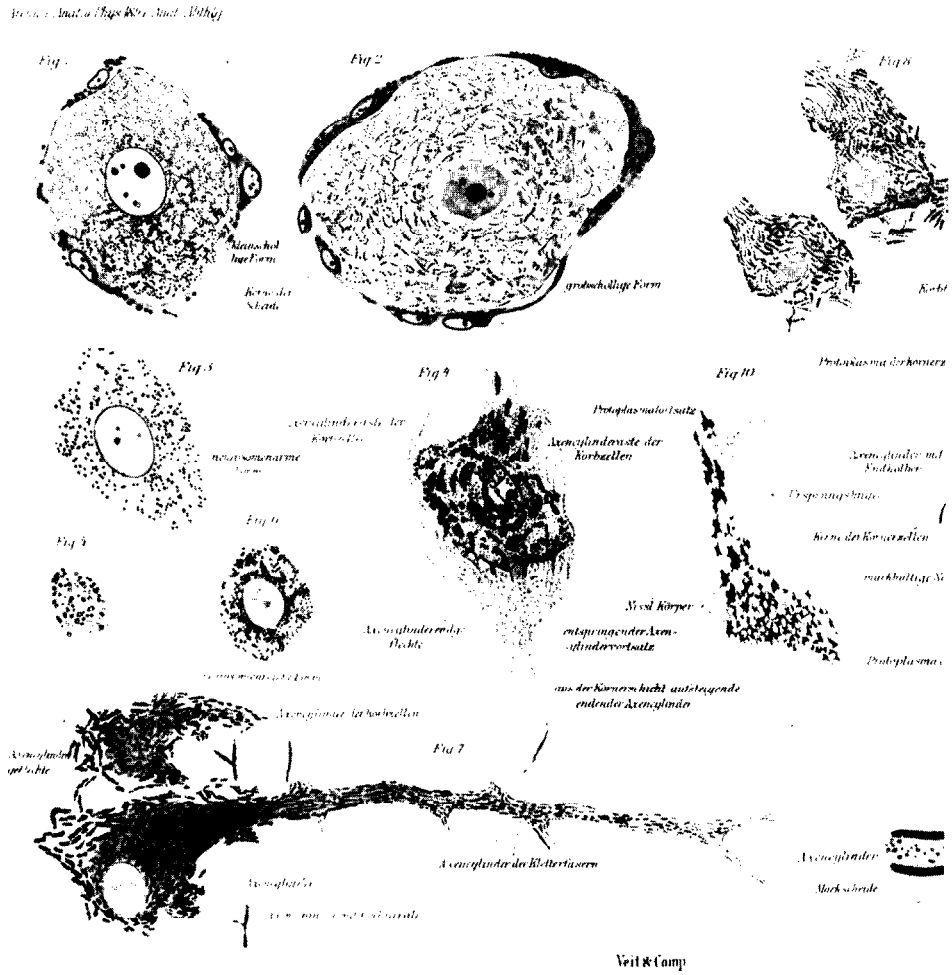

Figure 1. Drawings of neurons from rabbit trapezoid nucleus showing silver stained end-feet, which are considered one of the first demonstrations by light microscopy of synapses. Note particularly figures 1, 2, 4,7,8. These illustrations are from Held (3, his plate XI).

The term synapse has been used, firstly to summarise a number of physiological characteristics of the relationships of nerve pathways, and, secondly, to describe an anatomical structure, which is believed to be the locus of the latter physiological features. Nowadays, the existence of the anatomical synapse is regarded as central to all hypotheses about transmission (9, 10, 11, 12). This paper is an examination of the evidence for the existence of the anatomical as distinct from the physiological synapse.

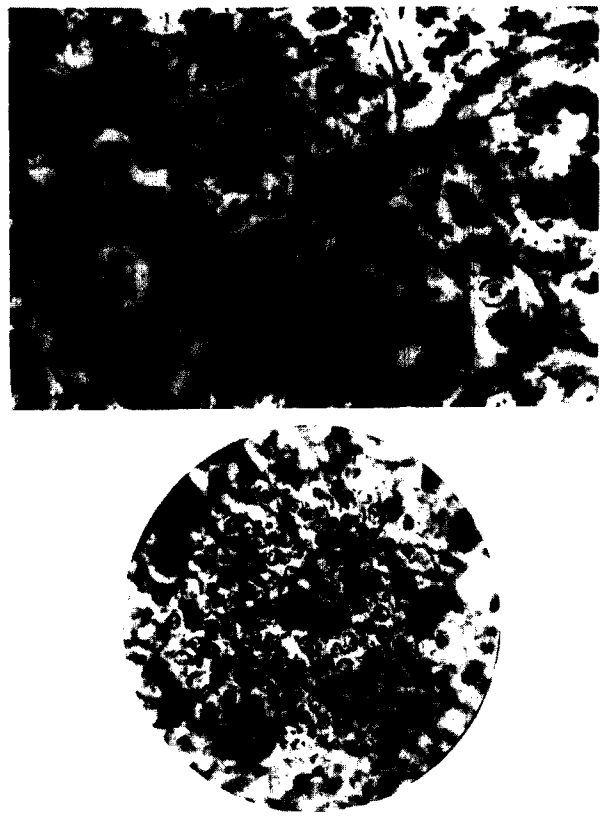

Figure 2. A neuron in the acoustic tubercle of an old guinea-pig. This was one of the first micrographs of what were subsequently described as synapses The original legend indicates that the cel body is surrounded by a "wreath" containing small knobs The cell body is about 50 μm in maximum diameter, and the distance of these knobs from the cell body should be noted (from Auerbach, 4).

The anatomical synapse has become so completely integrated into current belief that drugs having pre- and post-synaptic actions have been defined by the use of pharmacological blockers of each of these sites, and pharmacologists today confidently speak of drugs which act at each of these alleged sites. The existence of the anatomical site has not been questioned for many years, nor have the anatomists, physiologists, pharmacologists or biophysicists reviewed critically how one could with confidence assert that one could know the location of the tip of a micropipette, or action of a drug down to the nearest 1-3 μm.

CURRENT VIEWS ON THE SYNAPSE

The synapses appear by light microscopy as knobs on the surface of the neurons, occasionally with fibres attached to them (3,4,13,14). They are usually seen stained by silver stains (fig. 3, reproduced by permission from (14)). By electron microscopy, they are characterised by pre- and post-synaptic thickenings with a clear synaptic cleft between them, and they contain synaptic vesicles (15-19).

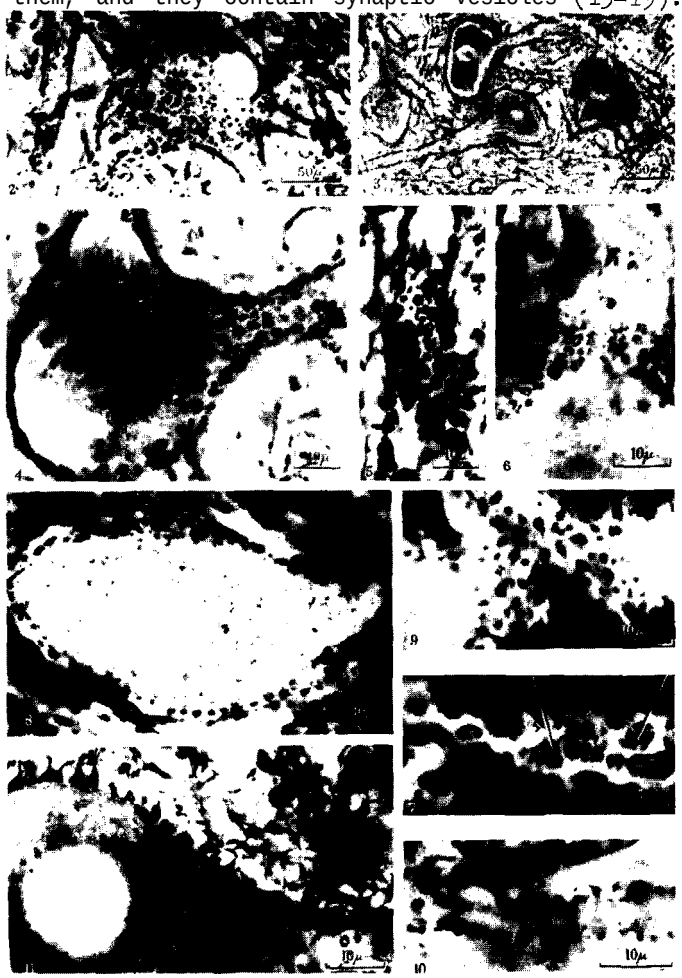

Figure 3. 'End feet' (synapses) on the neurons of the ventral horn of the thoraco-lumbar region of the cat. The 'end feet' were stained with silver stains. Please note the following features: the diameters of the end feet; the rarity, except on a few end feet in 11, of fibres attached to the end feet; the frequent presence in regions not obviously on the neuron surface, of silver-stained granules and fibres. This figure is reproduced from Wyckoff and Young (14) by kind permission of Professor Young and the Royal Society.

THE SYNAPSE BY LIGHT MICROSCOPY

A. Silver preparations were widely studied by light microscopy, and they all show granules on or near neuronal somas (figs 1 - 3). However, close scrutiny of micrographs (fig. 3; table 1) - as distinct from drawings or diagrams - shows: (a) some granules at a considerable distance from the neuron somas; (b) granules in the plane of the section close to the somas, but at sufficient distance that the 'synaptic cleft' can be seen clearly by low power light microscopy; (c) granules sometimes evenly distributed over the surface of the somas, end axons in histological sections.

Tissue	Reference
mammalian spinal cord	(65)
cat ventral nucleus of acoustic nerve	(66)
cat ventral horn cells	(67)
cat cervical sympathetic ganglion	(68)
cat ventral horn cells	(69)
human cerebral cortex	(70)
mammalian nervous system	(71)
cat visual cortex	(34)
cat ventral horn cells	(14)
rat superior cervical ganglion	(72)
kitten ventral horn cells	(73)
rat Deiters nerve cell	(24)
dog ventral horn cells	(74)
cat ventral horn cells	(75)
kitten ventral horn cells	(76)
cat reticular formation	(77 p.11)
cat medulla oblongata	(78)
rat reticular formation)	(79)
cat ventral horn cells)	
rat ventral horn cell	(80)

Table 1. Illustrations by light microscopy showing knobs of similar size to synaptic knobs outside the cell body. The references are arranged in date order.

These observations must be considered in detail.

(a) the following explanation for the appearance of granules some distance from the neuron are possible: they are synapses which have been displaced during section or histological preparation. This explanation would implicitly cast doubt on any localisation of granules

of those dimensions after histological preparation; or, the granules are synapses on somas or axons which are themselves not in the focal plane; or have been missed by the microtome. This is possible, but if it were so, one would expect to see the granules in the same sort of close groupings or in the rows, as they are seen on the somas or axons. The granules away from the somas or axons nearly always appear single (please see also the micrographs in publications listed in table 1 or elsewhere).

(b) One usually sees what appear to be synaptic clefts at the periphery of neurons by relatively low power light microscopy, which would give a resolution not better than 3 - 5 μm. This is about two orders of magnitude greater than the width of the cleft of 15 - 40 nm seen by electron microscopy (table 2).

Tissue	Width (nm)	Reference
rat abducens nucleus	20	(81)
rat abducens nucleus	20	(24)
rat dorsal cochlear nucleus	20	(54, p.17)
monkey lateral geniculate nucleus	20	(82)
cat subfornical organ	20	(83)
rat cerebral cortex	30 - 40	(79, p52)
rat prepyriform cortex	20 - 30	(15, p39)
rat cerebral cortex	20	(84)
cat oculomotor nucleus	20	(85)
rat ventral horn of spinal cord	20	(86, p370)
neurons from various species	20-30	(17, p135)
neurons from various species	15-25	(77, p11)

Table 2. Distance between pre and post synaptic membranes seen by electron microscopy.

(c) If one can see the maximum diameter of the nucleus, or the nucleolus in a histological section, it is evident that the section must have been made fairly near the equator of the cell body. Since the diameter of the cell body is usually several times greater than the thickness of the section, it follows that a section through the equator of the cell is unlikely to contain a surface of a cell, unless the cell had been flattened considerably. Therefore, in a section - as opposed to a whole isolated neuron soma - one would not expect to see synapses evenly spread over the surface of the cell body or axon; they should appear in greater density at the edge of the soma or axon. They should only be seen evenly distributed in sections cut thicker than the diameters of somas, - or if the granules of silver stain had been not only displaced, but also randomly distributed over the cell body and axon, after such displacement.

Two possibilities exist for granules of silver or silver-tissue complexes which are commonly described as synapses. It is conceivable that the granules on the cell bodies and axons are indeed synapses, but the granules appearing away from these sites are not synapses. Although this is possible, acceptance of such a view would then render it difficult to define any histological criteria by which the two types of granules could be differentiated, other than by their locations. If the latter is the only criterion, one must suppose that the sites of the 'artifact' granules have the same affinity for stain as the synapses have; this poses considerable problems in relation to the biochemistry, immunochemistry and pharmacology of receptors.

The heavy metal stains usually used in histology and electron microscopy make insoluble complexes with tissue. Therefore, one must entertain the possibility that the granules are precipitates of the heavy metals or their salts, with substances in the nervous tissue which in life were liquid or consisted of a suspension too fine tobe seen by light microscopy - that is to say, they are an artifact of preparation not present in the living tissue.

B. Several authors have been aware of the difficulty of seeing synapses in the mammalian central nervous system (see, for example 13, 14, 20). However, when structures were seen by electron microscopy all over the central nervous system and they were given the same name, the newer findings rapidly became accepted as providing confirmatory evidence for the existence of the synapses, even in the locations in which they could not be seen by light microscopy (see, for example, Johnston and Roots, (15) page 35; Peters, Palsy and Webster (16) page 124).

LIGHT MICROSCOPY AND ELECTRON MICROSCOPY

c. There is an important discrepancy between the images seen by light microscopy and those seen by electron microscopy. A minority but highly significant proportion of synapses appear to be connected to fibres two to five times as long as the diameters of the synapses, (fig. 3, from (14). By electron microscopy, one should see approximately the same appearance as by light microscopy, except, of course, larger. However, it is extremely rare to see any attachment to synapses either radial or tangential by electron microscopy (fig. 4). This will be considered in greater detail below,

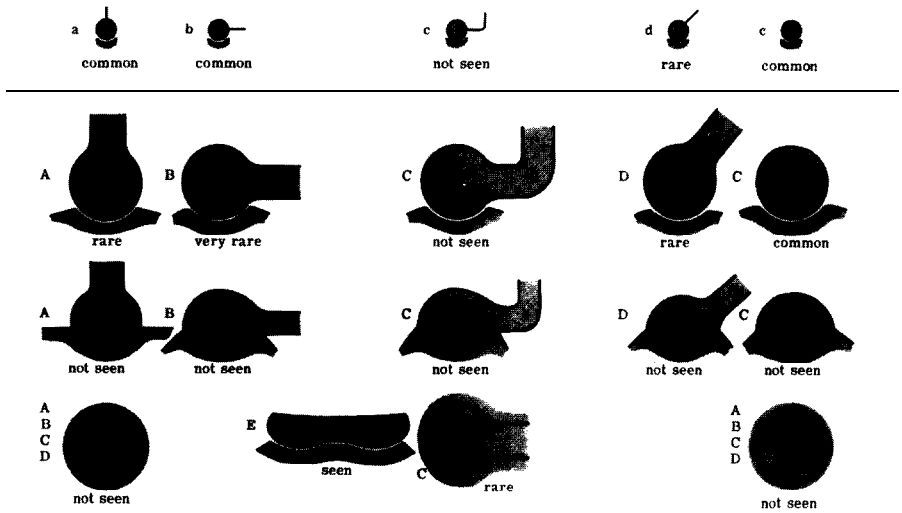

Figure 4. Drawings of possible appearances of synapses by light microscopy (top line, a, b, c, d) and electron microscopy (lower lines, A, B, C, D, E); the same letters should refer to the expected views of the same shaped synapse at different magnifications and orientations. The black lines and shading indicate cell membranes and pre- and post-synaptic thickenings; the grey shading indicates the cytoplasm of the synapses and the neurons. These diagrams are based on the assumption that the synapses are randomly distributed over the surface of the neurons. The appearances of the synapses by light microscopy should be completely compatible with their appearances by electron microscopy. Geometry dictates unequivocally that all the appearances of the electron microscopic images indicated by a particular capital letter should be seen, and they should occur in approximately equal frequencies. Examination of the considerable literature on the structure of synapses fails to show a significant incidence of any of the images of the third line down (with oval pre- or post-synaptic thickenings), nor of the segmental sections of the synapses as seen at the bottom extreme left and extreme right. Light microscopic images corresponding to the 'double' synapse seen on electron microscopy (E) do not seem to have been reported either (please see references on page). It should also be noted that the common views by light microscopy a, b, do not correspond to the common view by electron microscopy, C, in the right hand column.

D. The geometry of the synapses seen by electron microscopy shows a number of serious anomalies (fig. 4). Firstly, the synaptic cleft nearly always appears normal to section. If simple geometry were obeyed, and if the synapses were distributed randomly over the surfaces of the somas and axons, one would expect to see the synaptic cleft clearly very rarely. The whole synapse should be seen as a circular or

an oval solid object, and it should appear to encroach often on both the pre- and the post-synaptic regions much more frequently. Most of the views of the synaptic cleft which solid geometry would require are hard to find in the electron microscope or in the literature (fig. 4).

The usual explanation given for this is that electron micrographs are selected for submission for publication on the basis of a sharp synaptic cleft. However, examination of relatively large fields in which several synapses can be seen in the same micrograph reveals a vast preponderence of clefts normal to the surface, even sometimes when the pre- and post-synaptic thickenings are blurred (see, for example, 16-19).

One may proffer an explanation for the high incidence of the two-dimensionality of the synaptic cleft. The stain deposits on a part of the neuronal membrane, either because it has particular affinity for the chemical constituents in that particular region, or because particles of a precipitate must settle somewhere (since the dye particles represent only a small proportion of the volume of the stain-tissue suspension); the conditions of high vacuum and electron bombardment in the electron microscope are likely to make the underlying membrane vaporise. This would occur on the surface of the preparation during the evacuation of the electron microscope and when the electron beam is turned on. The vapour from the tissue would cause a crack in the overlying stain-tissue granule, which would appear on the surface as a cleft.

Electron microscopists who -believe that the frequency of the appearance in publications of the sharpness of the cleft is due to selection for publication could demonstrate this by examining electron micrographs of a particular neuronal preparation selected at random, and measuring the apparent width of all the synaptic clefts or gaps; they should show a normal distribution.

An equation for calculating the expected incidence of the appearance of two parallel lines normal to a section, from knowledge of the apparent distance between the two lines, and the thickness of the section, has been given (21).

Tissue	Width (nm)	References
mammalian nervous system	500 – 7000	(87)
monkey ventral horn cell	950 X 800 – 3330 x 2600	(65)
cat ventral horn cell	500 x 100 – 4000 x 5000 (Mean 1500 X 2000)	(67)
cat ventral horn cell	7000	(68)
cat ventral horn cell	1000 – 5000	(88)
cat cerebral cortex	2500	(89)
cat ventral horn cell	15000 x 8000	(90)
rat ventral horn cell	1000 – 5000 (Mean 2000)	(14)
human cerebrum	800 – 2500	(91)
rat visual cortex	2000 – 4000	(20)
cat ventral horn cell	1000 – 4000	(82)
cat reticular formation	500 – 3000	(77)
rat cerebellum	1500 – 6000	(92)
cat ventral horn cell	1000 – 3000	
cat granule and stellate cells in cortex and cerebellum	500	(79)

Table 3. Diameters of synapses seen by light microscopy in the literature. The values were stated by the Authors. The references are in date order.

E. There is a rather small overlap between the widths of the synapse seen by light microscopy (table 3) and the widths seen by electron microscopy (table 4). The former appear usually more than 1000 nm. while the latter usually appear less than 1000 nm. One must be a little careful about comparing these dimensions, since a variety of staining and microscopic techniques has been used in the various studies, and usually the figures given by authors or measured from their illustrations, are general estimates, rather than calculations from large numbers of measurements; the latter would require: firstly, a random selection of micrographs prepared using a particular technique; secondly, knowledge of the thickness of the sections; thirdly, assumptions about the shapes of the synapses; fourthly, knowledge of the effects of different techniques of preparation on these dimensions.

The information in the publications cited in tables 3 and 4 is not sufficient to compare directly the size of the synapses in a particular neural tissue, but the general lack of overlap should be

Tissue	Width (nm)	References
rabbit ventral horn cell	1200	(14)
cat upper lumbar ventral horn cell	600 – 1000	(53, 94)
rat hippocampus	500	(95)
ox brain	500	(96)
rat prepyriform nucleus	800	(8:)
rat medial mammillary nucleus	800	(97)
rat cerebellum	1500	(98)
cat lateral vestibular nucleus	1300	(77)
cat subformical organ) monkey ventral horn cell)	500 – 800 500	(83)
rat cerebellar cortex	500 – 2000	(99)
rat prepyriform cortex	1300	(15, p.38)
rat cerebral cortex cat oculomotor nucleus cat sensorimotor cortex cat hippocampus	1200) 1000 – 1500) 650 – 1000) 1200)	(85)
rat median septal nucleus	500 – 2500	(100)
rat olfactory glomerulus	600 – 1000	(101)
monkey lateral geniculate body	1500	(102)
rat ventral cochlear nucleus	250 – 600	
rat cerebral cortex rat auditory cortex rat visual cortex	250) 500 – 750) 400 – 900)	(16, pps 118 – 135)
monkey visual cortex	600	(103)

'Table 4. Diameter of total width of synapses as measured from illustrations of electron micrographs in the literature. The accuracy of measurement would be about 50 nm from the printed micrographs.

a cause for concern, when one realises that a linear diameter, which is double another, contains a volume eight times as great. It is difficult to believe that differences of preparation technique, – for example, the use of silver compared with osmium staining, both in tissues which are dehydrated – could explain the apparent differences in dimensions of synapses. If this were alleged to be the explanation, it may have been demonstrated hitherto, or could be demonstrated relatively easily.

Neuron	Synapses per cell	Reported or counted	Reference
guinea-pig trapezoid body nucleus	54	c	(104)
	48	c	
	46	c	
	36	c	
	26	c	
mammalian ventral horn cell	1-10	s	(65)
cat ventral horn cell	14	c	(66)
	38	c	
	38	c	
	22	c	
	30	c	
cat ventral horn cell	1250	s	(67)
cat ventral horn cell	192	c	(68)
	388	c	
	156	c	
	130	c	
	416	c	
	68	c	
	106	c	
	148	c	
	114	c	
	234	c	
	150	c	
	70	c	
cat cervical sympathetic ganglion	> 13	s	(69)
monkey lateral geniculate body	1	s	(103)
	1	c	
human dorsal sensory and internuncial neuron	97	s	(106)
human intermediate sensory and internuncial neuron	152	s	
human ventral sensory and internuncial neuron	116	s	
human dorsal nucleus	348	s	
human intermediolateral nucleus	122	s	
human posteromedian nucleus	362	s	
human anteromedian nucleus	657	s	
human snterolateral nucleus	1,624	s	
human posterolateral nucleus	3,990	s	
human cerebral cortex	16	c	(70)
	24	c	
cat ventral horn cell	> 2000	s	(14)

rat superior cervical ganglion)	12	c	(71)
)	14	C	
dog lumbosacral ventral horn cell)	800	s	(74)
dog ventral horn cell)	600~10000	s	
cat ventral horn cell	84	c	(75)

Table 5. Number of synapses per neuron soma reported (s), or counted (c), from their publications, as detected by light microscopy. The references are in date order.

Neuron	Synapses per cell	References
rat spinal cord	~ 2000	(22,107)
mammalian	(many hundreds)	(54, p.15)
rabbit Deiters cell	~ 10,000	(108)
rat lateral geniculate nucleus)	133	(109)
)	83	
guinea pig cerebral cortex	800	(94)
mouse visual cortex)	7,000)	
mouse motor cortex)	13,000)	(110)
monkey visual cortex)	5,600	
monkey motor cortex)	60,000)	
vertebrates	10,000	(15)
cat cerebral cortex	870	(111)
cat spinal cord	(several thousands)	(112)
mammalian spinal motor neuron	500	
mammalian cortical pyramidal cell	30,000	(113)
mammalian cerebellar Purkinje cell	80,000	

Table 6. Number of synapses per neuron soma reported by electron microscopy.

F. An equally serious discrepancy occurs when one compares the number of synapses per neuron cell body seen by light (table 5) and by electron microscopy (table 6). The former – with some notable exceptions – reveals hundreds or less; the latter usually shows thousands or more; they are thus generally showing magnitudes of differences of incidence. Once again, one must bear in mind the reservations listed above, (E).

The explanation for the apparent discrepancy would be that either or both techniques do not necessarily stain all the synapses on cell bodies. or that either or both stain other elements in addition to synapses. This may be true; it would be difficult to prove unequivocally. If it were so, it would throw into doubt many measurements of numbers of synapses made hitherto, and would require a comprehensive and rigorous study of the whole question. Many calculations of the chemistry, physiology and pharmacology of transmissior would have to be re-evaluated.

Nevertheless, if indeed, the diameters and synapses of the knobs seen by light microscopy are grossly and significantly different from those seen by electron microscopy – taking into account the reservations entered – one must conclude, either that one or both technique is inappropriate, or that each technique demonstrates different objects, or that one or both of the objects shown by them is an artifact of preparation.

DO THE SYNAPSES CONNECT WITH OTHER CELLS?

G. Allusion has already been made briefly to the observation that very few of the synapses by light microscopy, and virtually none by electron microscopy, show a 'stalk' – presumably a dendrite of another neuron – by which the presynaptic knob is attached to another cell. The whole physiological concept of a synapse requires such an attack ment, since otherwise there could be no route for synaptic trans-mission. Occasionally one sees a fibre, whose length is two to three times that of the diameter of the synaptic knob, issuing from it (fig. 3). De Robertis, (22) has illustrated it beautifully in a well-known diagram, in which he puts 'telodendroglia' or stalks on all the synaptic knobs. The stalks stop abruptly, and even in his diagram they are not attached to any dendrite. The fact that one can sometimes see them shows that their dimensions are within the resolution of the light microscope, and that they are in the plane of the section; only those fibres normal to the surface would not be seen, but others should be observed coursing across the field from most synapses (as shown in fig. 5). Such fibres can be seen in commissures of the brain, in the spinal and in the cerebellar white matter. A micrograph of a section of the latter tissue shows fibres turning from the plane of the section towards a plane normal to it (fig. 6), so that one must conclude that if such connections were there, they could be seen. Many synapses are shown in thick silver stained preparations, which would give one a much greater possibility than would 4 – 6μm sections of seeing fibres in planes not parallel to those of the sections.

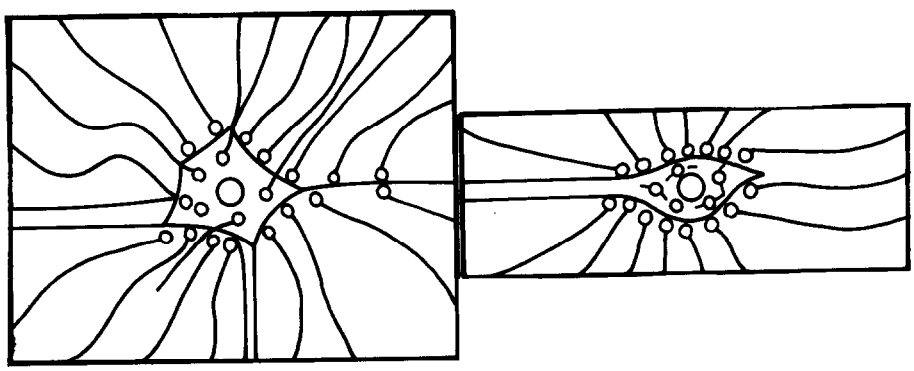

Figure 5. Expected plan (left) and lateral (right) views of synapses seen by light microscopy on the soma of a neuron. Please note the synaptic connections spreading to the edge of the field. This should be compared with figure 6.

Figure 6. White matter of the human cerebellum stained with Palmgren's stain. Please note how the fibres in the plane of the field in the lower half swing up towards a transverse orientation at the upper half of the field. The bar is 100 pm.

In the literature, I can find no reference to this problem, and would be interested to hear from any neurohistologist who would either show me a preparation with connections to the synapses, or has an explanation of why they are not seen. However, it should be stressed that theories of chemical transmission across synapses depend crucially upon the existence of such connections. Furthermore, it would be dangerous to argue for the existence of conducting fibres which did not stain, and could not be seen unstained, as this would open up a Pandora's box of supposition.

EXAMINATION OF UNSTAINED NEURONS AND TISSUE CULTURES

H. If there is a possibility that the appearance of synapses could arise from precipitation of metal and tissue, it would be appropriate to examine unstained neurons, to see if synapses could be seen on them. Neurons were isolated from rabbit medulla by the technique of Hyden (23, 24) and placed in 0.25 M sucrose; they were viewed by phase contrast microscopy, without being stained (fig. 7). This elegant technique has been used, among others by Hyden in Sweden, Hamberger in Sweden, Hertz in Denmark, Hillman in Sweden and Britain, von Neuhoff in Germany, Osborne in Germany and Britain, and Pavlin in Yugoslavia, and their collaborators. Many publications have resulted showing isolated neurons from the medulla, spinal cord, and sympathetic and dorsal root ganglia. These appear granular on the surface, but the grains are very small and do not have stalks; some workers believe these granules to be synapses.

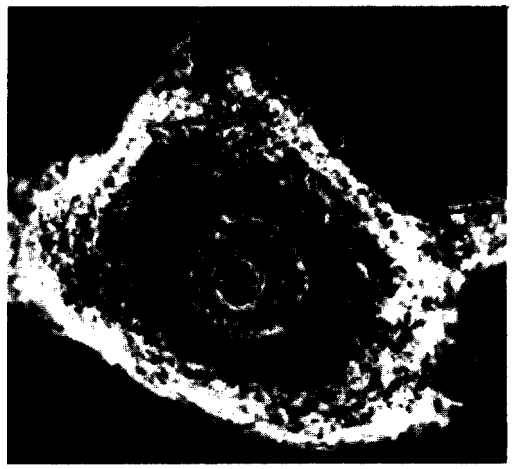

Figure 7. Rabbit neuron isolated by the technique of Hyden (23) placed in 0.25 M sucrose, and *seen* by phase contrast microscopy. Note the granular appearance. *Are* these granules synapses? Please see text. The maximum diameter of the soma is approximately **60** μm.

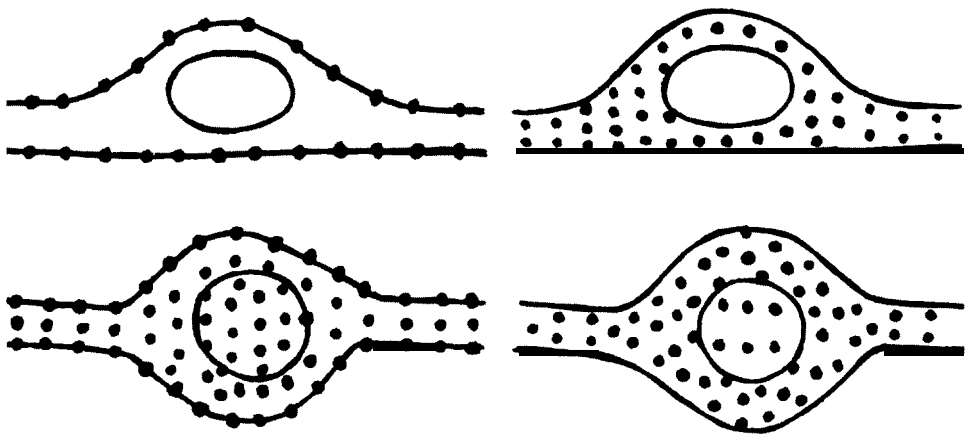

Figure a. If one sees granules in a cell under the microscope, one can determine if they are on the membrane of the cell (left-hand figures) or in the cytoplasm (right hand figures), that is, whether they are synapses or mitochondria. The cell is viewed in transverse section (upper) and then from above (lower figures). If the granules are on the surface, they will appear in only slightly less density over the nucleus (lower left). If they are in the cytoplasm but not in the nucleus (upper right), from above, they will appear in much lower density over the nucleus than over the cytoplasm (lower right). Under low power magnification when the depth of focus is several micrometers, many less granules are visible over the nucleus than over the rest of the cytoplasm, suggesting that the granules are intracytoplasmic, that is, they are mitochondria.

It has been argued that the reason for which the synapses do not appear on unfixed neurons, is that during the isolation the membranes are pulled off (25). This is unlikely because firstly, the cytoplasm would flow away when the cell bodies were placed in aqueous media, and, secondly, potential differences have been recorded on penetrating the cells, and these are assumed to have arisen across the membrane; these voltages fall reversibly on making the incubating media hypoxic or adding excess potassium salts (26). It is unlikely that neurons would maintain abrupt potential differences, if their membranes were not intact.

human cerebral and cerebellar cortex	(114)
human cerebral cortex	(115)
rat and cat cerebellum*	(116)
rabbit cerebral hemispheres and cerebellum	(117)
chick and rat dorsal ganglion	(118)
mouse ventral horn cells and dorsal root ganglion*	(119)
mouse dorsal root ganglion	(120)
mouse ventral horn cell*	(121)

Table 7. Various living tissue cultures seen by low power light microscopy showing the nucleus clearer than the cytoplasm, indicating that the granules are probably intracellular and not extracellular. The asterisks* indicate that the cells are excitable in culture.

One can decide whether or not they are likely to be extracellular, and therefore probably synapses, or intracellular, and therefore probably mitochondria (fig. 8). If one views a cell from above at low power magnification, any granules on the surface of the membrane will appear to be approximately evenly distributed, whereas granules within the cytoplasm would appear to be less dense over the region of the nucleus, since they are not present within it. Living neurons in cell culture nearly always appear less granular over the nucleus (please see micrographs in publications in table 7). Therefore, these particles must be intracellular; that is, they must be mitochondria, and not synapses. In addition, both in isolated neurons and in neurons in tissue culture, one does not see any attachment to the cell membranes where synapses might be.

I. ANOTHER LOOK BY LIGHT MICROSCOPY

Clearly, it is as difficult to tell if axons or dendrites of a cell connect with each other by looking at a micrograph, as it would be to tell if tendrils of ivy were attached to each other from a photograph since a two-dimensional picture cannot distinguish between structures in the plane of the picture which pass in front of each other, and those which make contact. Thus, in the beautiful drawings or micrographs by the pioneers of architectonics (27-34), it is difficult to determine whether fibres make contact, where they appear to overlap. It is also quite clear that if one has sufficient density of arborisations, overlap will occur. Van der Loos and Glaser **(35, 36)** have shown that fibres do pass close to each other, and they believe that they synapse. However, in my own observations of thick Golgi-Cox preparations of rat and guinea pig, whenever I have seen apparent overlap, careful focussing through the thickness of the section has revealed that the two fibres, while close, were not touching. In **1954,** Ramon y Cajal summarised observations he had made over 50 years. "We have made careful investigations of the courses and connections of nerve fibres in the cerebral and cerebellar convolutions of man, monkey, dog, etc., and we have never seen an anastemosis between the ramifications of two different protoplasmic expansions, nor have we observed them between filaments emanating from the same expansions of Deiters. The fibres intermingle in a most complex manner, producing a thick and intricate plexus, but never a net... It could be said that each element is an absolutely autonomous physiological canton "(32).

J. CONNECTIONS SEEN BY ELECTRON MICROSCOPY

The latter view of Ramon y Cajal has been usually interpreted as meaning that the neurons are not physically joined together as a reticulum, and the modern interpretation is that it implies that although not joined, the fibres are in contact with each other at the synapse. Indeed, Ramon y Cajal in the same work (32) lists all possible contacts between neuron and neuron. Peters, Palay and Webster **(17,** pps 159-180) list the following contacts: axo-dendritic; axo-somatic; axo-axonal; dendro-dendritic; somato-dendritic; dendro-somatic; somato-axonic; somato-somatic; dendro-axonic; axo-glomerular; mixed; those involving neuroglial cells. In addition, they also quote references to 'puncta adhaerentia', which they define as non-synaptic junctions, because they have a cleft but no synaptic vesicles; they consider these 'purely adhesive', and list them between the following structures: neighbouring dendrites; dendrites and axons; dendrites and somata; adjacent somata; axon terminals and initial segments of axons; neuronal processes and astrocytes; axon membranes and their surrounding sheaths. They add "Although puncta adhaerentia are considered to be simple points of attachment, it is not uncommon for them to occur at interneuronal interfaces."

From these lists, drawn up from references to electron microscopical studies, one may conclude that it is currently believed that virtually any part of a neuron can make contact with another part of a neuron or a neuroglial cell, and that such contact is characterised

by a cleft, usually with a thickening on either side. The presence of synaptic vesicles is regarded as diagnostic of them being a 'functional site', that is, presumably, the site of transmission. One must suppose that the electron microscopists take the view that this rich repertoire of contacts has not been identified by light microscopy because the magnification is not sufficient, but would it not be reasonable to expect to identify all such contacts more-easily at the lower magnification if they were present, even if one could not discern their finest structure?

If it is accepted that the presence of vesicles in a region lined by a synaptic cleft is so crucial to the identification of the synapse, it behoves one to ask 'How do we define a synaptic vesicle?? Please see Section L.

Furthermore, one is in a difficult logical position since the identification of a region as synaptic depends upon seeing vesicles within it, while the vesicles are defined by their presence in regions believed to be synapses, because they are lined by clefts. One should also bear in mind that the only criterion the electron microscopists use to relate one of the parts of the neuron to another, is the siting of synaptic vesicles in a region whose pre-synaptic nature is defined by them, so that they can add a further type of synapse to their lists. In doing so, they provide the physiologist with a task to investigate the alleged relationships, and the biochemists with duties to try to separate and characterise the types of synapses, - without minimising their differences during these endeavours (37).

However, I think that I should state quite unequivocally that, in my opinion, the presence or absence of synaptic vesicles - coupled with the uncertainty about their origin - is totally insufficient evidence to draw all the afore-mentioned conclusions.

K. SYNAPTOSOMES

This is the name given to a subcellular fraction which can be separated from nervous tissue preparations, and looks similar by electron microscopy to regions in the brain believed to be synapses (16, 38, 39, 40-43). The lack of connections of the synaptosomes has generally been explained by the proposition that they have been pulled off, and that the holes represented by the stalks have sealed themselves. The following evidence was advanced for this proposition: the synaptosomes metabolise and increase their ATP and creatine phosphate; they take up Na^+ and Ca^{2+} in a carrier mediated fashion; they extrude K^+; they take up choline, acetylcholine, adrenalin and aminoacids; they respire; they are osmotically sensitive; they contain glycolytic and mitochondrial enzymes; a preparation after gel filtration with equilibrated isomotic sucrose loses K^+ into aqueous fluids (44).

All these findings can be interpreted as meaning that sealing has taken Place, but alternatively they could mean that the various substances discussed have different affinities for the fraction, which are affected by the total chemical environment in which one studies them.

However, important questions are raised by the assertion that synaptosomes seal themselves off. What is the mechanism by which a hole in a sphere generally believed to consist of proteins and lipids is repaired? Where are the proteins and lipids synthesised to carry out the repair so rapidly? Have the enzymes for doing so been located in the synaptosomes? How is it possible during a period in which the tissue is being homogenised, centrifuged and put onto a column? Where is the fraction of stalks?

The identification of the synaptosome with the synapse depends upon their similar dimensions and shapes and their contents of synaptic vesicles: also, the synaptic vesicle fractions seen in the whole brain and in the synaptosomes have similar appearances, but this must be interpreted with caution, since the preparation procedure for both studies is virtually identical.

It is of considerable interest that synaptosomal fractions - in a similar fashion to cerebral slices (for review, see 45) - increase their oxygen uptake, phosphate metabolism, potassium ion loss, sodium ion concentration, calcium uptake, lipid and protein synthesis, etc., when stimulated by the application of electric currents, or the addition to the incubation medium of high potassium ion concentration, or of veratridine (for review, see 42). This may be interpreted as meaning that the biochemical responses to stimulation, which are found in cerebral slices, occur mainly in those elements of the slices which can be isolated in the synaptosomal fraction (41, 39). Unfortunately, it is difficult to prove the widespread assumption that the overall biochemical changes in cerebral slices or in synaptosomal fractions resulting from stimulation, reflect only the biochemical changes in, or adjacent to, the synapse itself - more specifically in the region of the pre- and post-synaptic thickenings. Furthermore, one cannot show that physiological excitability, i.e. the generation of action potentials as a result of stimulation, occurs either across the whole synapse, or specifically across the synaptic cleft, in the synapse visible in the synaptosomal fraction.

One may also generalise that there is no histological technique by which one can be certain that the same sites are the locations of, receptors, actions of transmitters, synapses, or immunochemical reactions. The identity of any sites can only be established by classical pharmacological criteria.

L. SYNAPTIC VESICLES

The presence of synaptic vesicles is characteristic of synapses. Vesicles appear in electron micrographs as circles 15 - 70 nm in diameter (table 8). When one looks in the cytoplasm of neurons of cat, rat and rabbit, in the cerebrum, cerebellum, spinal cord, and dorsal ganglia, one frequently sees circles of the same range of diameters. These are called neurotubules or microtubules (46 - 52). These are not called synaptic vesicles, because they are not located apparently in synapses, but, of course, a section which cut the corner of a neuron which contained neurotubules, and from which a dendrite was arising,

would have the same appearance as a synapse. How can one distinguish between a synaptic vesicle and a neurotubule or microtubule? What is the significance of the claim that neurotubules are said to be present in the pre-synaptic region? (53).

Tissue	width (nm)	Reference
guinea-pig forebrain	50	(122)
rat abducens nucleus	20 – 65	(24)
rat cerebral cortex	CC – 70 mean 40	(107)
mammalian central nervous system	30 – co	(54, p.15)
rat cerebellar cortex	15 – 60	(123)
rat prepyriform cortex	30 – 60	(82)
cat dorsal nucleus of lateral geniculate body	30 – 50	(124)
rat ventral horn	20 – 65	(86, p.370)
cat reticular formation	30 – 60	(77)
rat cerebellum	<40 50–60 x 25	(94)
rat cerebral cortex	10 – 20	(16, p.36)
cat nculomotor nucleus	40 – 60	(85)
mammalian various	40 – 50	(125 p. 86)

Table 8. Diameters of synaptic vesicles (nm) from the literature.

Synaptic vesicles suffer also from a geometrical disability, which can be appreciated immediately on examination of the micrographs in any of the publications cited in tables 2,4,6,8. That is, they appear very uniform in diameter in any particular publication. Since these electron micrographs are of thin sections, one should see a normal range of diameters in a particular synapse, even if the individual vesicles were all of uniform size. One could not argue that these illustrations have been selected to show clear images of vesicles, because one could not select any random collection of spheres of equal size so that nearly all of them would be cut in the plane of the micrograph, even from one single section. In order to gainsay this assertion about the uniformity of the size of appearance of vesicles, one would have to study them quantimetrically and statistically, and show them to exhibit a normal range of distributions of diameters.

The likelihood that the vesicles do not obey the laws of solid geometry in respect of three dimensional spheres leads one to propose that they are two-dimensional. They are probably bubbles on the surface of the specimens caused by the vacuum and the heat liberated when the electrons bombard the heavy metal-tissue complexes in the

tissue compartments.

M IONOPHORESIS

Since the pioneering work in the early 1950's of Eccles and his collaborators in Australia, substances have been applied by micropipettes to cells in the central nervous system (for review, see Eccles, 54). In mammals one cannot see the cells under direct vision, so that the belief that the tip of the pipette is penetrating a neuron is confirmed by stimulating the cell and producing an action potential. The intracellular penetration is detected by a sudden change of potential difference recorded at the tip of a micro electrode. The application of substances extracellularly is usually regarded as a presynaptic event. A large number of drugs, as well as excitatory, inhibitory and putative transmitters have been applied, and their effects have been interpreted in relation to receptors and synapses on the neuron surfaces. However, one's inability to see the tip of a classical micropipette or the synapse in vivo in experiments means that one cannot assert, or draw diagrams showing the tip accurately poised over a single synapse. Since it would be generally agreed that even in the presence of the maximum density of synaptic knobs on a neuron surface, most of the cell surface is not covered by synapses, it follows that any substance applied ionophoretically is liable to reach regions between the synapses first, before diffusing laterally into the synaptic clefts. An intracellular penetration with a micropipette would be a relatively rapid route to the presynaptic membrane on a neuron if, indeed, the synapses were connected by dendrites to cell somas. On the other hand, an extracellularly applied substance would reach the surface of the membrane not covered by the synapse more quickly. It must be clear that any extracellularly applied substance would take some time to diffuse into the synaptic cleft if it had the width measured by the electron microscopists (table 2). It could accumulate there at a higher concentration than it would at non-synaptic regions of the membrane, only if the former site had a higher affinity for the substance than the latter site had. In either case it would always diffuse to both sites and reach the non-synaptic regions first.

N. SYNAPSES AND NEUROMUSCULAR JUNCTIONS

When a muscle is denervated, the excitability spreads from the localised region of the 'boutons terminaux' to the whole of its surface (55-58). By light microscopy the number of neuromuscular junctions has not been found to increase (59), and I can find no reference in theelectron microscopic literature claiming that it does, although Gauthier (60, p. 481) says "ultrastructural manifestations of receptor synthesis have been observed in our laboratory." However, there are several reports of binding sites of α-bungarotoxin, which is regarded as a marker for acetylcholine receptors, spreading over the surface of the muscle (61 - 63). The point here is that after denervation, the sensitivity to acetylcholine and electrical stimulation, which are physiological properties, are dissociated from the anatomical structure, in that the function of transmission is

occurring in a region where no boutons are visible. Therefore, one must suppose that the anatomical neuromuscular junction is not necessary for transmission in this denervated tissue, and that this kind of excitability outside the neuromuscular junction is normally inhibited by the neurotrophic influence in the intact tissue. The neuromuscular junction is frequently regarded as a model of the synapse.

0. BIOCHEMICAL AND PHARMACOLOGICAL FINDINGS

There is a huge literature in which the biochemical properties of synapses, synaptosomes, receptors, transmitters and drugs have been interpreted in relation to belief about the existence of the synapse as a structure. However, logically, these dependent interpretations cannot be used to argue for or against the existence of the anatomical synapses. It would be a circular argument to attempt to do so. Nevertheless, if the structure is shown not to exist, then all interpretations dependent upon its existence would have to be modified.

FALSIFIABILITY OF PRESENT HYPOTHESIS

The present conclusions throw considerable doubt on the existence of synapses as anatomical structures. The minimal evidence which would negate this assertion would be as follows:

(a) Anyone alleging the existence in life of anatomical synapses would have to show them on unfixed, unstained neurons;

(b) An indication should be given of where the structures currently believed to be synapses connect to other neurons;

(c) The serious discrepancies between the observations by light and by electron microscopy should be resolved.

(d) It should be demonstrated rigorously that synapses and synaptic vesicles could exist in three dimensions.

IMPLICATIONS OF PRESENT CONCLUSIONS

If synapses do not exist as structures, or do not connect to other neurons, then:

1. The current hypotheses on chemical transmission at synapses would have to be changed, since they depend upon the existence of the structure. It seems unlikely that chemical transmission without synapses adjacent to other neuron cell bodies could occur, since diffusion *is a slow* process *relative to* the rates of transmission *in* the nervous system, Therefore, the most likely mechanism of transmission would be a process by which excitation reaches the tip of dendrites and is then conducted electrically by the tissue between neurons. Cerebral tissue has a high conductivity (for review, see 64). The distance between neurons is frequently tens of micrometres (H. Hillman, to be submitted for publication),

2. The role of transmitters, hormones, peptides, amino-acids, K^+, Ca^{2+} and Mg^{2+}, would be to alter the conductive properties, the relative affinities of constituents in compartments and organelles, and the metabolism, within each of the following parts of the tissue: the space between neurons, the membranes, the cytoplasm, the nucleoplasm, and the pars amorpha. In view of the facts that the cell membranes have high resistances and impedances, and also they react very rapidly to electrical stimulation and ionophoresis, it seems likely that transmitters are those substances which can alter the neuron membrane permeability very rapidly. The membrane of the cell should be looked upon asabi-stable structure, which can oscillate very easily between the more stable and the less stable states. This would fit in very well with the 'gating' mechanisms detected by voltage clamps applied across virtually all the membranes of excitable tissues. Such propositions are all open to experimental examination.

3. Discussion about whether each synapse contains one or several transmitters (the Dale principle) becomes irrelevant. They were always untestable.

4. Synaptic thickenings on both sides of the membranes may represent a particular chemical region in life, or they may be a precipitate, which happened to deposit during preparation of electron microscopic sections. There is no way of deciding between these two possible explanations.

5. The same uncertainty affects the location in life of receptors, ligands and immunochemical reactions, which are usually studied in dead, non-metabolising and drastically processed tissues. There have regrettably been no systematic comprehensive studies of the effects of preparation procedures on the biochemical properties of tissues. Until they are executed, the status of the huge volume of findings from incomplete experiments must continue to remain, at best, unproved.

6. No purpose is served by studying the biochemistry of synaptic vesicles, since the only criterion identifying the similarity in structure between the circles seen in whole synapses by electron microscopy and those seen in the subcellular fraction is the diameter, and neither of their shapes in either location is compatible with solid geometry.

7. The vesicle hypothesis (8) requires the existence of the anatomical synapse and synaptic vesicles, and would be grossly weakened if either or both of them do not exist.

8. It must be stressed that demonstration that synapses do not exist as structures affects the concept of transmission, the theory that neural transmission is chemical, and the vesicle hypothesis of transmission, but has no implications whatsoever for the validity of the findings upon which these theories are based.

CONCLUSIONS

It has been shown that: the anatomical synapse was first seen in stained neurons; the granular appearance on cell soma is probably due to mitochondria; that there are such serious discrepancies between the observations by light and electron microscopy in respect of the shapes of the synapses, the widths of the clefts, the diameters of the synapses and their incidence on the neuron somas, that it is extremely unlikely that the structures observed by the two techniques are identical; that synapses are not seen on unstained neuron somas; that even in tissues stained with silver salts, the synapses are not seen to be attached to other neuronal bodies; that neither ionophoresis, nor synaptosomal fractions, nor biochemical nor pharmacological techniques are accurate enough to define whether or not one is looking at synapses - as distinct from other regions of the cell; that the only certain criteria that the electron microscopists have to identify synapses is the presence of synaptic vesicles; these vesicles are probably bubbles.

It is concluded that the anatomical synapse does not exist in living neurons, that a new location of the physiological events of transmission must be sought, and that a new theory of transmission should be proposed which is not dependant upon the existence of synapse.

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