Early electron microscopic observations of synaptic structures in the cerebral cortex: a view of the contributions made by George Gray (1924–1999)

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The first clear account of synaptic structures in the cerebral cortex was provided by George Gray in 1959. Here we look at the background to this study, which established some clear structural distinctions between synaptic types, and enquire about the extent to which these distinctions can be related to contemporary knowledge of synaptic organization.

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THE IMAGE OF a cortical pyramidal cell, with apical and basal dendrites richly endowed with dendritic spines, has appeared on book covers, sweatshirts and symposium programs to represent any number of neuroscientific endeavors. It represents the beauty and the mystery of the cerebral cortex, with the numerous spines collecting the many and varied inputs and the single axon representing the pathway over which the cortex can dominate the rest of the brain, serving us in many simple and all of our most complex acts. An essentially complete picture of the dendritic tree and its spines has been available for well over 100 years. However, the synaptic relationships on cortical cells, including those on the spines, were not revealed until 1959 when George Gray, whose death last year was recorded in only one not widely noticed obituary[1], first studied them with the electron microscope[2]. He provided the first clear evidence of synaptic contacts upon the dendritic spines, stems and cell bodies of cortical neurons and, in addition, drew a significant distinction between synapses that are still called 'Gray's type I and Gray's type II'. The former are asymmetrical, having a postsynaptic density that is thicker than the presynaptic one and are now commonly associated with excitatory junctions on spines; the latter are symmetrical and normally characterize inhibitory junctions. Today, when the postsynaptic spines can be observed in real time, changing in response to afferent activity[3,4] and functional significance can be ascribed to some of the structures at these synapses, Gray's observations still serve to raise important questions about the organization of cortical synapses.

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Synaptic structure as seen at University College London in the 1950s

George Gray came to the Anatomy Department at University College London (UCL) in 1955 to work as a postdoctoral assistant to John Z. Young. He had just completed a PhD on control of pigment cells of fish. His view of the synapses of the CNS was significantly influenced by what was then a lively interest in central synapses within the Anatomy Department at UCL. The focus was on synapses contacting lower motor neurons. Young, who had earlier worked with members of Charles Sherrington's Oxford laboratory, was himself undertaking an early electron microscopic study of the ventral horn with R.W.G. Wykoff[5]. Sherrington, arguing from a knowledge of Cajal's material, had much earlier introduced the term 'synapse', and in his laboratory there had long been a major interest in synapses on motor neurons[6]. These had been illustrated by Cajal[7,8] and also later in Oxford, by Hoff[9], showing the synaptic 'boutons', stained using reduced silver methods, scattered on ventral horn cells. These were the synaptic structures that were most closely related to the electrophysiological studies of central synapses at the time (see, for example, Ref. 10).

In 1955, Palay and Palade[11] published their strikingly clear picture of an individual central synapse, showing the presynaptic axonal terminal on a nerve cell in the abducens nucleus, another motor neuron. Pre- and postsynaptic membranes were clearly distinct, as well as the synaptic thickening, synaptic vesicles and presynaptic mitochondria. A year later, Wykoff and Young[5] also showed electron micrographs of synapses on the surface of ventral horn cells. Their pictures were not good by the standards established by Palay and Palade but both studies were a surprise in relation to earlier, hotly debated descriptions proposed by supporters of the neuron doctrine on the one hand and their opponents (the reticularists) on the other.

Although the electron microscope has often been celebrated as finally establishing the neuron doctrine, in reality it illuminated just the one part concerned with the discontinuity between nerve cells and at the same time it revealed the accuracy of some of the observations upon which the reticularists had based their interpretations. The neuron doctrine was widely accepted at this time, and its main purpose, to provide a tool for analysing the nervous system in terms of its parts, was well on the way to fulfilment. So confirmation of the reticularists descriptions was generally ignored. However, when Palay and Palade[11] showed that the space separating the pre- from

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the postsynaptic membrane was only 20 nm, they demonstrated a gap that could not have been visible to light microscopists and must have looked like continuity to them. When, perhaps more disconcertingly, Wykoff and Young showed the very dense synaptic covering of ventral horn cells, they confirmed earlier illustrations produced by the reticularists, Held[12] and Auerbach[13]. The then triumphant neuronists, including Young had earlier regarded those pictures, as artefacts of histological processing (see Ref. 8).

Keith Richardson, the then histologist at UCL, was stimulated by the study of Wykoff and Young to look more closely at the surprisingly dense synaptic covering of ventral horn cells[14]. He was probably not concerned with doctrinaire views of the synapse and perhaps enjoyed tweaking the tails of dogmatic neuronists. He developed a light microscopical preparation, similar to Auerbach's that matched the electron microscopical image of a dense synaptic covering on ventral horn cells that the reduced silver methods had failed to show. Gray[15] later explored this difference, but at the time these studies showed that views of synaptic structure needed re-evaluation, even in the spinal cord where synapses had been most closely studied.

Whereas the structure of synapses on motor neurons began to be appreciated, the synapses of the cerebral cortex were still unexplored. At that time, Donald Sholl, also one of Young's recruits to UCL and a statistician by training, was studying the cerebral cortex. He was looking at Golgi preparations, which at that time were preserved under Canada Balsam with no cover slips, ruling out the use of oil immersion lenses. Sholl puzzled long and hard about where the synapses in cortex might be. Reduced silver methods, such as those used by Hoff and Cajal, showed virtually no synaptic structures in cortex and Richardson's method were equally unhelpful either because it did not show the finer dendrites and their spines. Sholl speculated that the dendritic spines might be synaptic specializations, but, similar to his predecessors (see, for example, Ref. 7), he lacked the resolving power to solve the problem. Earlier workers had expressed varying views on the dendritic spines (summarized at the time by Fox and Barnard[16]), some treating them as artefacts or, even though this seems improbable to us now, as axon terminals adhering to the dendrites[17]. Others considered them as possible postsynaptic sites, without, however, any clear view as to whether the afferent axons contacted the spine heads or the spine necks[7,16].

Gray's account of cortical synapses

When Gray joined the Anatomy Department at UCL he first studied the muscle spindle[18] and then moved on to study the fine structure of the locust's ear[19,20], which involved considerable struggles to obtain adequate fixation for electron microscopy. At this time several members of the Anatomy Department would regularly join for lunch and it was then that Gray, listening to Richardson and Sholl, realized that the synapses of the cerebral cortex were *terra incognita*, waiting for an electron microscopist. This was at a time when fixation for the electron microscope was by immersion not perfusion, using osmium tetroxide or potassium permanganate, both somewhat poor at penetrating tissues. So a surface structure like the cortex was ideal for rapid fixation, easier, and for Gray at the time, far more rewarding than the locust's ear. Gray rapidly moved ahead on this new project with enormous enthusiasm and produced a brief and concise account[2] that is still profitable to read.

In this study Gray used phosphotungstic acid after primary fixation in osmium tetroxide, whereas today

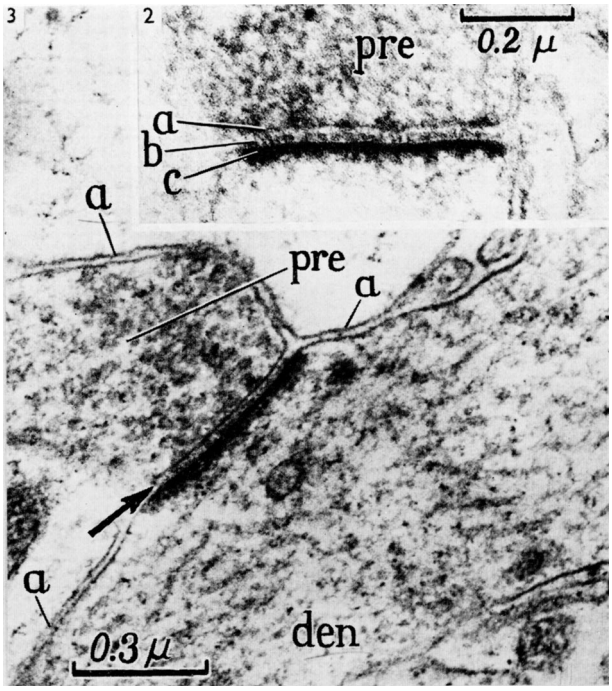

Fig. 1. ***A type 1 (asymmetrical) synapse from Figures 2 and 3 of Gray's 1959 paper[2].*** *'a' shows the presynaptic thickening in the upper figure and shows the non-thickened region of membranes in the lower figure. 'b' shows the extracellular dense material in the synaptic cleft and 'c' shows the postsynaptic thickening. Abbreviations: den, dendrite; pre, presynaptic process.* Reproduced, with permission, from Ref. 2.

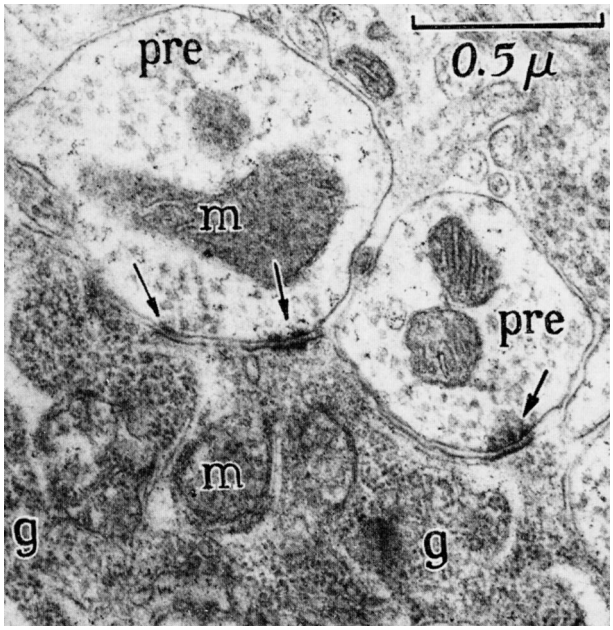

Fig. 2. ***Type 2 (symmetrical) axosomatic synapses from Figure 8 of Gray's 1959 paper[2].*** *Abbreviations: g, granules of endoplasmic reticulum; m, mitochondria; pre, presynaptic process.* Reproduced, with permission, from Ref. 2.

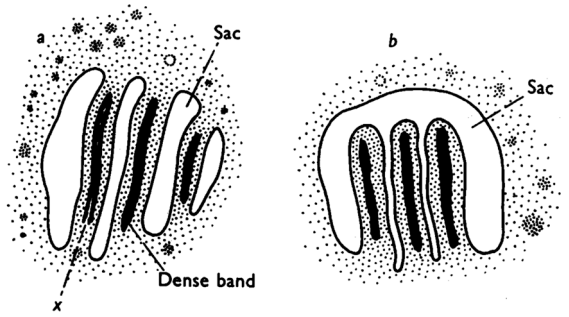

Fig. 3. ***The spine apparatus.*** *Illustration of the spine apparatus from Gray's 1959 paper (text fig. 1)*[2]*. Membranous sacs are shown alternating with electron dense bands or plates, whose function is still undefined. The right-hand panel 'b' shows a cross-section through the x-axis represented in 'a'.* Reproduced, with permission, from Ref. 2.

lead staining after aldehyde fixation and post-osmication is mainly used. The resulting images (Figs 1 and 2) that Gray obtained differ from most of the currently used preparations and showed some of the synaptic structures even more clearly. Gray's 1959 paper showed that spines were postsynaptic specializations and that synapses on spines were distinguished from many of those on dendritic stems and cell bodies by the asymmetry of the synaptic thickenings. He identified some irregular dense presynaptic projections as well as a curious structure of electron dense plates and membranous sacs, the 'spine apparatus' (Fig. 3).

The identification of the dendritic spines as a prime site for postsynaptic specializations in the cerebral cortex was the most significant observation at the time, and probably still is. Almost equally significant was the distinction between the asymmetrical and the symmetrical junctions (Gray's type 1 and type 2, respectively), the former onto spines, the latter onto cell bodies and dendritic stems. Gray noted the different postsynaptic densities and also showed that the asymmetrical junction had a wider extracellular space which contained electron-dense extracellular material (Fig. 1). Further, whereas the asymmetrical synapses tended to occupy most of the juxtaposed axonal and dendritic membranes, the symmetrical synapses tended to occupy only a fraction of the apposed membranes. The strength of this classification was that it was based on several correlated variables and although exceptions have been noted (see below) the distinction has proved functionally significant for many later studies. In addition to these details, Gray noted that at the damaged surface of his small blocks, where cytoplasmic processes were often ruptured, the postsynaptic thickenings remained attached to the presynaptic element, demonstrating the adhesive properties of the synapses.

The extent to which some of these structural features can now be related to functional and molecular properties of synapses is summarized by De Camilli *et al.*[21] and Sheng[22]. The presynaptic thickening and the presynaptic dense projections, which Gray later showed form a regular hexagonal array[23], are now thought to be involved in the complex mechanisms concerned with vesicle release (see for example Ref. 24), although it is still unclear exactly what the dense projections represent. The extracellular material in some synaptic clefts probaby relates to adhesive functions of synapses and might provide a mechanism for providing some highly specific connectivity patterns[25]. This can lead to questions about the differences in adhesive properties of synapses: are the type 2 synapses less adhesive than the type 1 synapses? And if, as appears probable, they are, then what is the molecular basis and the functional significance of this difference? The postsynaptic thickening is thought to represent receptors and the molecules that serve to anchor them to the postsynaptic membrane and the cytoskeleton[22,26,27]. In general, the details of how molecular components at synapses relate to the differences between the synaptic types that Gray described are not yet clearly defined. These will probably prove important for helping to understand the significance of the electron microscopic images and, more importantly, they will allow attention to focus on how the structural heterogeneity of synapses relates to differences in their known molecular and functional properties.

A further important, but more often neglected part of Gray's account was the description of the 'spine apparatus'. Figure 3 shows it as an electron dense plate or series of plates adjacent to membranous sacs. Although the structure has been more closely defined[28], we still have no clear idea of what it does. The direct evidence that dendritic spines rapidly change their shape[3,4] in the living brain, and also recent observations[29] relating the plates to actin in the spines, suggest that the spine apparatus might play a role in the plastic responses. Here again, however, Gray's eye for significant differences between structures might yet prove critical, because he noted in a later study[30] that cerebellar Purkinje cells lacked a true spine apparatus, often having the membranous sacs but not the electron dense plates.

Gray's 1959 study established him as a profoundly observant microscopist and provided structural clues or signposts that can still serve to guide the contemporary student of synapses. Perhaps the most widely used of these signposts is the symmetry or asymmetry of the contact zone. This is often used to identify putative excitatory (asymmetric) or inhibitory (symmetric) synapses, although the experimental evidence for this is often forgotten. The link was indirect, and was not made until aldehydes replaced osmium tetroxide as the primary fixative for electron microscopy. In aldehyde fixed tissues, membrane bound structures, including synaptic vesicles, are still osmoreactive[31]. Provided that tissues are exposed to an appropriate osmolarity before transfer to osmium tetroxide, the shape of the vesicles can be modified. In hypo-osmolar solutions they appear regularly spherical, whereas in hyper-osmolar solutions they are more shrunken, appearing irregularly flattened or 'pleomorphic'. The degree of this 'flattening' depends not only on the osmolarity of the solutions, but also on the nature of the vesicles or (more probably) their contents. Soon after aldehydes had been introduced as primary fixatives for electron microscopy, Uchizono[32] observed that in the cerebellum, where the excitatory and inhibitory nature of distinct presynaptic populations had been clearly established[33], the excitatory synapses had round vesicles and the inhibitory synapses had flattened or pleomorphic vesicles. Subsequently, the more or less general association of such flattened vesicles

with GABA or glutamic acid decarboxylase (GAD) immunoreactivity supported Uchizono's original suggestion (see for examples Refs 34,35), although not all published figures show this flattening, presumably because the relevant osmolarities were not used and the relationship to glycine appears to be more variable[36].

Establishing the link of Gray's type I and type II synapses to the shape of the fixed vesicles, although suggested by Uchizono, was an extra step taken by Marc Colonnier[37], who was studying cortex with Gray. The link was not absolute; there is a range of asymmetries in the contact zones in cortex. In general, the symmetrical synapses are associated with flattened vesicles in the cerebral cortex and the asymmetrical synapses with round vesicles. This association has also been found widely in other parts of the brain. (For discussion and examples of exceptions see Ref. 38.)

Gray's later studies

George Gray carried out several other important studies. In collaboration with Victor Whittaker he published methods for isolating 'synaptosomes'[39], the pinched off axon terminals, with their vesicular and mitochondrial contents intact and the thickened postsynaptic membrane still attached. These allowed a novel attack on the nature of vesicular contents. When Eccles and colleagues[40] showed that depolarization of primary afferent fibers in dorsal roots could be produced by the action of one axon upon another in the dorsal horn, Gray showed such 'axo-axonal' contacts in electron micrographs[41]. Michael Kidd[42], working in the same laboratory had earlier shown the serial (dendro-dendritic) contacts made by amacrine cells in the retina, so that the dogmatic view of axons as always presynaptic and dendrites as postsynaptic (the so-called law of dynamic polarization, formulated by Cajal[7,8]) had already lost some of its power (but for a current view see Ref. 43). The acceptance of such serial axo-axonal or dendro-dendritic junctions provided a new freedom for thinking about neuronal connections.

Gray later made sense of the different densities of synapses on motor neurons shown by the reduced silver methods of Cajal and Hoff on the one hand, and by the methods of Held and Auerbach on the other (see above)[44]. The methods of Cajal and Hoff show a cytoskeletal element (Fig. 4) that characterizes some, but by no means all axon terminals in the spinal cord, this feature is never seen in the cerebral cortex[15], where these methods stain virtually no synapses.

Gray continued his interests in synaptic structures in later years. Figure 5 shows a picture of Gray circa 1990. With Willis[45] he described what are now recognized as the clathrin coats of recycling vesicles and proposed that the coats provide a scaffold that determines the relatively constant vesicle sizes seen in axon terminals; a view that is still of interest[46]. He looked at the distribution of microtubules at synapses using unusual methods of fixation[47]. In general he was an avid student of anything relating to the structure of the nervous system, later taking an interest in spongioform encephalopathies and Alzheimer's disease by looking at fine structural changes in postmortem brains. His early years as an electron microscopist, however, were the most exciting for him and for those of us who were lucky enough to have worked with him. At that time he was an enthusiast, who enjoyed the technically difficult investigations that he undertook and who was able to pass his enthusiasm on to others. There were new things to be found wherever one looked. Gray enjoyed looking and persuading others to join in the hunt. In later years he suffered long bouts of deep depression, and wrote a brief account of this experience[48]. His early enthusiasm was lost. Occasionally one could still see a gleam of the early enjoyment but, sadly, his illness got the better of him and essentially we lost a stimulating colleague long before he died in August last year.

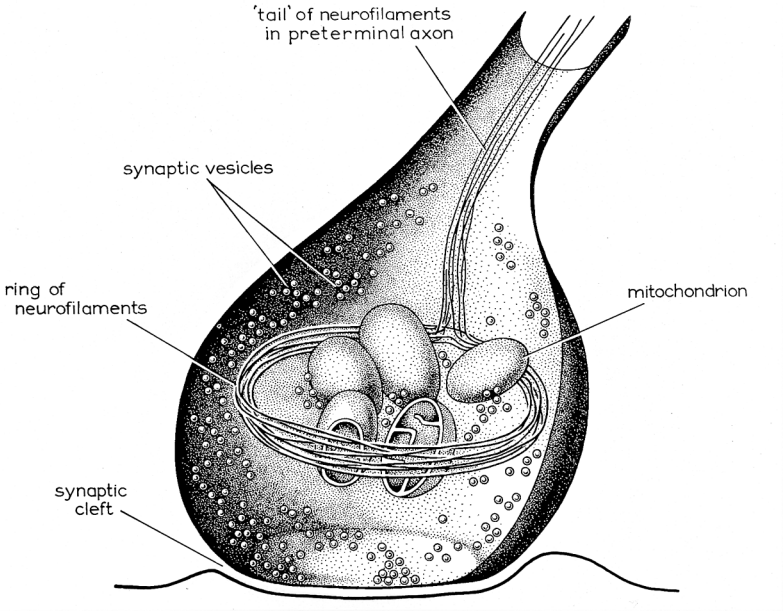

Fig. 4. ***George Gray's sketch of an axonal ending showing the ring of neurofilaments that is stained using the reduced silver methods used by Cajal[7,8] and Hoff[9].*** *Because only some axons contain such rings this method gives only a partial view of all synaptic terminals and shows a relatively large gap between the stained presynaptic (axonal) component and the postsynaptic membrane.* Reproduced, with permission, from Ref. 44.

Fig. 5. ***George Gray (circa 1990).***

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