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AN ELECTRON MICROSCOPIC STUDY OF THE DEVELOPMENT OF AXONS AND DENDRITES BY HIPPOCAMPAL NEURONS IN CULTURE

II. Synaptic Relationships[1]

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Abstract

We have studied by electron microscopy the synaptic relationships which develop between hippocampal neurons in dissociated cell cultures. Neurons, obtained from fetal rats at 18 to 20 days of gestation, were plated at high cell density onto polylysine-treated coverslips and maintained in serum-free medium. After 3 to 4 weeks, the cells were interconnected by an extensive network of processes which made frequent synaptic contacts with one another. Certain of the processes could be readily identified as dendrites by their branching pattern and content of polyribosomes. Often individual dendrites could be followed for 100 to 200 μm from their cells of origin. In every instance observed, the dendrites were postsynaptic. The presynaptic processes were quite different in appearance; they lacked ribosomes, their microtubules were spaced more closely together, and they were thinner than even the distal dendrites except at synaptic sites where they formed varicosities. Because of their small diameter, the presynaptic processes could not be traced through the dense neuropil to their origins, but in all other respects they resembled the axons identified in younger cultures.

There were differences in the features of the synapses present on different portions of the cell. The great majority of synapses on dendritic spines made asymmetric junctions, whereas those on cell bodies made symmetric junctions. Both types were observed on dendritic shafts, but asymmetric junctions were predominant. These results show that synapses do not form indiscriminately between the processes which develop in culture, but rather that axons and dendrites acquire distinct synaptic polarities, just as when they develop *in situ*.

In the preceding paper (Bartlett and Banker, 1984), as well as in previous work (Banker and Cowan, 1979), we have presented morphological evidence that processes with the distinctive features of axons and dendrites can develop in dissociated cell cultures from the hippocampus. A more important distinction between axons and dendrites, recognized since the time of Cajal, is in their function. Cajal proposed that dendrites were receptive elements and hence postsynaptic, whereas the axon conveyed information to succeeding cells and hence was presynaptic. It is now clear that both axons and dendrites can enter into many other synaptic relationships and, in particular, dendrodendritic and axoaxonal synapses have been reported in many regions of the nervous system (see, e.g., Shepherd, 1979). Nevertheless, in most areas of the nervous system, and in the hippocampus specifically, axodendritic and axosomatic synapses are by far the most numerous. The principal objective of the experiments presently described was to determine if axons and dendrites express a similar selectivity when synaptogenesis occurs between dissociated cells in culture: Do axons and dendrites, identified on structural grounds, exhibit a distinct synaptic polarity, with axons predominantly presynaptic and dendrites predominantly postsynaptic, or are synapses formed indiscriminately between the two types of processes?

A second question concerns the localization of synapses over the cell surface. In general, particular populations of presynaptic fibers terminate preferentially on particular portions of the dendrites or on the cell body. Even along a particular portion of the dendrite, some fibers tend to terminate on dendritic spines, whereas others terminate directly on the dendritic shaft. These aspects of synaptic localization, which are shared by most neurons, are especially characteristic of the pyramidal cells in the hippocampus where particular populations of terminals are restricted to rather discrete zones of the apical and basilar dendrites or to the somatic surface (Gottlieb and Cowan, 1972). We were interested to determine if there was any indication that synaptic terminals which form in culture are also selectively localized to particular portions of the cell surface.

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To investigate these questions, we have studied hippocampal cell cultures established at high cell densities and maintained for 3 to 4 weeks. At that stage the cells have well formed dendritic trees and are interconnected by a dense plexus of fine fibers. Such cultures were examined by electron microscopy in order to determine the features of their processes and to study their synaptic relationships.

Materials and Methods

Preparation and examination of cultures. Methods for establishing the hippocampal cell cultures and for preparing them for electron microscopy were essentially as described in the preceding paper. To increase the opportunity for contacts between cell processes, about 400,000 viable cells were added to each 60-mm dish containing six coverslips, a plating density of 15,000 cells/cm^2. The cultures were fixed after 3 to 4 weeks *in vitro*. Areas of the cultures which contained a dense network of neurites were chosen for examination by electron microscopy.

Analysis. Two different approaches were used for studying the processes which established synaptic connections in these cultures. The first set of experiments focused on those processes which could be followed from their cells of origin for at least 50 to 100 μm. Material for this analysis was selected from four different cultures which had been maintained for 3 weeks *in vitro*. Montages were prepared illustrating the full length of such processes until they left the section, and segments for analysis were selected at equal intervals along each process as described in the preceding paper. For each analysis segment, we determined the process width and the density of polyribosomes and of microtubules. We also identified every synaptic contact along these processes.

In the second set of experiments, features of pre- and postsynaptic processes were examined based on a random selection of synapses from seven different cultures which had been fixed after 21 to 25 days *in vitro*. This sample included 400 synapses involving 383 different presynaptic processes and 150 different postsynaptic processes. Each of the pre- and postsynaptic elements in this sample was assessed for the presence of polyribosomes. Each process in this sample which participated in more than one synaptic contact was examined and categorized as presynaptic at each site, postsynaptic at each site, or mixed. Included in this sample were 65 profiles of presynaptic processes and 40 profiles of postsynaptic processes which were long enough so that their features could be studied outside of the immediate synaptic zone. For these examples we determined the process width and the density of polyribosomes and of microtubules as described above.

Both sets of cultures were used to study the features of the synaptic junctions present on different portions of the cells. Examples were selected in which the postsynaptic element could clearly be identified as a nerve cell body, as the shaft of a process, or as a spine which could be seen arising from a process. To avoid synapses cut tangentially, only examples where the synaptic cleft was clearly visible along the full length of the junction were included. Photographs of these synapses, masked so that only the immediate junctional zone was visible, were classified by three independent observers as asymmetric (postsynaptic membrane obviously denser and thicker than the presynaptic membrane), symmetric (pre- and postsynaptic membranes of comparable density and thickness), or intermediate (postsynaptic membrane denser but not clearly thicker than the presynaptic).

Results

The appearance of cells after 3 weeks in culture. An example of a "dense" hippocampal culture which had been maintained 24 days is illustrated in Figure 1. The individual nerve cell bodies are enmeshed within an extensive network of fibers. By comparison with hippocampal neurons after only a week in culture (as described in the preceding paper), the cell bodies are substantially larger in diameter, up to 20 to 25 μm. Processes which correspond to the dendrites described in younger cultures are readily apparent. However, they are thicker at their bases, more complexly branched, and considerably longer. The tapering of the dendrites and their decrease in diameter at branch points are especially obvious in such mature cultures. As *in situ* (Hillman, 1979), the length of each dendrite is roughly proportional to its "stem" diameter near its point of origin from the soma. The distal portions of the dendrites become lost among the plexus of fine processes, which most probably are axons.

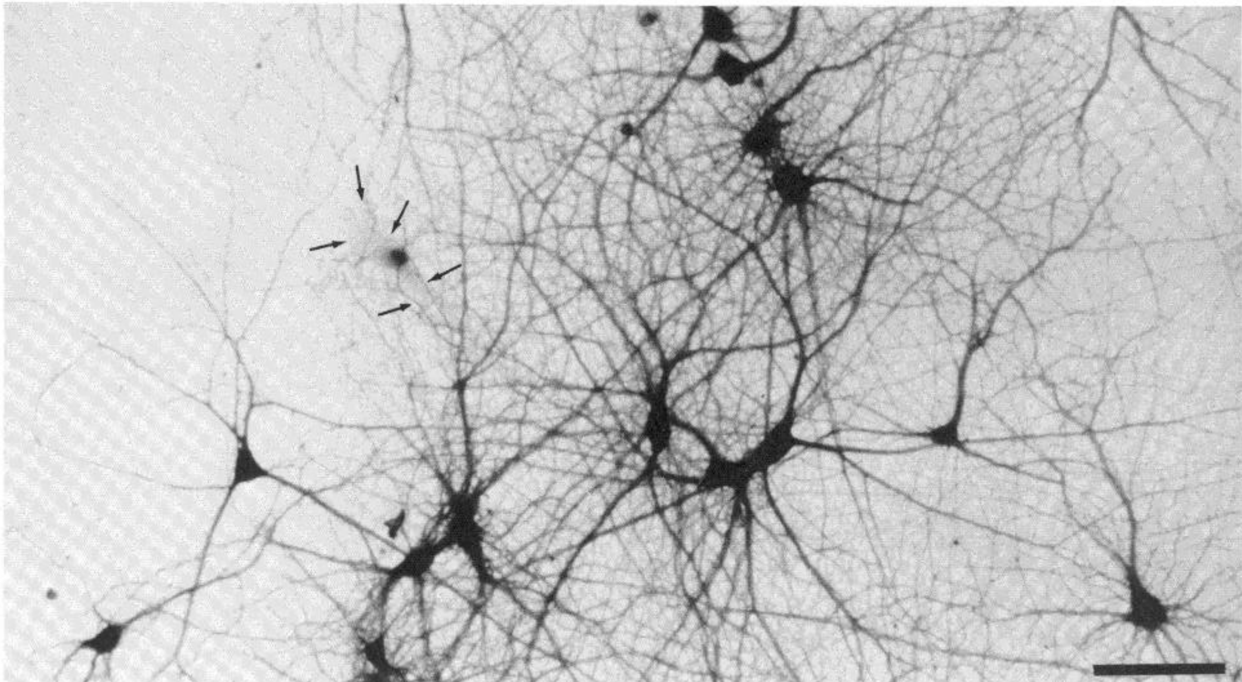

Figure 1. A photomicrograph illustrating hippocampal cells which have been maintained in culture for 24 days. Several dendrites, thick and tapering, arise from each of the nerve cells and can be followed for some distance. In denser regions of the culture, as the dendrites branch and become thinner, they become lost among the other processes in the neuropil. The electron micrographs which follow were obtained from such regions. In less dense regions, as at *left*, the dendritic arborizations of individual cells can be more readily followed. This field also contains a single flattened glial cell (outlined by *arrows*) whose nucleus is densely stained. Toluidine blue stain. *Scale bar*: 100 μm.

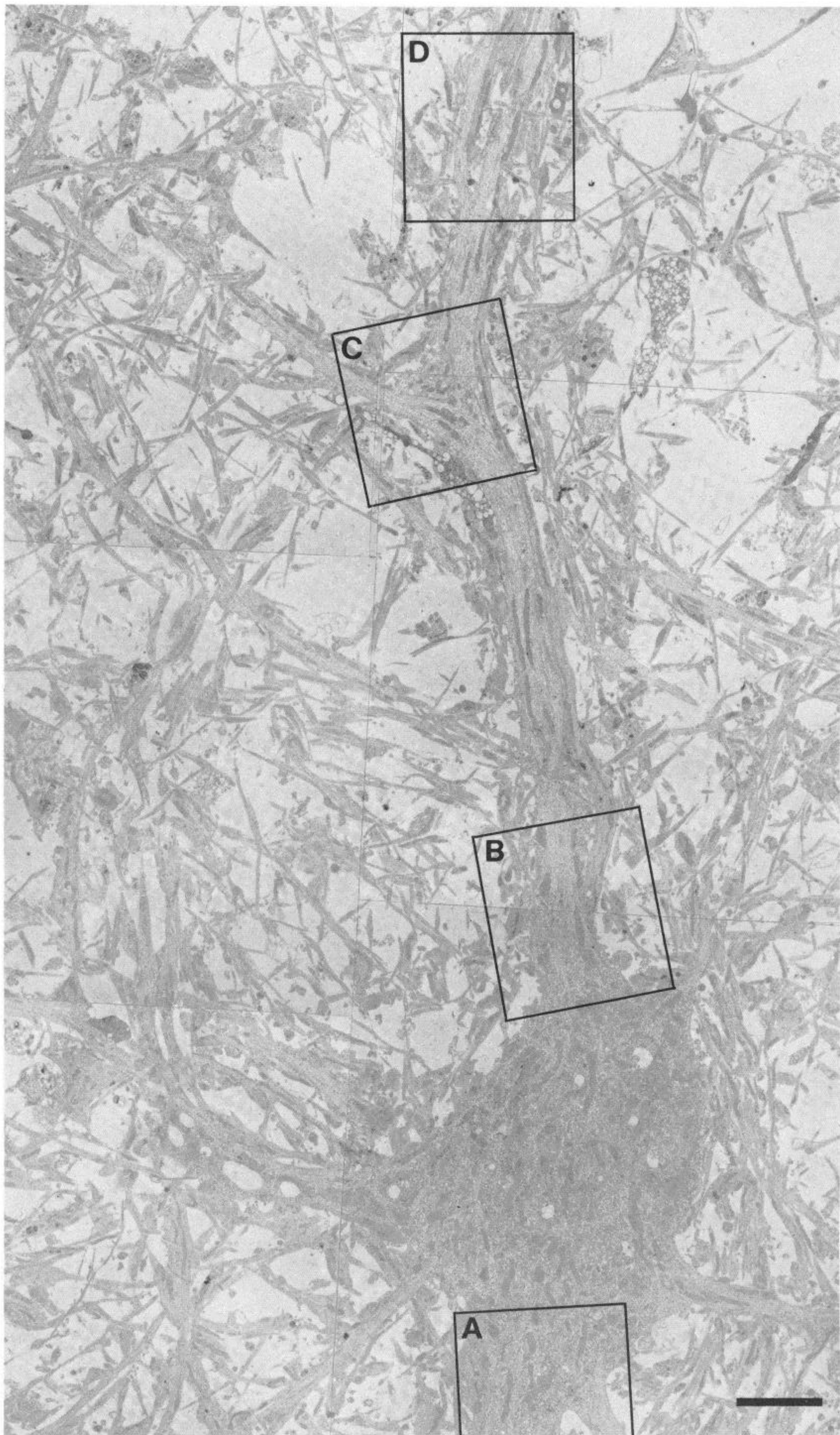

Figure 2. A low magnification electron micrographic montage of a hippocampal neuron and the processes surrounding it (25 days *in vitro*). The thick dendritic processes which arise from this neuron lie within a plexus of much thinner processes which sometimes run along the surface of the cell body and dendrites. This section was cut very near to the substrate, below the level of the nucleus of the cell shown. The regions enclosed within the *boxes* are shown at higher magnification in Figure 4. *Scale bar*: 3 μm.

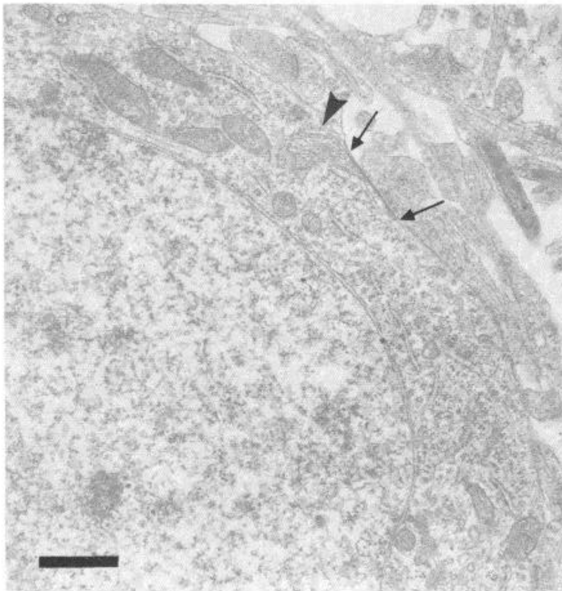

Figure 3. An electron micrograph illustrating a portion of the cell body of another neuron after 25 days in culture. This cell shows many of the features typical of a maturing neuron, including a prominent Golgi apparatus (*arrowhead*), many polyribosomes, and several cisterns of rough endoplasmic reticulum. A subsurface cistern is present just beneath an apparent presynaptic terminal (between *arrows*); two other synapses can be seen in the neuropil. The nucleus is relatively clear and lacks a rim of dense heterochromatin. *Scale bar*: 1 μm.

Non-neuronal cells, probably mostly astrocytes, are occasionally scattered among the neurons (*arrows*) or are present in large confluent patches which are set apart from the neurons (not shown).

Areas of such cultures which contained dense neuronal networks were selected for study. A low magnification electron micrographic montage from such a region is shown in Figure 2. This shows a neuronal cell body, which has been sectioned below the level of the nucleus, the thick processes which originate from this cell, and the dense network of fine processes which surround it. Figure 3 shows a portion of another nerve cell body at somewhat higher magnification. Both cells show the features characteristic of maturing neurons. They contain an abundance of free polyribosomes, many profiles of rough and smooth endoplasmic reticulum, and a well developed Golgi complex, often associated with coated vesicles. Synapses were seen both on the somata of the cells and among the surrounding processes.

The ultrastructural appearance of the flattened, non-neuronal cells in such cultures was quite different (not shown). In non-neuronal cells, intermediate filaments were present throughout the perikaryon and extended out into the processes in densely packed, parallel arrays (see also Lim et al., 1976). Most probably these represent astroglial filaments composed of glial fibrillary acidic protein (GFAP) since comparable cells are intensely stained by immunofluorescence using anti-GFAP antibodies (A. Hodson and G. A. Banker, unpublished observations). The processes of non-neuronal cells were readily distinguished from those of neurons, which predominantly contained microtubules.

Processes which correspond to proximal dendrites could be readily identified in electron micrographs of such cultures, and, because they were relatively thick, they could be followed for a considerable distance. However, these processes could not be followed to their tips because as they became thinner, they became interwoven among other processes and passed out of the plane of section. Individual axon-like processes, which were quite thin, could be traced only for short distances; we were never successful in tracing an axon to the cell from which it originated. Therefore, two different approaches were used to characterize the processes in these cultures. The first approach focused on those dendritic processes which could be traced for a considerable distance from their cells of origin. In the second set of experiments, processes which were involved in synapses were selected at random to determine whether there were characteristic differences in the ultrastructural features of the pre- and postsynaptic processes.

Identified dendrites. The processes which could be followed for substantial distances from their cells of origin were clearly dendrites and in all of their essential features resembled the dendrites of younger cells, as described in the preceding paper (Figs. 2 and 4). They have a gradual origin and at their base contain many of the same organelles which are present in the perikaryon. With increasing distance a clear reorganization of cellular organelles takes place (Fig. 4). Microtubules, which funnel into the base of the processes, become densely packed and form the dominant cytoskeletal element. Neurofilaments are rare. Polyribosomes decrease in number and become largely confined to scattered pockets, especially along the periphery of the process. They also appear to be concentrated in the angles of branch points and at the bases of spines (Fig. 4, *B* to *D*). The Golgi complex and cisternae of rough endoplasmic reticulum are rarely found beyond the first dendritic branch point. The mitochondria within these processes are usually long and thin and are oriented along the long axis of the process.

Seven neurons, which gave rise to 13 dendrites that could be followed for considerable distances, were chosen for analysis, as described under "Materials and Methods" (Table I). The most significant characteristic of these dendritic processes, which was obvious without the need for analysis, concerned their role in synapses; every identified dendrite was exclusively postsynaptic along its entire length (Table I). In all, these 13 dendrites were postsynaptic to 314 presynaptic terminals over the lengths included in our sample, amounting to an average of two synapses for every 10 μm of dendritic length. The terminals occurred directly on the dendritic shafts as well as on a variety of dendritic spines (see below). As with younger cells, the density of polyribosomes declined with distance from the cell body, but some polyribosomes were consistently found as far distally as the processes could be followed (Fig. 5). The distribution of polyribosomes within the dendrites, as well as their absolute density, were essentially the same as in 7-day-old cells. The dendrites of older cells also tapered in diameter with distance, as described for younger cells (data not shown).

Finally, the density of microtubules was measured at varying distances from the soma. In neocortical pyramidal cells *in situ*, the density of microtubules is relatively constant throughout the dendritic tree (Hillman, 1979). Our results, shown in Figure 6, indicate little if any change in the density of microtubules within the proximal 100 to 200 μm of the dendrites. Over this distance the width of the dendrites decreases by about 2-fold. On the average, the density of micotubules in these dendritic processes was $7.0 \pm 2.6/\mu m$ (mean ± SD),[4] about the same as in 1-week-old hippocampal neurons (Bartlett and Banker, 1984). In processes believed to be more distal dendrites (see

[4] The mean density of microtubules in adult neocortical pyramidal cells *in vitro* has been reported to be about $70/\mu m^2$. Assuming our sections are 600 to 900 Å, 11 to 17 sections would be required to span a thickness of 1 μm, giving a density of microtubules of 75 to $120/\mu m^2$; because of double counting, these figures are overestimates. Considering the crudeness of this calculation, there appears to be reasonable agreement between our estimates of microtubule density based on sections cut parallel to the long axis of the fiber and the reported density of microtubules in cross-sections of dendrites *in situ*.

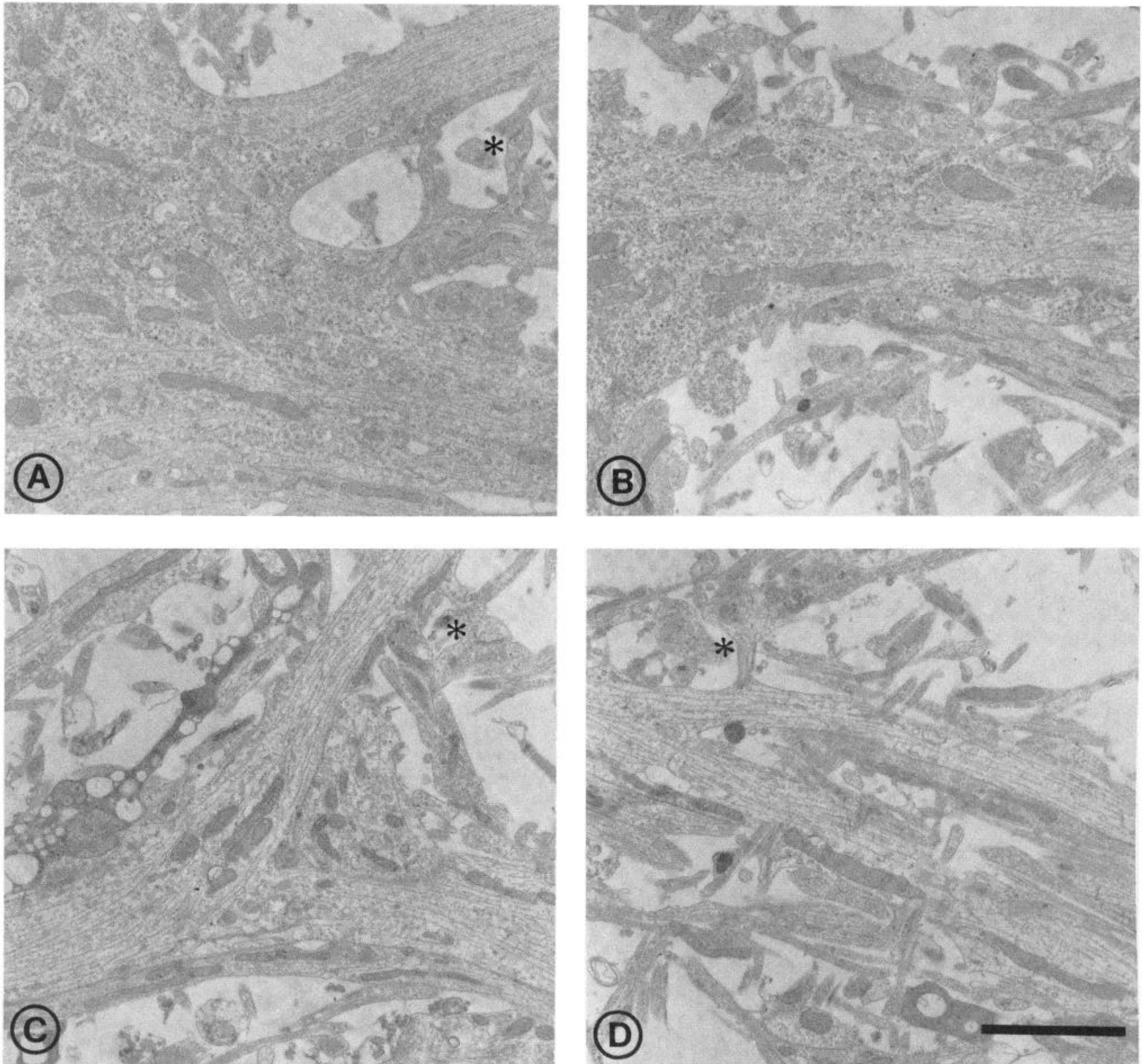

Figure 4. Higher magnification views of the neuron shown in Figure 2, illustrating the appearance of several of its dendrites at different distances from the cell body. The origins of three of the dendrites are seen in *A* and *B*. Within a short distance of their origin, there is a marked decline in the number of ribosomes and rough endoplasmic reticulum, and the microtubules have assumed a parallel alignment. More distal portions of the dendrite in *B* are shown in *C* and *D*. Microtubules are the dominant cytoskeletal element, with mitochondria, ribosomes, and an occasional cistern of smooth endoplasmic reticulum scattered among them. As with younger cells, there is some accumulation of ribosomes and mitochondria at branch points. Even at this magnification, apparent synapses can be seen both along the shafts of the dendrites and on dendritic spines (*). *Scale bar*: 3 μm.

below), which are even thinner, the density of microtubules is still about the same.

In summary, in every respect these processes have features which correspond to those of dendrites, both as seen *in situ* and as described for less mature hippocampal neurons *in vitro* (Bartlett and Banker, 1984). Most important, in every instance the dendrites which developed in culture were found to be exclusively postsynaptic.

Characteristics of the pre- and postsynaptic processes. Although large dendrites can be followed for some distance in this material, the great majority of processes, both pre- and postsynaptic, are seen only as short segments. Examples of such processes were selected at random from neuropil regions of several cultures. To determine whether individual processes had a consistent synaptic polarity, examples of processes which were involved in two or more synapses were examined (Fig. 7). In the total sample of 400 synapses examined, 50 postsynaptic processes were involved in two or more synapses (Table II). Because the presynaptic processes were quite thin and so were difficult to follow, only 15 unambiguous examples of presynaptic processes making two or more synapses were found. In every instance, processes which were postsynaptic at one site were postsynaptic at all other sites, and processes which were presynaptic at one site were presynaptic at all sites. No examples of individual processes which were both pre- and postsynaptic were found. Thus synapses are not formed indiscriminately between the processes in these cultures; rather, each individual process has a distinct synaptic polarity.

To compare the features of pre- and postsynaptic processes, examples of synapses in which a considerable length of both the presynaptic and postsynaptic processes could be seen were examined (Fig. 8). In general, in regions away from the immediate synaptic zone, the axons are quite thin and microtubules form their principal constituent. Their diameter increases somewhat in the synaptic zone where vesicles are accumulated. In contrast, the postsynaptic processes are somewhat thicker and appear to have a more electron lucent cytoplasm. Polyribosomes are common in the postsynaptic elements but not in

TABLE I
Identified processes studied

Process[a]	Length	No. of Segments Analyzed	Total No. of Synapses[b]
	μm		
A	160	11	12
D_1	150	11	16
D_2	90	6	12
D_3	210	16	14
E	90	9	12
F	80	8	15
G	140	11	23
H_1	80	4	25
H_2	180	16	50
H_3	170	12	56
H_4	60	4	11
I_1	120	10	38
I_2	150	14	30
Total sample	1680	142	314

[a] Each letter refers to a different nerve cell.
[b] This refers to the total number of synapses along the entire length indicated.

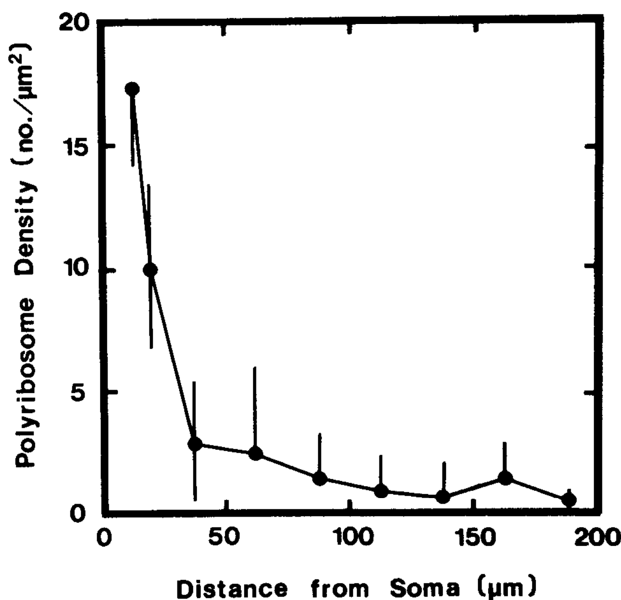

Figure 5. The change in the density of polyribosomes in dendritic processes with increasing distance from the cell body. There is a rapid decrease in the density of polyribosomes in the transition region from cell body to process proper (proximal 10 to 25 μm). The density of polyribosomes continues to decrease gradually at greater distances from the cell body, but occasional polyribosomes are present even in the most distal segments that could be traced. Mean ± SD.

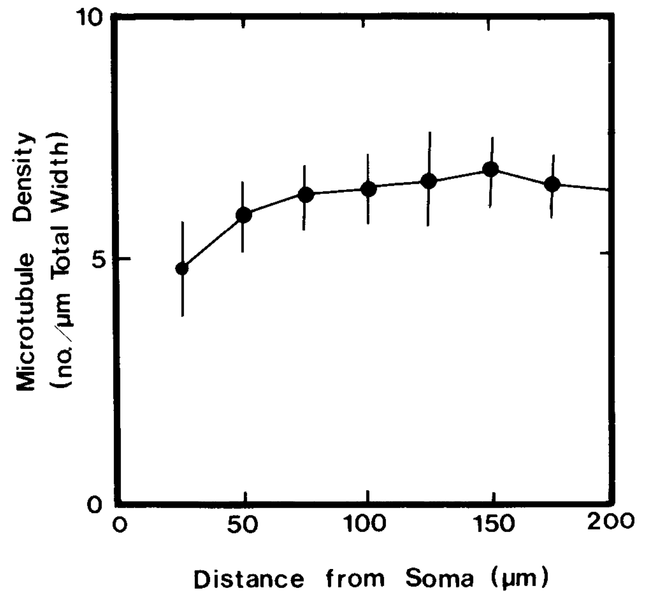

Figure 6. The density of microtubules in dendritic processes at different distances from the cell body. Beyond the base where microtubules begin to acquire their parallel orientation, there is little change in their density. Mean ± SD.

the presynaptic elements. The density of microtubules also appears somewhat higher in the presynaptic processes.

Figure 9 compares the width of the pre- and postsynaptic processes observed in these cultures. Since the presynaptic process are enlarged in the immediate synaptic zone, measurements of their width were made away from the synaptic region where they were more uniform in diameter. The presynaptic processes were in general quite thin and all fell within a rather narrow range (0.28 ± 0.1 μm, mean + SD). The postsynaptic processes were larger and more variable in width, ranging from about 0.5 μm to over 2 μm (1.1 ± 0.5 μm, mean ± SD).

Figure 10 shows the density of microtubules in the randomly selected sample of pre- and postsynaptic processes. The density of microtubules in the identified dendrites listed in Table I is also included. The microtubule density in the two groups of postsynaptic processes is similar and is significantly less than the microtubule density in presynaptic processes (t test, $p < 0.001$).

The distribution of polyribosomes in presynaptic and postsynaptic processes is summarized in Table III. Of the 383 profiles of presynaptic processes which were examined, none contained ribosomes. In contrast, 82% of the postsynaptic profiles contained ribosomes. Since the distribution of ribosomes within individual dendrites is uneven, one would expect that in some sections ribosomes would not be seen.

Features of the synapses. Synapses are quite common in hippocampal cultures which have been maintained for 3 weeks or more *in vitro* and are found in a variety of configurations (Figs. 7, 8, and 11). They occur on nerve cell bodies, on dendritic shafts, and on dendritic spines and are associated with both asymmetric and symmetric junctions. Some spines have a long, thin neck, containing membranous cisternae and amorphous flocculent material, and an enlarged head (Figs. 8*B* and 11, *D* and *E*). Other spine-like projections are flattened and sessile (Fig. 11*G*). Still others are quite large protrusions, irregular in outline and filled with polyribosomes (Figs. 7*D* and 11*F*). Frequently these large protrusions are contacted by several synaptic terminals.

Presynaptic fibers run along the surface of dendrites as well as at right angles to them. Synapses *en passant* are common (e.g., Figs. 7*D* and 11, *A* and *E*); *boutons terminaux* are also

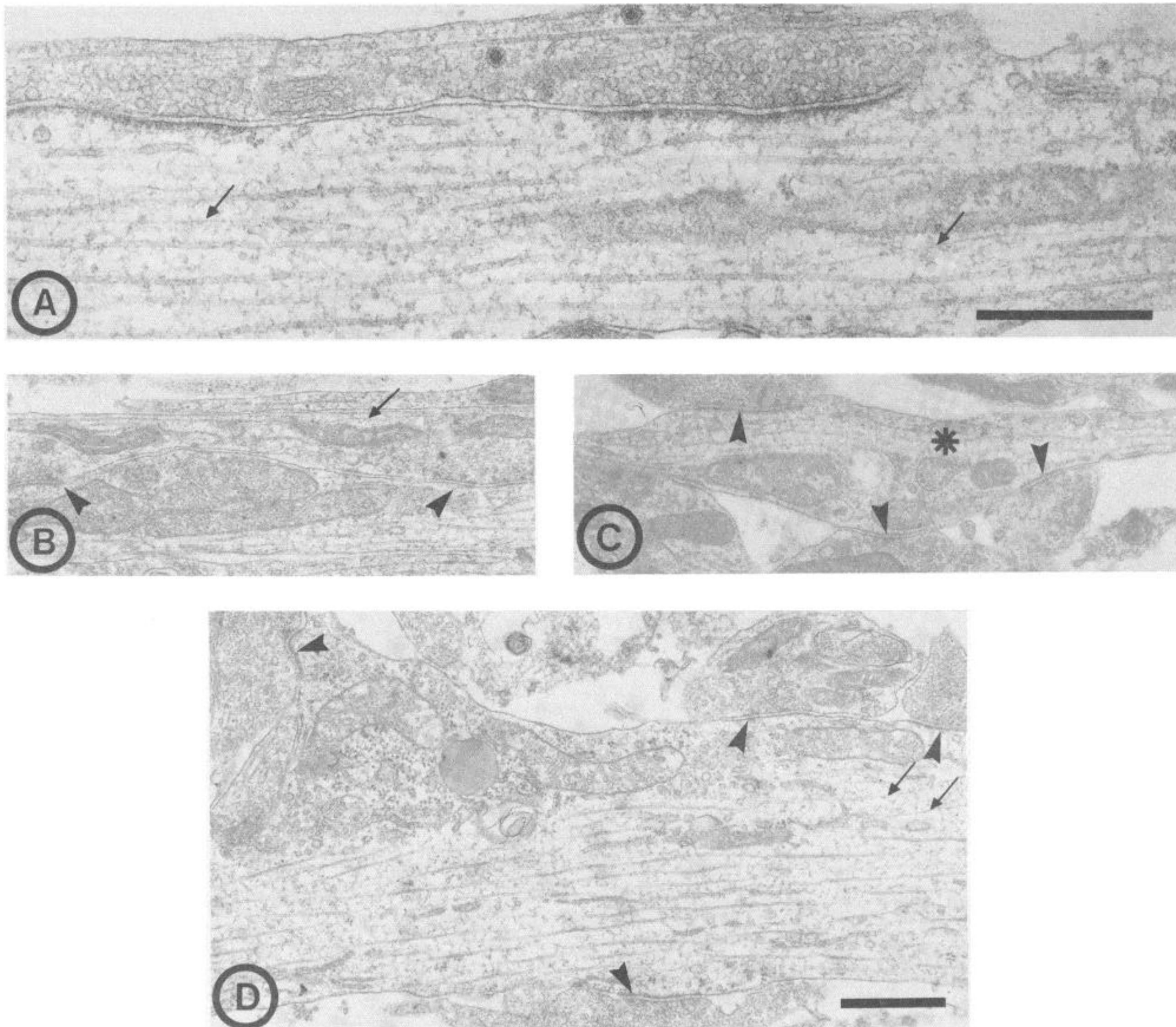

Figure 7. Several examples of neuronal processes which make more than one synapse. *A* and *B* illustrate presynaptic processes which each form two synapses (*arrowheads*). The postsynaptic processes in each of the figures are involved in two, or in some cases several, synapses. Polyribosomes are commonly found in postsynaptic processes, sometimes in large clusters (*D*). They are not found in presynaptic processes. Microtubules form the principal cytoskeletal element in both pre- and postsynaptic processes, although occasional neurofilaments are also present (*arrows*). Note also the multivesicular body present in the postsynaptic process in *C* (*). *Scale bar*: *A*, 0.5 μm; *B*, *C*, and *D*, 1 μm.

TABLE II
Processes involved in multiple synapses

	Total No. of Processes	Total No. of Synapses
Exclusively presynaptic	15	32
Exclusively postsynaptic	50	150
Mixed	0	0

seen (Fig. 11*C*). No systematic attempt was made to identify different classes of presynaptic terminals, although there did appear to be differences in terminal size and in vesicle density. Terminals associated with symmetric junctions often seemed to contain fewer synaptic vesicles than did those associated with asymmetric junctions (e.g., Fig. 11*B*). Terminals with obviously flattened vesicles were not observed, presumably because a hyperosmotic fixative was not used (see Valdivia, 1971).

Synapse distribution. Synapses onto process shafts were by far the most common type seen. Synapses onto nerve cell bodies were relatively infrequent, since the somatic membrane represents only a small fraction of the total cell surface. The frequency of synapses on spines is difficult to estimate accurately. They are probably relatively common, but the continuity of the spine with the dendritic shaft can be seen in only a small fraction of the cases.

In the hippocampus *in situ*, somatic synapses are predominantly associated with symmetric junctions, whereas synapses onto spines are associated with asymmetric junctions (Gottlieb and Cowan, 1972). Both types are observed on dendritic shafts, but asymmetric junctions predominate. A similar distribution of junctional types was observed in the cultures. Examples of synaptic junctions found on the cell somata, dendritic shafts, and dendritic spines of hippocampal neurons in culture are shown in Figure 12. When the distribution of asymmetric and symmetric synapses was analyzed quantitatively (Table IV), we found that they were not uniformly distributed on the different portions of the nerve cells (χ^2 test, $p < 0.001$). The great

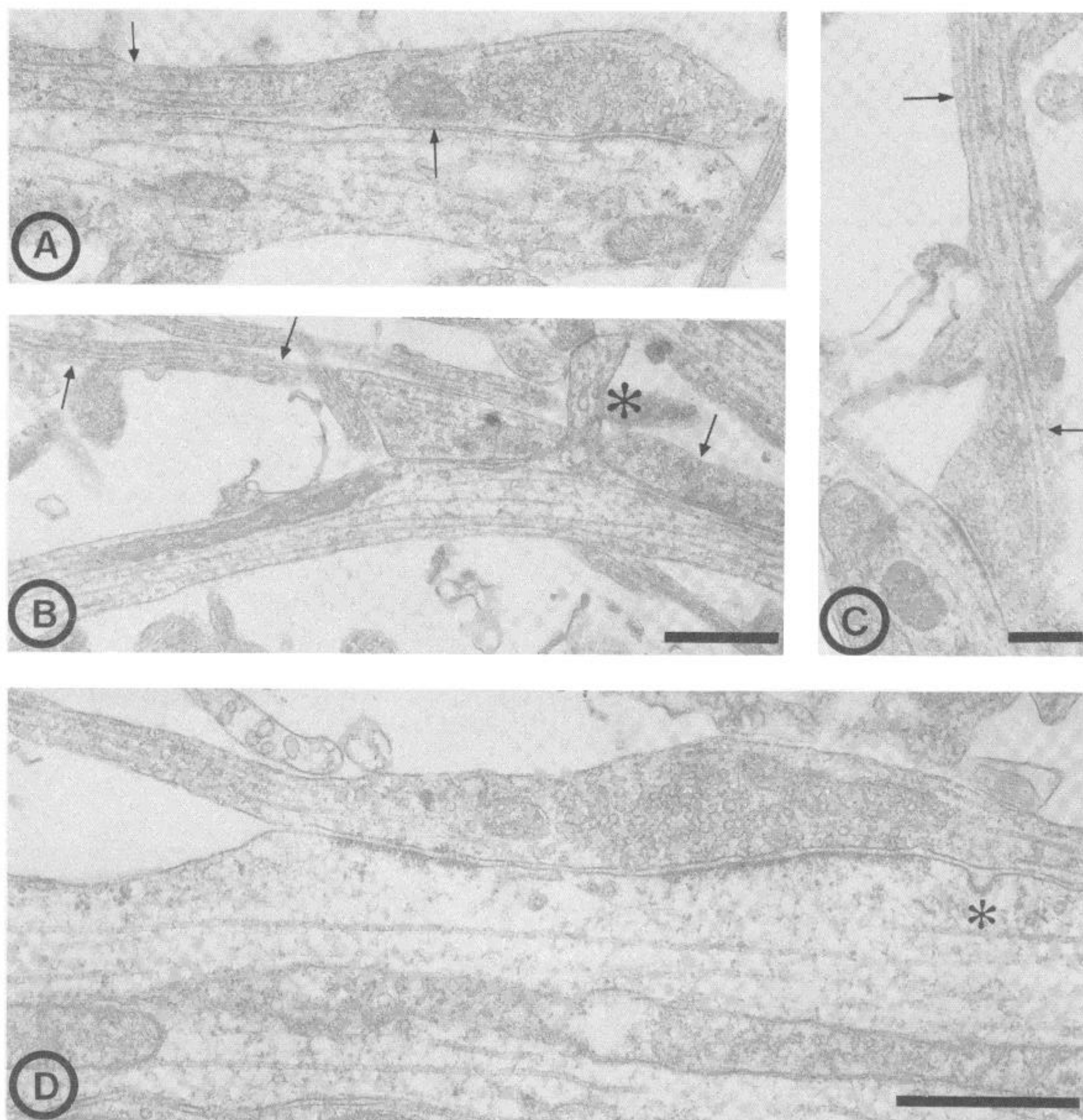

Figure 8. Electron micrographs illustrating the differences in appearance of pre- and postsynaptic processes. These micrographs were selected because they show a relatively long continuous length of the presynaptic processes (*arrows*), which tend to be considerably thinner than postsynaptic process. Scattered polyribosomes were frequent postsynaptically but were not observed in presynaptic processes. The overall electron opacity of the cytoplasm seems somewhat greater in the presynaptic processes, and presynaptic microtubules seem to be more closely spaced. Note the dendritic spine in *B* (*), with dilated cisternae of smooth endoplasmic reticulum within its neck. Also note in *D* the coated pit (*) adjoining the postsynaptic density. *Scale bars*: *A*, *C*, and *D*, 0.5 μm; *B*, 1 μm.

majority of somatic junctions were symmetric, whereas the great majority of junctions on spines were asymmetric. Both types were present on dendritic shafts, the majority being asymmetric (57%).

Discussion

Synaptic relations of axons and dendrites. The most important conclusion from the observations presented here is that the axons and dendrites of hippocampal neurons which develop in culture establish synapses with one another selectively. Axons are predominantly presynaptic and dendrites postsynaptic, indicating that the cells are able to develop their normal functional polarity *in vitro*. The evidence in support of this conclusion is especially strong in the case of dendrites. In most instances, dendritic processes can be identified unambiguously. Some can be followed continuously for 100 to 200 μm from their cells of origin; in other cases only short segments can be seen, but most can be positively identified because they contain ribosomes. We encountered no instance where an identified dendrite was presynaptic, and none of the presynaptic terminals observed in these cultures contained ribosomes.

The presynaptic processes are distinctly different in morphology. They lack ribosomes, their microtubules are on average more closely spaced together, they are thinner even than the distal dendrites, and they expand to form vesicle-filled varicosities in the immediate synaptic region. These processes undoubtedly correspond to the axons identified in the younger cultures described in the preceding paper, but by 3 weeks they have formed a plexus so dense that individual fibers cannot be traced to their cells of origin. We have not observed a clear instance of an axon which was postsynaptic. Some of the ribosome-free postsynaptic profiles might have been axons, although none was as thin as the average axon. Since synapses on the initial segments or terminal portions of axons are relatively common *in situ*, one might expect to observe occasional axoaxonal synapses in hippocampal cultures.

We also observed evidence of further specialization within the somatodendritic domain of hippocampal cells in culture. This was manifest by the presence of dendritic spines, which take on a variety of shapes, and by differences in the type of synaptic junctions which occur on somata, spines, and dendritic shafts. For the most part, the distribution of junctional types observed *in vitro* closely parallels that seen in the hippocampus *in situ* (Gottlieb and Cowan, 1972), symmetric junctions predominating on the cell body and asymmetric junctions predominating on dendritic spines and shafts. The presence of even a small number of symmetric junctions on dendritic spines is unusual. This may simply reflect the difficulty of accurately identifying and categorizing possible synaptic contacts. It could also reflect a transitory stage in synaptic development; *in situ* some fibers initially form symmetric junctions which become asymmetric with maturation (Adinolfi, 1972; Crain et al., 1973; Hinds and Hinds, 1976).

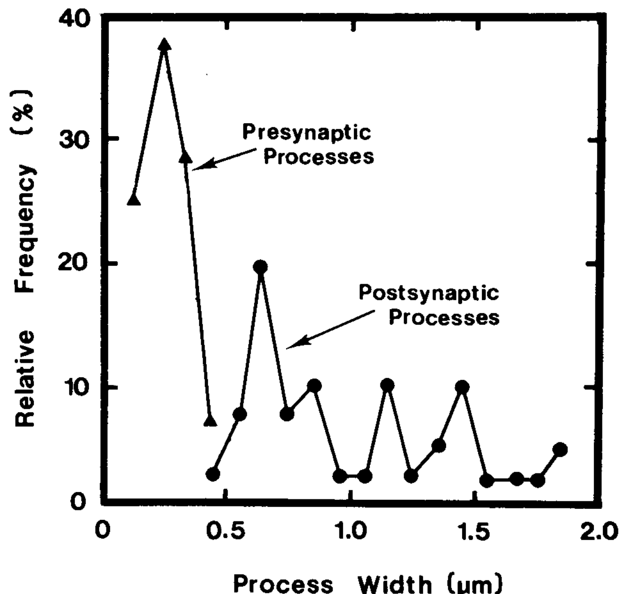

Figure 9. The width of randomly selected segments of pre- and postsynaptic processes. Typically the presynaptic processes (*triangles*) were quite thin (usually between 0.15 and 0.4 μm). The width of postsynaptic processes (*circles*) fell over a much broader range, but their minimum width was about 0.5 μm. The analysis was based on 65 presynaptic and 40 postsynaptic processes.

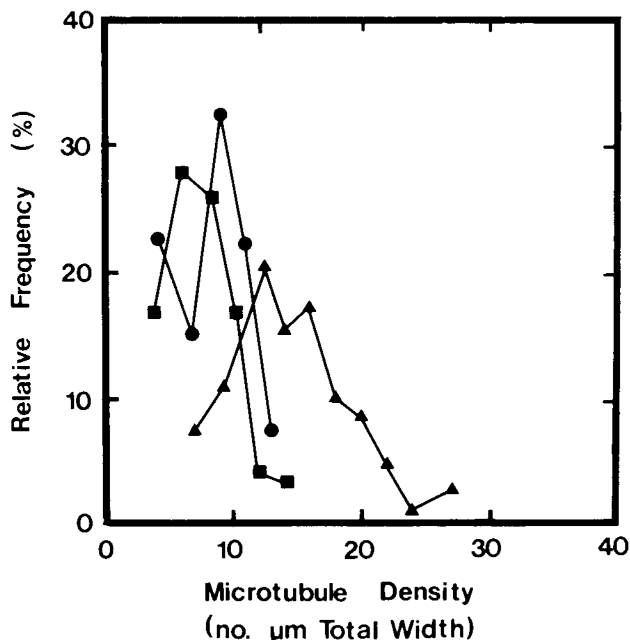

Figure 10. The relative density of microtubules in pre- and postsynaptic processes. The density of microtubules was measured in postsynaptic processes selected at random (*circles*) as well as in those dendrites which could be followed from their cells of origin (*squares*). Both have a similar microtubule density. In contrast, the density of microtubules in presynaptic processes (*triangles*) is significantly higher. The random sample included 40 postsynaptic processes and 65 presynaptic processes.

Symmetric and asymmetric junctions are usually associated with synapses which arise from different afferent populations, and frequently they differ in physiological function, symmetric junctions being most often associated with inhibitory synapses and asymmetric junctions with excitatory synapses. In the hippocampus, pyramidal cells receive monosynaptic excitatory inputs from other pyramidal cells via Schaffer collateral and commissural fibers which terminate on the dendrites (Shepherd, 1979). Many of the asymmetric junctions observed in culture could be associated with such synaptic terminals interconnecting pyramidal cells, which are the predominant cell type present in these cultures (Banker and Cowan, 1979). GABAergic cells have also been identified in hippocampal cultures (Walker and Peacock, 1982; Seifert et al., 1983; G. A. Banker, unpublished observations), and many of the symmetric junctions are likely to be associated with their terminals. *In situ* fibers from GABAergic interneurons such as basket cells terminate at symmetric junctions on the somata, and possibly on the dendritic shafts, of pyramidal cells (Ribak et al., 1978; Andersen, 1979).

Comparison with other types of neurons in culture. These findings concerning hippocampal cells are generally consistent with observations of several other types of neurons in dissociated cell culture. Intracellular injection of fluorescent dyes or of horseradish peroxidase has been used to trace the processes of single neurons in cultures of central (Neale et al., 1978; Kriegstein and Dichter, 1983) and peripheral (Wakshull et al., 1979) neurons. In each instance it was possible to distinguish presumptive dendrites, which are thick, tapering, and local, from presumptive axons, which are much longer and more uniform in diameter. In spinal cord cultures it has been shown by electron microscopy that the presumptive axons can be presynaptic and the presumptive dendrites can be postsynaptic (Neale et al., 1978), although these results do not exclude the possibility that other synaptic relationships also occur. In electron microscopic studies of single sympathetic neurons cocultured with heart cells, Landis (1977) has described presumptive axons and dendrites, which differ in their ultrastructural appearance and in their synaptic polarity.

The ultrastructural features of synapses which develop in culture have been described for cells from several regions of the CNS (Burry and Lasher, 1978; Neale et al., 1978; Romijn et al., 1981), including the hippocampus (Peacock et al., 1979). Although these studies have generally not attempted to categorize individual processes as axons or dendrites, the postsynaptic processes frequently contain ribosomes and otherwise resemble

TABLE III
Distribution of ribosomes in pre- and postsynaptic processes

	Ribosomes Present	Ribosomes Absent
	%	%
Presynaptic processes (*N* = 383)	0	100
Postsynaptic processes (*N* = 152)	82	18

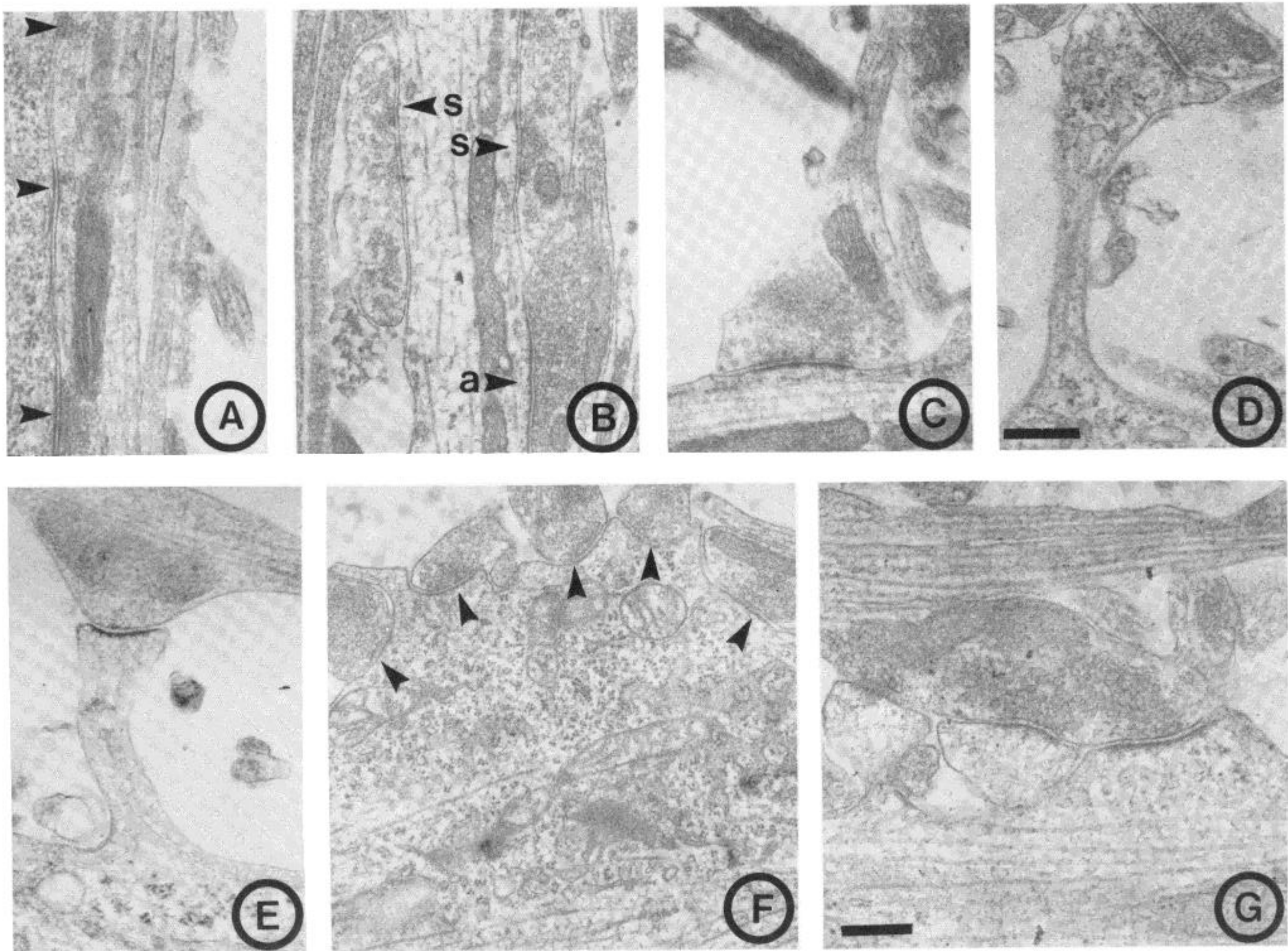

Figure 11. Illustrations showing the variety of synaptic types found in hippocampal cultures. *A* shows an example of synapses found on a neuronal cell body. In this instance a single presynaptic process makes three symmetric junctions (*arrowheads*), marked by membrane densities and accumulations of vesicles. *B* illustrates three synapses along a single dendritic shaft. Two of these are symmetric (*s*), and one is asymmetric (*a*). In this example, the density of synaptic vesicles is much greater in the terminal associated with the asymmetric junction. *C* illustrates a presynaptic process apparently ending in a *bouton termineau*, forming an asymmetric junction on the shaft of a process. *D* to *G* illustrate the variety of spine and spine-like projections seen in the cultures. Some spines have a relatively long and narrow neck and a somewhat enlarged head (*D* and *E*). Others, as in *F*, are broad and flat, containing numerous polyribosomes, and are frequently contacted by several separate presynaptic terminals (*arrowheads*). *G* illustrates a single presynaptic terminal which contacts three separate spines arising from a single postsynaptic process. One of these (at *left*) has a narrow stalk and broad head; another (at *right*) is of the sessile variety. Note that polyribosomes are frequently located at the base of these spines and occassionally are present within the spine itself (*D*). *Scale bar*: *A*, *B*, *D*, and *E*, 0.3 μm; *C* and *G*, 0.5 μm.

the dendrites seen in hippocampal cultures. Synapses on dendritic spines have been observed in cultures from hippocampus (Peacock et al., 1979) and neocortex (Romijn et al., 1981) and at least in some cases are associated with asymmetric junctions. Examples of somatic synapses with symmetric junctions have also been described (Peacock et al., 1979), but the relative frequency of each junction type on different portions of the cell has not been determined. It may well be that the selective distribution of synapses observed in hippocampal cultures will prove to apply to cultures from other regions of the CNS as well.

Axonal and dendritic features in vitro and in situ. In a previous description of hippocampal neurons in culture (Banker and Cowan, 1979), we argued that certain of the processes must be dendrites based solely on their similarity in form to dendrites *in situ* as judged by light microscopy. Because the electron microscopic observations described in this and the preceding paper strongly support this interpretation, it is worth considering in more detail the nature of this resemblance between dendrites which develop *in vitro* and *in situ*. In some instances this extends to the pattern of branching of the dendritic tree as a whole, so that cells with a characteristic pyramidal branching pattern can be identified. Often the orientation of the dendritic processes does not follow this pattern, but the shape and length of individual processes still mark them unmistakably as dendrites. Presumably the reason that individual neuronal processes can be recognized as dendritic in form is because certain constraints influence dendritic growth and branching, both *in situ* and *in vitro*. Although even *in situ* the size and pattern of dendritic arborizations can vary widely, some parameters of dendritic shape are relatively invariant among different classes of neurons (Hillman, 1979). These include the relative decrease in diameter which occurs at branch points, the relationship between stem diameter and overall dendritic length, and the diameter of terminal dendritic branches. Although largely qualitative, our observations suggest that some of these fundamental parameters of dendritic shape are shared by dendrites which develop in culture.

One quantitative difference between axons and dendrites *in situ*, which has so far received little attention, relates to the

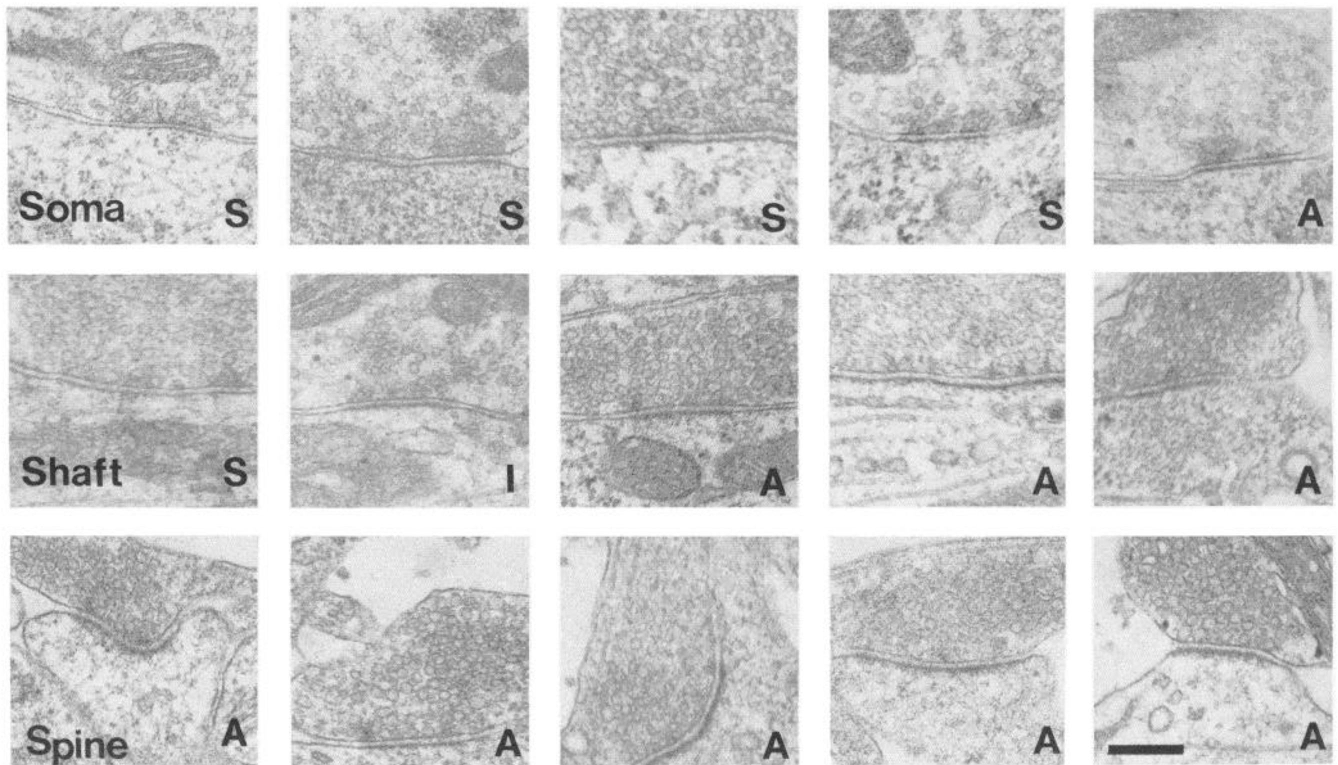

Figure 12. Examples of the types of synaptic junctions found on neuronal somata, shafts, and spines. The junctions shown were classified as asymmetric (*A*), intermediate (*I*), or symmetric (*S*). The junctions found on cell somata are principally of the symmetric type, whereas those found on dendritic spines are principally asymmetric. Both asymmetric and symmetric junctions are found on process shafts, but asymmetric junctions predominate. *Scale bar*: 0.25 μm.

TABLE IV

The relative frequency of occurrence of symmetric and asymmetric junctions on different portions of the postsynaptic cell[a]

Postsynaptic Element	No.	Frequency of Occurrence		
		Asymmetric	Intermediate	Symmetric
		%	%	%
Dendritic spines	20	85	5	10
Dendritic shafts	35	57	17	26
Somata	20	15	10	75

[a] $X^2 = 24.7$; $p < 0.001$.

minimum diameter which they can attain. The diameter of the most distal segments of dendrites, which has been determined for several different classes of neurons, ranges from 0.5 to 1.0 μm (Hillman, 1979). Apparently, dendrites do not ordinarily become thinner than this. In contrast, unmyelinated axons can be as thin as 0.1 to 0.15 μm (e.g., Peters et al., 1976). This difference obtains in culture as well. Measurements of distal dendrites in 1-week-old cells (Bartlett and Banker, 1984) or of postsynaptic processes in 3-week-old cultures (Fig. 12) indicate a minimum diameter of about 0.5 μm. By contrast, presynaptic processes often were as thin as 0.15 μm.

The density of microtubules is somewhat greater in the axons of hippocampal neurons in culture than in their dendrites. This might be related to differences in the microtubule-associated proteins (MAPs) present in axons and dendrites (Matus et al., 1981). One of these, MAP 2, is preferentially associated with dendritic microtubules, both *in situ* (Caceres et al., 1984b) and in hippocampal cultures (Caceres et al., 1984a). The presence of MAP 2, which protrudes as a side arm, decreases the packing density of microtubules polymerized *in vitro* (Kim et al., 1979).

It seems surprising that cells which in other respects appear quite mature and which bear considerable resemblance to their counterparts *in situ* have axons so thin (0.15 to 0.4 μm) and that the average diameter of the axons actually decreases between 1 and 3 weeks in culture. If, as has been suggested, neurofilaments play an important role in determining the diameter of axons (Hoffman et al., 1983), the small diameter of axons in hippocampal cultures could be related to the small number of axonal neurofilaments which develop in these circumstances. Perhaps certain cellular interactions which are necessary for axonal maturation and neurofilament systhesis are disrupted in such cultures. One factor could be the paucity of oligodendrocytes present (A. Hodson and G. Banker, unpublished observations), since pyramidal cell axons normally become myelinated.

The development of neuronal form must arise from an interplay between intrinsic and extrinsic determinants (for reviews, see Rakic, 1975; Hillman, 1979; Berry, 1982). A number of experimental approaches, including deafferentation (Parks, 1981), x-irradiation (Altman and Anderson, 1972), and the study of neurological mutations (Caviness and Rakic, 1978; Stanfield and Cowan, 1979), have established the importance of cellular interactions on neuronal development. Even in such circumstances, however, neuronal form remains sufficiently normal so that axons and dendrites can be readily distinguished and nerve cell types can be identified based on dendritic pattern. In culture, the environment for neuronal development is even more severely disrupted: growth is confined to two dimensions instead of three, the geometric alignment of cells and processes is scrambled, and the opportunity for contact between nerve cells is reduced or in some cases eliminated entirely. Nevertheless, some aspects of neuronal form, including those features which distinguish axonal from dendritic processes, are

faithfully expressed; these features of neuronal form must be intrinsically specified (see also Hillman, 1979). Present evidence indicates that the cytoskeleton is one of the important endogenous determinants of cell shape (Solomon, 1981) and that the molecular composition of the cytoskeleton differs in different classes of nerve cells and in different portions of a single cell (Shaw et al., 1981; Goldstein et al., 1983; Lazarides and Nelson, 1983; Caceres et al., 1984a). Further studies of nerve cells in culture may prove useful in elucidating the role of endogenous determinants, such as the cytoskeleton, in specifying neuronal form and the mechanisms by which they regulate axonal and dendritic growth.

References

Adinolfi, A. M. (1972) The organization of paramembranous densities during postnatal maturation of synaptic junctions in the cerebral cortex. Exp. Neurol. *34:* 383–393.

Altman, J., and W. J. Anderson (1972) Experimental reorganization of the cerebellar cortex. I. Morphological Effects of elimination of all microneurons with prolonged x-irradiation started at birth. J. Comp. Neurol. *146:* 355–406.

Andersen, P. (1979) Factors influencing functional connectivity during hippocampal development. Prog. Brain Res. *51:* 139–147.

Banker, G. A., and W. M. Cowan (1977) Rat hippocampal neurons in dispersed cell culture. Brain Res. *126:* 397–425.

Banker, G. A., and W. M. Cowan (1979) Further observations on hippocampal neurons in dispersed cell cultures. J. Comp. Neurol. *187:* 469–494.

Bartlett, W. P., and G. A. Banker (1984) An electron microscopic study of the development of axons and dendrites by hippocampal neurons in culture. I. Cells which develop without intercellular contacts. J. Neurosci. *4:* 1944–1953.

Berry, M. (1982) Cellular differentiation: Development of dendritic arborizations under normal and experimentally altered conditions. Neurosci. Res. Program Bull. *20:* 451–461.

Burry, R. W., and R. S. Lasher (1978) Electron microscopic autoradiography of the uptake of (^{3}H)GABA in dispersed cell cultures of rat cerebellums. II. The development of GABAergic synapses. Brain Res. *151:* 19–29.

Caceres, A., G. A. Banker, O. Steward, L. Binder, and M. Payne (1984a) MAP2 is localized to the dendrites of hippocampal neurons which develop in culture. Dev. Brain Res. *3:* 314–318.

Caceres, A., L. I. Binder, M. R. Payne, P. Bender, L. Rebhun, and O. Steward (1984b) Differential subcellular localization of tubulin and the microtubule-associated protein MAP2 in brain tissue as revealed by immunocytochemistry with monoclonal hybridoma antibodies. J. Neurosci. *4:* 394–410.

Caviness, V. S., Jr., and P. Rakic (1978) Mechanisms of cortical development: A view from mutations in mice. Annu. Rev. Neurosci. *1:* 297–326.

Crain, B., C. Cotman, D. Taylor, and G. Lynch (1973) A quantitative electron microscopic study of synaptogenesis in the dentate gyrus of the rat. Brain Res. *63:* 195–204.

Goldstein, M. E., L. A. Sternberger, and N. H. Sternberger (1983) Microheterogeneity ("neurotypy") of neurofilament proteins. Proc. Natl. Acad. Sci. U. S. A. *80:* 3101–3105.

Gottlieb, D. I., and W. M. Cowan (1972) On the distribution of axonal terminals containing spheroidal and flattened synaptic vesicles in the hippocampus and dentate gyrus of the rat and cat. Z. Zellforsch. *129:* 413–429.

Hillman, D. E. (1979) Neuronal shape parameters and substructures as a basis of neuronal form. In *Neurosciences: Fourth Study Program*, F. O. Schmitt and F. G. Worden, eds., pp. 477–498, MIT Press, Cambridge, MA.

Hinds, J. W., and P. L. Hinds (1976) Synapse formation in the mouse olfactory bulb. II. Morphogenesis. J. Comp. Neurol. *169:* 41–62.

Hoffman, P. N., R. J. Lasek, J. W. Griffin, and D. L. Price (1983) Slowing of the axonal transport of neurofilament proteins during development. J. Neurosci. *3:* 1694–1700.

Kim, H. L., I. Binder, and J. L. Rosenbaum (1979) The periodic association of MAP2 with brain microtubules *in vitro*. J. Cell Biol. *80:* 266–276.

Kriegstein, A. R., and M. A. Dichter (1983) Morphological classification of rat cortical neurons in cell culture. J. Neurosci. *3:* 1634–1647.

Landis, S. (1977) Morphological properties of the dendrites and axons of dissociated rat sympathetic neurons. Soc. Neurosci. Abstr. *3:* 525.

Lazarides, E., and W. J. Nelson (1983) Erythrocyte and brain forms of spectrin in cerebellum: Distinct membrane-cytoskeletal domains in neurons. Science *220:* 1295–1296.

Lim, R., S. S. Troy, and D. E. Turriff (1976) Fine structure of cultured glioblasts before and after stimulation by a glia maturation factor. Exp. Cell Res. *106:* 357–372.

Matus, A., R. Bernhardt, and T. Hugh-Jones (1981) High molecular weight microtubule-associated proteins are preferentially associated with dendritic microtubules in brain. Proc. Natl. Acad. Sci. U. S. A. *78:* 3010–3014.

Neale, E. A., R. L. MacDonald, and P. G. Nelson (1978) Intracellular horseradish peroxidase injection for correlation of light and electron microscopic anatomy with synaptic physiology of cultured mouse spinal cord neurons. Brain Res. *152:* 265–282.

Parks, T. N. (1981) Changes in the length and organization of nucleus laminaris dendrites after unilateral otocyst ablation in chick embryos. J. Comp. Neurol. *202:* 47–57.

Peacock, J. H., D. F. Rush, and L. H. Mathers (1979) Morphology of dissociated hippocampal cultures from fetal mice. Brain Res. *169:* 231–246.

Peters, A., S. L. Palay, and H. deF. Webster (1976) *The Fine Structure of the Nervous System: The Neurons and Supporting Cells*, W. B. Saunders Co., Philadelphia.

Rakic, P. (1975) Role of cell interaction in development of dendritic patterns. Adv. Neurol. *12:* 117–134.

Ribak, C. E., J. E. Vaughn, and K. Saito (1978) Immunocytochemical localization of glutamic acid decarboxylase in neuronal somata following colchicine inhibition of axonal transport. Brain Res. *140:* 315–332.

Romijn, H. J., M. T. Mud, A. M. M. C. Habets, and P. S. Wolters (1981) A quantitative electron microscopic study on synapse formation in dissociated fetal rat cerebral cortex *in vitro*. Dev. Brain Res. *1:* 591–605.

Seifert, W., B. Ranscht, H. J. Fink, F. Forster, S. Beckh, and H. W. Muller (1983) Development of hippocampal neurons in cell culture: A molecular approach. In *Neurobiology of the Hippocampus*, W. Seifert, ed., pp. 109–135, Academic Press, New York.

Shaw, G., M. Osborn, and K. Weber (1981) An immunofluorescence microscopical study of the neurofilament triplet proteins, vimentin, and glial fibrillary acidic protein within the adult rat brain. Eur. J. Cell Biol. *26:* 68–82.

Shepherd, G. S. (1979) *The Synaptic Organization of the Brain*, Ed. 2, Oxford University Press, New York.

Solomon, F. (1981) Specification of cell morphology by endogenous determinants. J. Cell Biol. *90:* 547–553.

Stanfield, B. B., and W. M. Cowan (1979) The morphology of the hippocampus and dentate gyrus in normal and reeler mice. J. Comp. Neurol. *185:* 393–422.

Valdivia, O. (1971) Methods of fixation and the morphology of synaptic vesicles. J. Comp. Neurol. *142:* 257–274.

Wakshull, E., M. I. Johnson, and H. Burton (1979) Postnatal rat sympathetic neurons in culture. I. A comparison with embryonic neurons. J. Neurophysiol. *42:* 1410–1425.

Walker, C. R., and J. H. Peacock (1982) Development of GABAergic function of dissociated hippocampal cultures from fetal mice. Dev. Brain Res. *2:* 541–555.