

**MORPHOLOGICAL CHANGES IN THE NEURITIC GROWTH
CONE AND TARGET NEURON
DURING SYNAPTIC JUNCTION DEVELOPMENT IN CULTURE**

ROSEMARY P. REES, MARY BARTLETT BUNGE, and
RICHARD P. BUNGE

From the Department of Anatomy, Washington University School of Medicine, St. Louis, Missouri
63110

ABSTRACT

Our object was to characterize the morphological changes occurring in pre- and postsynaptic elements during their initial contact and subsequent maturation into typical synaptic profiles. Neurons from superior cervical ganglia (SCG) of perinatal rats were freed of their supporting cells and established as isolated cells in culture. To these were added explants of embryonic rat thoracic spinal cord to allow interaction between outgrowing cord neurites and the isolated autonomic neurons. Time of initial contact was assessed by light microscopy; at timed intervals thereafter, cultures were fixed for electron microscopy.

Upon contact, growth cone filopodia became extensively applied to the SCG neuronal plasmalemma and manifested numerous punctate regions in which the apposing plasma membranes were separated by only 7–10 nm. The Golgi apparatus of the target neuron hypertrophied, and its production of coated vesicles increased. Similar vesicles were seen in continuity with the SCG plasmalemma near the close contact site; their apparent contribution of a region of postsynaptic membrane with undercoating was considered to be the first definitive sign of synapse formation. Tracer work with peroxidase and ferritin confirmed that the traffic of coated vesicles within the neuronal soma is largely from Golgi region to somal surface. Subsequent to the appearance of postsynaptic density, the form and content of the growth cone was altered by the loss of filopodia and the appearance of synaptic vesicles which gradually became clustered opposite the postsynaptic density. As the synapse matured, synaptic vesicles increased in number, cleft width and content increased, presynaptic density appeared, branched membranous reticulum became greatly diminished, and most lysosomal structures disappeared. Coated vesicles continued to be associated with the postsynaptic membrane at all stages of maturation. The incorporation of Golgi-derived vesicles into discrete regions of the cell membrane could provide the mechanism for confining specific characteristics of the neuronal membrane to the synaptic region.

Electron microscope studies of the development of synaptic contacts between neurons have been made in various regions of the amphibian, avian, and mammalian nervous systems, both in vivo and in

vitro. One aspect of this work has been concerned with the temporal relationship between the appearance of synaptic vesicles and the development of membrane specializations in axon terminals and

their related postsynaptic elements. In available reports the question of whether the appearance of plasma membrane specializations precedes, is concurrent with, or follows the appearance of synaptic vesicles is contended.

Glees and Sheppard (27), Bunge et al. (16, see also for earlier references), Altman (5), and Stelzner et al. (81) asserted that the appearance of plasma membrane-related specializations is the primary event initiating the formation of a synapse. Bodian (10), Larramendi (50), and Adinolfi (2) observed the appearance of membrane specializations to be concurrent with the appearance of a small number of synaptic vesicles whereas Oppenheim and Foelix (64) and Hayes and Roberts (32) noted that the appearance of vesicles in the presynaptic process precedes demonstrable membrane changes (see also 49). Several of these workers reported that the presence of transient attachment sites between neuritic processes, many of which were in uncertain relationship to small clumps of vesicles, made delineations of the steps in synaptic development difficult. Also, in some cases identification of pre- and postsynaptic elements in the immature neuropil presented additional problems. Thus, further study of synapse development in well defined systems is needed.

Previous observations in this laboratory have established that synapses are formed in culture between single or small groups of dissociated fetal rat superior cervical ganglion (SCG) principal neurons and neurites growing from adjacent short segments of fetal rat thoracic spinal cord (SC) (17). The synapses, seen on the SCG neuronal somas and processes, have the morphology of the cholinergic synapse found in the intact rat SCG and were interpreted as being derived from the axons of preganglionic visceral motor neurons in the SC explant. This synapse connecting SC and SCG neurons in culture has now been shown to have physiological and pharmacological properties very similar to its *in vivo* counterpart.¹ In addition, the SCG neurons form synapses with one another (62, 72).

This culture system possesses several advantages for studying synapse formation. The morphological and physiological properties of the SC-SCG synapse *in vivo* have been studied extensively. Pre- and postsynaptic elements can be readily established in culture and maintained for several weeks,

allowing synaptic maturation to occur. This culture system does not contain the small intensely fluorescent interneuron that is present in the rat SCG *in situ* (see discussion in 72). *In vivo*, cholinergic synapses are present primarily on principal neuron dendrites and rarely on their somata. Thus, when these neurons are removed from the animal and dendrites lost (as well as the glial ensheathment), few or no areas of former synaptic membrane specialization remain on their now completely exposed surfaces. This is an important advantage because in a number of *in vivo* systems it is known that postsynaptic specializations are retained for several months following denervation or may be formed without directly apposed presynaptic elements (69; see also 37, 43). Also, because the axons of the SC segment have been severed at the time of explantation, the newly formed fibers and their leading growth cones lack synaptic specializations. Thus, both the pre- and postsynaptic specializations must be formed *de novo*.

Lack of glial ensheathment *in vitro* allows visualization of the surface of the cultured SCG somas and their processes over their entire extent. The approach of the presynaptic element, the SC neuritic growth cone, to the SCG neuronal soma can be observed under the light microscope, and appropriate times for fixing the specimen for electron microscopy can be selected; the observed areas can be relocated after embedding for serial sectioning. Because each culture also contains uncontacted SCG neuronal somata and SC neurites, an intrinsic control is provided for relating observed morphological changes to synapse formation.

We report here a series of observations at various stages of synaptic development in this culture system, emphasizing the sequence of appearance of synaptic specializations, the origin of the membrane specialization known as the postsynaptic density, and the modification in organelle content attending the conversion of the growth cone to a synaptic bouton.

MATERIALS AND METHODS

Combination cultures of SCG neurons and SC segments were prepared by the following method. SCG were obtained from 20–21-day fetal or newborn Holtzman rats and dissociated by the mechanical method of Bray (12). The initial culture medium, a modification of that originally used (12), contained 90% Leibovitz (52) L 15 medium, 10% human placental serum, 15 mM added KCl (77), 6 mg/ml methylcellulose, 6 mg/ml added

¹ Ko, C.-P., H. Burton, and R. P. Bunge. Manuscript submitted for publication.

glucose, 0.01–0.1 $\mu\text{g/ml}$ purified nerve growth factor (NGF) (courtesy of Dr. R. Bradshaw, Washington University), and 125 U/ml penicillin. The neurons were grown in small collagen-coated Aclar inserts which were carried in modified plastic dishes (18). The SCG neurons were sparsely seeded (500–1,000 neurons/dish or 1 or 2 neurons/ mm^2) to minimize contact between them during the first days in culture.

About 48 h later, after the SCG neurons had settled and begun to grow, fetal SC explants were prepared from 15-day rat fetuses. The thoracic SC portion was selected, stripped of meninges, and cut into 0.5-mm lengths to provide small explants of the "booklet" type (18). Four explants were placed in each dish to increase the chance of contact between SC neuritic outgrowth and SCG somas. At this time the initial medium was replaced by a standard medium consisting of 88% Eagle's minimum essential medium, 2% 9-day chick embryo extract (50% in balanced salt solution, BSS), 10% human placental serum, 6 mg/ml added glucose, 20 U/ml NGF, 10^{-5} M 2-deoxy, 5-fluorouridine (to suppress the outgrowth of nonneuronal elements from the SC explant), 10^{-5} M uridine, and 15 mM added KCl. The cultures were incubated at 35–36°C, in air when the initial medium was used or in 5% CO_2 with the secondary medium, and refed every 2–3 days.

The position of the SCG neurons, uncontacted by other SCG neurons but near SC neuritic growth cones, was noted with the light microscope (Fig. 1). Cultures were fixed at suitable intervals after initial contact had been observed.

Material was prepared for electron microscopy by one of the following methods. (a) 3% glutaraldehyde (TAAB Laboratories, Emmer Green, Reading, England) in 0.1 M cacodylate buffer at 35°C was introduced very slowly by means of tubing onto the bottom of the Aclar insert while medium was withdrawn at the meniscus until a complete replacement was attained (2–3 min) (15). After 30 min at room temperature, the cultures were maintained at 4°C for a minimum of 60 min with two additional changes of fixative. After two rinses in cold BSS, they were further fixed in cold 2% OsO_4 in BSS for 1 h. Dehydration was carried out in ethyl alcohol and propylene oxide followed by equilibration in a 1:1 mixture of Epon-Araldite and propylene oxide before embedding in Epon-Araldite (55).

(b) Preservation was somewhat improved by adding sucrose to the initial aldehyde fixative, as suggested by Ludueña (53). The cultures were rinsed briefly with BSS before fixing in 0.09 M arsenate buffer (Sigma Chemical Co., St. Louis, Mo.; 78), 2% glutaraldehyde, and 4.1% sucrose (final pH, 7.3) at room temperature. After 1.5 h, the cultures were placed at 4°C for $\frac{1}{2}$ h and thereafter handled as above except for the addition of *en bloc* staining with uranyl acetate (detailed in 15; from Karnovsky, 45).

(c) For *ethanolic-phosphotungstic acid* (E-PTA)-

stained material, the following modifications in procedure were made. After primary fixation and rinsing in BSS as in (a) above, the tissue was either further fixed in 0.5% OsO_4 in BSS or not (to allow maximum visibility of the E-PTA stain). After further rinsing in BSS and dehydration up to 95% ethanol, the tissue was stained for 1 h in 1% PTA (Fisher Scientific Co., Pittsburgh, Pa.) in absolute alcohol containing a trace of water (1 to 2 drops of 95% alcohol/10 ml absolute alcohol [9]). The tissue was further dehydrated and embedded as described above. Sections were examined without further staining.

(d) Type II or Type VI (dialyzed against BSS or L 15 for 24 h) *horseradish peroxidase* (HRP, Sigma Chemical Co.) was added to BSS, to L 15 with added glucose, or to initial culture medium minus serum in the amounts of 2, 3, or 5 mg/ml. After the culture was rinsed with the above without HRP at 36°C, the HRP-containing fluid was applied at 35°C for 4, 6, or 24 min. The cultures were then rinsed as quickly as possible and fixed with the initial aldehyde preparation described in (a) or (b) above or in the paper by Bunge (15). The culture was rinsed carefully in buffered solutions prefatory to treating with the buffered reaction mixture (according to Graham and Karnovsky, 28) with 0.05% 3,3'-diaminobenzidine, 0.01% H_2O_2 and, in some cases, 10 mM CaCl_2 . After rinsing in buffered fluids, the tissue was further fixed in buffered 2% OsO_4 , in some cases *en bloc* stained with uranyl acetate as above, dehydrated, usually equilibrated in propylene oxide, and embedded in Epon-Araldite or Epon alone.

(e) For exposure of the cultured cells to *ferritin*, the preparation was rinsed briefly in initial culture medium without serum at 36°C before incubating for 1 or 8 min in the same medium containing 9% of the tracer. To minimize toxicity to the culture, two times crystallized cadmium-free ferritin (Miles Laboratories, Inc., Elkhart, Ind.) was further purified by centrifugation (145,000 g) of the stock ferritin solution, suspension of the resulting pellet in 0.1 M phosphate buffer followed by gel filtration on a 2×180 -cm column of Biogel A5M in the same buffer, concentration of the main peak fractions in an Amicon device (Amicon Corp., Scientific Sys. Div., Lexington, Mass.), and dialysis against L 15 for 36 h (prepared by Dr. M.-F. Maylié-Pfenninger, Yale University). The culture was rinsed with L 15 as quickly as possible, or in one case rinsed for 10 min, and fixed in the arsenate-glutaraldehyde-sucrose mixture as in (b) above. The tissue was next rinsed in cold BSS, further fixed in 2% OsO_4 in BSS, *en bloc* stained with uranyl acetate, dehydrated in graded alcohols, equilibrated with propylene oxide, and embedded in Epon-Araldite as described above. Sections were stained with alkaline lead as mentioned below or with bismuth subnitrate (3).

After each procedure the cured plastic disc was removed from the Aclar insert and examined under the phase microscope to select specific SCG neurons for study. These were encircled with a diamond marking

tool, cut out of the disc, and remounted for sectioning parallel to the surface of the insert, beginning at the collagen surface. Sections were cut on an LKB-Huxley ultramicrotome (LKB Instruments, Inc., Rockville, Md.) with a diamond knife, mounted on grids coated with carbon-stabilized Formvar films and, except where noted, stained with 3% aqueous uranyl acetate and lead citrate (83). Sections were examined in a Philips 300 electron microscope.

RESULTS

At the time neurites appear at the margin of the SC explant, the SCG neurons have established a neuritic outgrowth and, when in proximity to other SCG neurons, may have begun to form a network. Over the following days, the SC neurites approach either elements of this network or SCG neuronal somata (Fig. 1; see also 72). To allow for unequivocal identification of pre- and postsynaptic elements, the observations reported here are derived from regions where small SC neuritic bundles had approached individual (or a small group of) SCG neuronal somata not yet contacted by other SCG or SC neurites.

Initial SC growth cone to SCG soma contact can be accurately observed. When cultures are maintained for more than a few hours after first contact, however, the system is complicated by the arrival of additional SC neurites into the selected area. Thus, in material fixed for more than 4–5 h after initial contact, presynaptic profiles in various stages of synaptogenesis are present on somata, and it is not possible to state unequivocally which ending had formed the initial observed contact. The order of later events in the SC neurites is postulated from the minimum time of appearance of that event after initial contact and can be considered to be accurate only within the range of a few hours. A summary of time periods required for certain events appears at the end of the section, "Changes in SC Cones After Contact." Postulated changes within SCG somata were obtained by direct comparison with uncontacted neurons of the same age in the same culture dish.

SCG Neurons before Contact

As initially described by Bray (12, 13), the SCG neurons settle, attach to the substrate, and form two or more neurites within 24 h of seeding in culture. Their nuclei are markedly eccentric (Fig. 2), and their cytoplasm is characteristic of that of developing neurons (Fig. 4), as previously de-

scribed (15). In contrast to the mature neuron, they display an abundance of polysomes with only scattered segments of granular or agranular endoplasmic reticulum. The Golgi apparatus is relatively small, having a few cisternae associated with vesicles that occasionally may contain a dense core or may be coated (Fig. 5); the coated vesicles may at times appear to be continuous with Golgi cisternae. Lysosomal structures are present in the form of dense bodies, myelin-figure containing bodies, or largely empty multivesicular bodies (that may exhibit a coated area on the limiting membrane; see Fig. 12 *b*). Microtubules and neurofilaments are sparse. Also visible, occasionally, are vesicles clustered inside a protuberance of the somal plasmalemma (as described by Bunge, 15; termed "mounds" by Pfenninger and Bunge, 67; see also 68).

The surfaces of the SCG neurons and their processes are free of glial ensheathment (Fig. 4). Cellular debris, apparently resulting from the mechanical dissociation procedure, is seen along the neuronal surface. No sign of previous synaptic membrane specialization is visible at the surface of the explanted SCG neurons although it is known that synaptic contacts had been made within the ganglion before explantation (63).

SC Growth Cones before Contact

Neuritic outgrowth from the SC is seen within 24–48 h after explantation. Neurites grow out individually or, more frequently, as bundles tipped by numerous growth cones. Under the phase microscope, growth cone filopodia can be seen moving about in the medium and on the collagen surface; their appearance, at one time spiky and dark and at another wider and more transparent, suggests that they are flattened rather than cylindrical processes which may turn 90° or more as they advance. A short distance from the cord explant, groups of neurites frequently separate from larger bundles to pursue an independent course. Filopodia of some SC growth cones become associated and keep pace with supporting cells or fibroblasts which advance across the collagen by means of ruffling membranes, as first described by Grainger and James (29).

Serial sections of single or multiple SC neuritic growth cones were examined in the electron microscope. All advancing neurites, including those in bundles, are tipped by growth cones; clustered

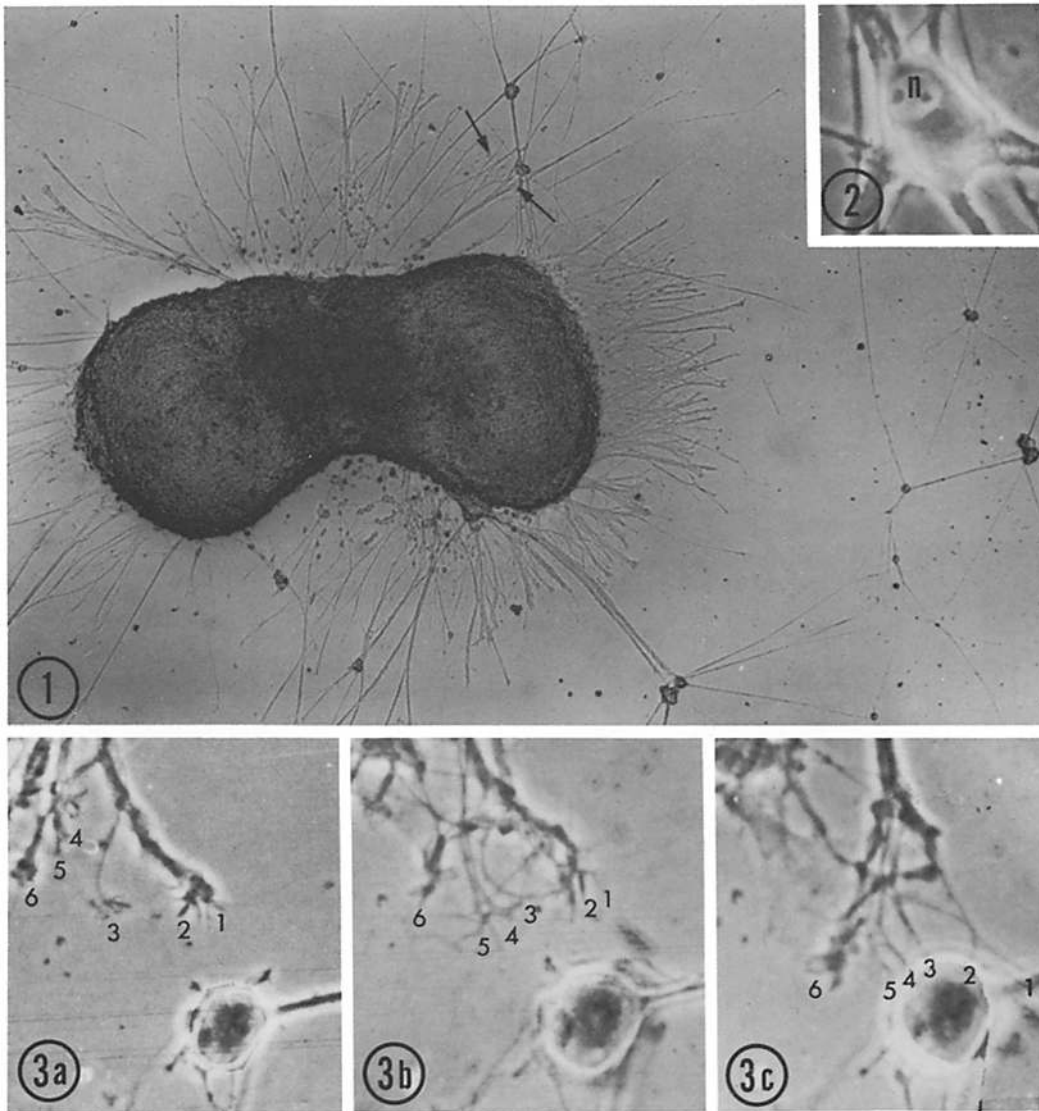


FIGURE 1 Light micrograph of a living culture shows neurites growing from a segment of SC explanted 3 days previously. Some of the neuritic tips (between arrows) are approaching isolated SCG placed in the culture dish 5 days earlier. Uncontacted neurons are seen at the right. $\times 40$.

FIGURE 2 Enlargement of a SCG neuron after 6 days in culture, showing the eccentricity of the nucleus (*n*). $\times 400$.

FIGURE 3 Portion of a series of photomicrographs illustrating the approach of a group of SC neurites to an isolated SCG neuron. Neurites nos. 1-5 made and maintained individual contacts. This culture was fixed 48 h later, and electron microscope examination showed these contacts to be synaptic in nature. Neurites in the bundle numbered 6 did not contact the neuron and continued to grow past the soma. (a) 98 min before initial contact by neurite 3; (b) 22 min later; (c) 160 min later. $\times 400$.

growth cones possess the typical organelle content, but the filopodia are more frequent at the free margins where they are not adjacent to other cones. Filopodia may be 2-5 μm long and 100 nm

in smallest diameter. Many of these are, in fact, seen in several consecutive serial sections, indicating a flattened, platelike form in agreement with the light microscope observations above. As is

typical for filopodia, they contain chiefly microfilamentous material, but, occasionally, coated vesicles, 70–80 nm in diameter, are seen at their surface, and mound areas may be visible along or at their base (Fig. 6). Synaptic vesicles or areas of synaptic membrane specialization are not present in this or other parts of the advancing growth cone.

Proximal to the filopodia the growth cone contains branched agranular endoplasmic reticulum, many vesicles including some with dense cores and an occasional mitochondrion (Fig. 6). Often, large numbers of empty-appearing, smooth, round vesicles (of varying diameter up to a maximum of about 200 nm) are clustered inside the plasma membrane which may protrude as a

mound up to 2 μm in diameter from the side of the cone (Fig. 6 *c*). More proximally, the cone also contains microtubules and numerous lysosomes which may contain disintegrating dense-cored vesicles, myelin figures, or areas of recognizable cytoplasm. Comparable observations as well as freeze-cleave data on similar SC growth cones and fibers have been reported recently by Pfenninger and Bunge (68).

Initial Contacts Made between SC Cones and SCG Somata

The following light microscope observations were made during the course of assessing the time

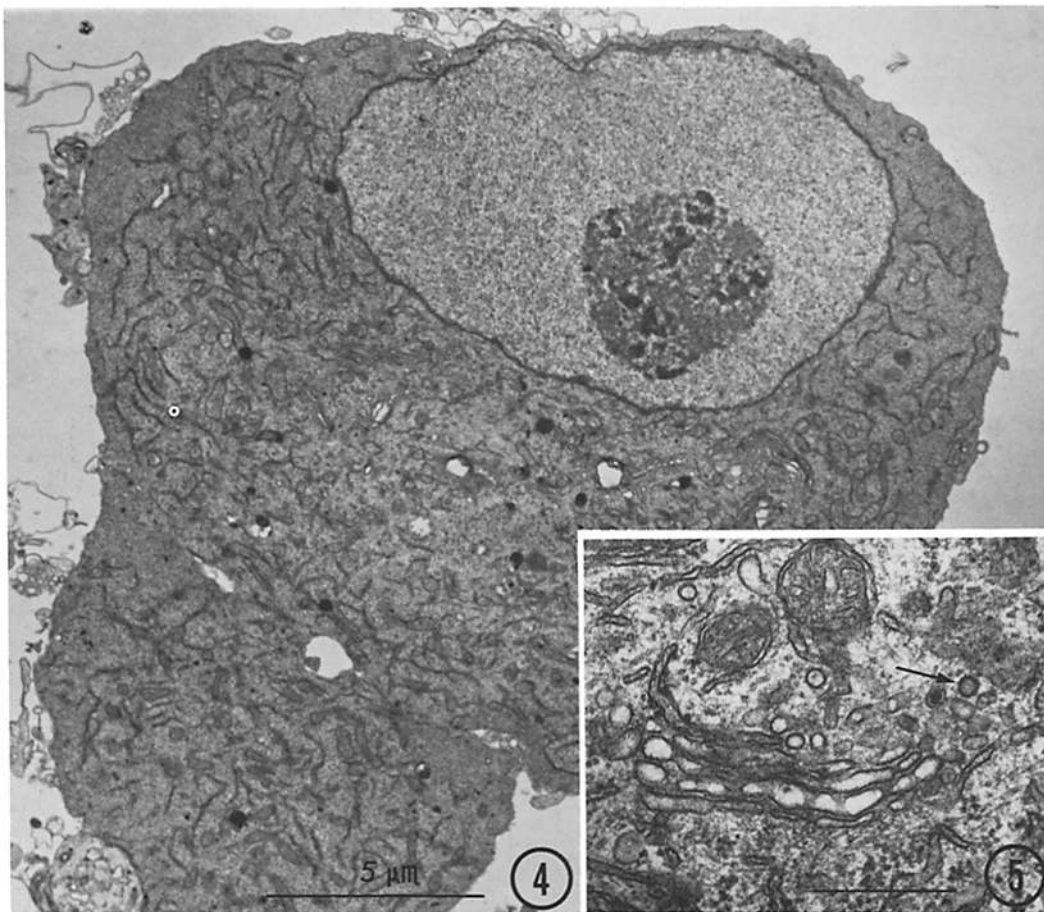


FIGURE 4 Low power electron micrograph of two SCG neuronal somata before contact by SC neurites. The cellular debris clinging to the plasma membrane is interpreted as being the remains of former neighboring cells disrupted in the isolation procedure. Former synaptic membrane specializations are not seen. 3 days in culture. $\times 6,600$.

FIGURE 5 Detail of the Golgi apparatus of an uncontacted SCG neuron. The arrow indicates a coated vesicle 100 nm in diameter. $\times 19,000$. Bar, 1 μm .

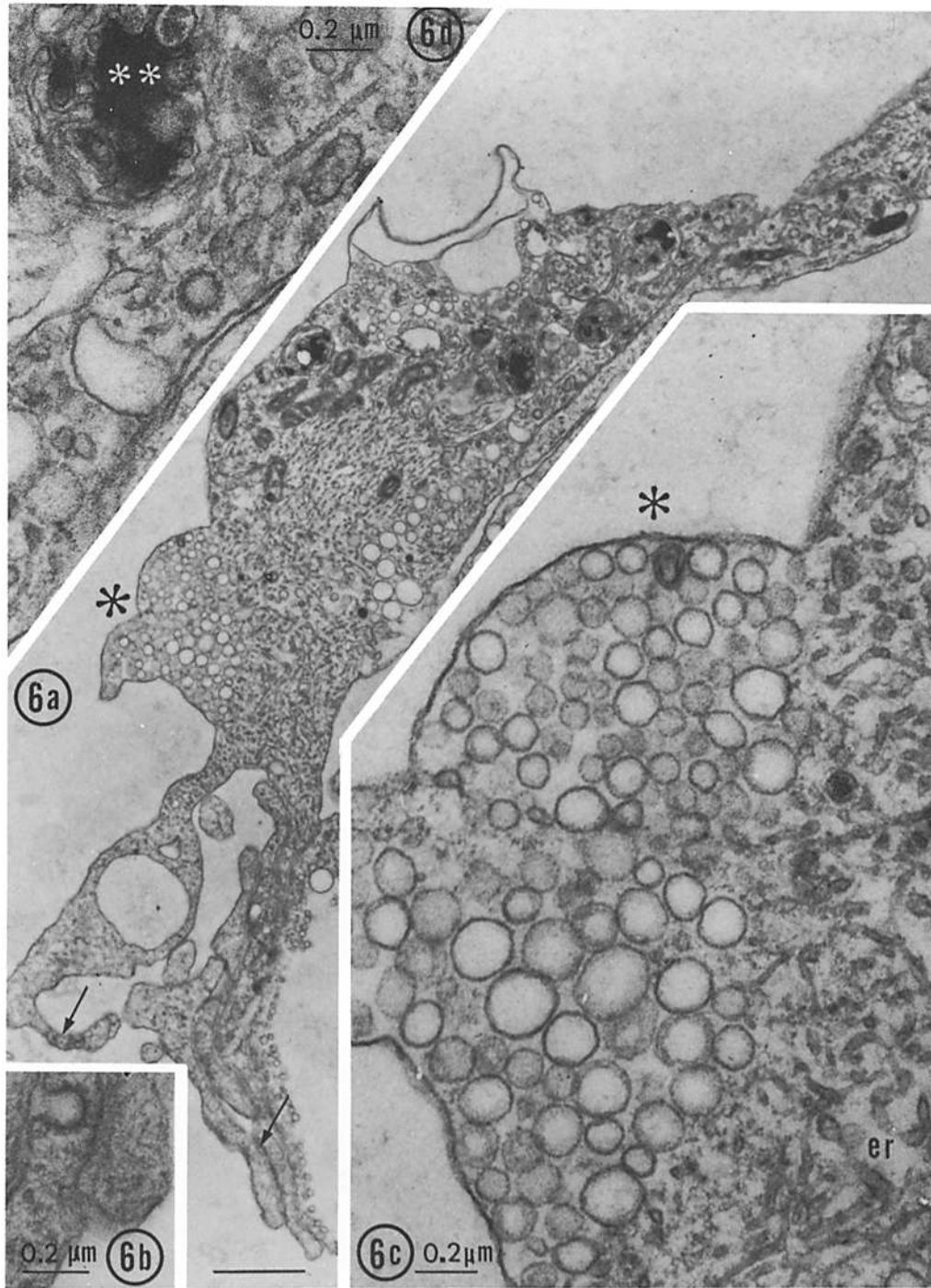


FIGURE 6 Electron micrographs of a SC neuritic growth cone before contact. The overall view of the growth cone is shown in (a) with enlargements of some areas in (c) and (d). In (a), note the filopodia (arrows) at the front of the cone and, farther back, the large mound area (*). Filopodia contain few organelles except filamentous material and, as shown here, an occasional coated vesicle (b). The interior of the cone is partly occupied by branched membranous reticulum (*er*) (c); moundlike protuberances containing clusters of vesicles are commonly observed (*, c). Farther back in the cone is an area containing numerous lysosomes (a, d); one of them contains dense-cored vesicles (**, d). Also in this area are microtubules and coated vesicles. (a) $\times 13,000$, bar, $1 \mu\text{m}$; (b) $\times 51,000$; and (c and d) $\times 49,000$.

of first contact between SC growth cones and SCG somata. Two types of initial contact are observed. In one type, the contact is made by one or more of the motile filopodia of the cone, and this contact is maintained. Within a few minutes the remaining filopodia cease their exploratory motion (or palpation, 56) and can no longer be observed as extensions. After a variable interval of contact (up to ½ h), filopodia may form anew on the growth cone adjacent to the contact point and the neurite may continue to grow while maintaining its initial contact. In the other type of initial contact, filopodia can be seen to move on the surface of the postsynaptic element for several minutes before the cone withdraws all filopodia and continues to grow. Actual contact between the periphery of the growth cone and SCG somata or processes seems essential for subsequent interaction. Neurites growing at random on the collagen may pass within several micrometers of a SCG soma, but if no contact is made they do not appear to deviate from their course in the vicinity of the neuron (Fig. 3). It was not possible to study further structural detail at the light microscope level because of the limits of resolution with our culture chamber and the tendency of the growth cone to make contact with the SCG soma at or near the level of the collagen where events are obscured by the bulk of the overlying cell body.

In cultures examined at or near the time of initial contact, no specialized membrane areas are seen either on the SCG somata or on the SC growth cones. A process from the growth cone appears to establish an extensive area of contact with the SCG soma (Fig. 7) and can be observed as a flattened sheet lying on the surface of the soma, extending through as many as 10–15 serial sections. The typical filamentous feltwork is seen within this sheet, together with occasional membranous elements. In the immediately adjacent soma the cytoplasm is typical, containing ribosomes, some scattered sacs of smooth endoplasmic reticulum and an occasional large dense-cored vesicle. The distance between the outer leaflets of the apposing membranes varies; at occasional points along the area of apposition, they come closer to each other over lengths of some 20–50 nm (Figs. 7 *b* and *h*). The space between outer leaflets in these punctate areas is 7–10 nm. Within the extracellular space near this point of close contact there is often a suggestion of intercellular material, but there is no pre- or postsynaptic density (Figs. 7 *b*, *d*, and *h*).

Changes in SCG Somas after Contact

In examining SCG somata during the first several days after contact by SC neurites and comparing these to uncontacted cells, we gained the impression that the Golgi regions of contacted cells are larger. We also noted an apparent increase in the number of coated vesicles of various sizes in these hypertrophied Golgi regions, as well as in relation to the plasmalemma of the neuronal soma and its proximal processes (Fig. 8). In that portion of the SCG soma adjacent to collagen, where most processes arise, coated vesicles are particularly numerous, within both soma and processes.

To better characterize this apparent response of the SCG somata to neuritic contact, coated vesicles of all sizes were measured. Charted results (Table I) indicate at least two coated vesicle populations, one most frequently exhibiting a diameter of 50 nm and another of 100 nm. We then counted coated vesicles of each size class in Golgi regions and a 0.5- μ m surrounding zone in both contacted and uncontacted SCG somata. These counts indicate that contacted neurons have an increased number of both classes of coated vesicles in this region (Table II). Vesicles appear either in continuity with the Golgi cisternae, often at the maturing face, or in the adjacent cytoplasm (Fig. 9).

The larger coated vesicles could also be seen near or continuous with the SCG plasmalemma, particularly in the area of developing junctions. In order to quantitate the relation between these vesicles and forming junctions, we selected (from 5-day contacted SCG neurons) 100 regions where development of a synaptic junction had begun; we then counted the number of coated vesicles at or within a 0.5- μ m zone of this site. 63% of these regions contained a coated vesicle. In regions *not* contacted by presynaptic elements, only 8 of 100 segments of neuronal surface (of comparable length) contained coated vesicles. These observations raise the question whether coated vesicles fuse with the plasmalemma at developing synaptic regions, thereby adding a region of membrane with an undercoating which may contribute to postsynaptic density. This site in the contacted soma is considered to be the first definitive sign of synapse formation; it occurs next to rather than in place of the close contact regions described above. Areas of close contact continue to be seen adjacent to synaptic regions in a more advanced state of

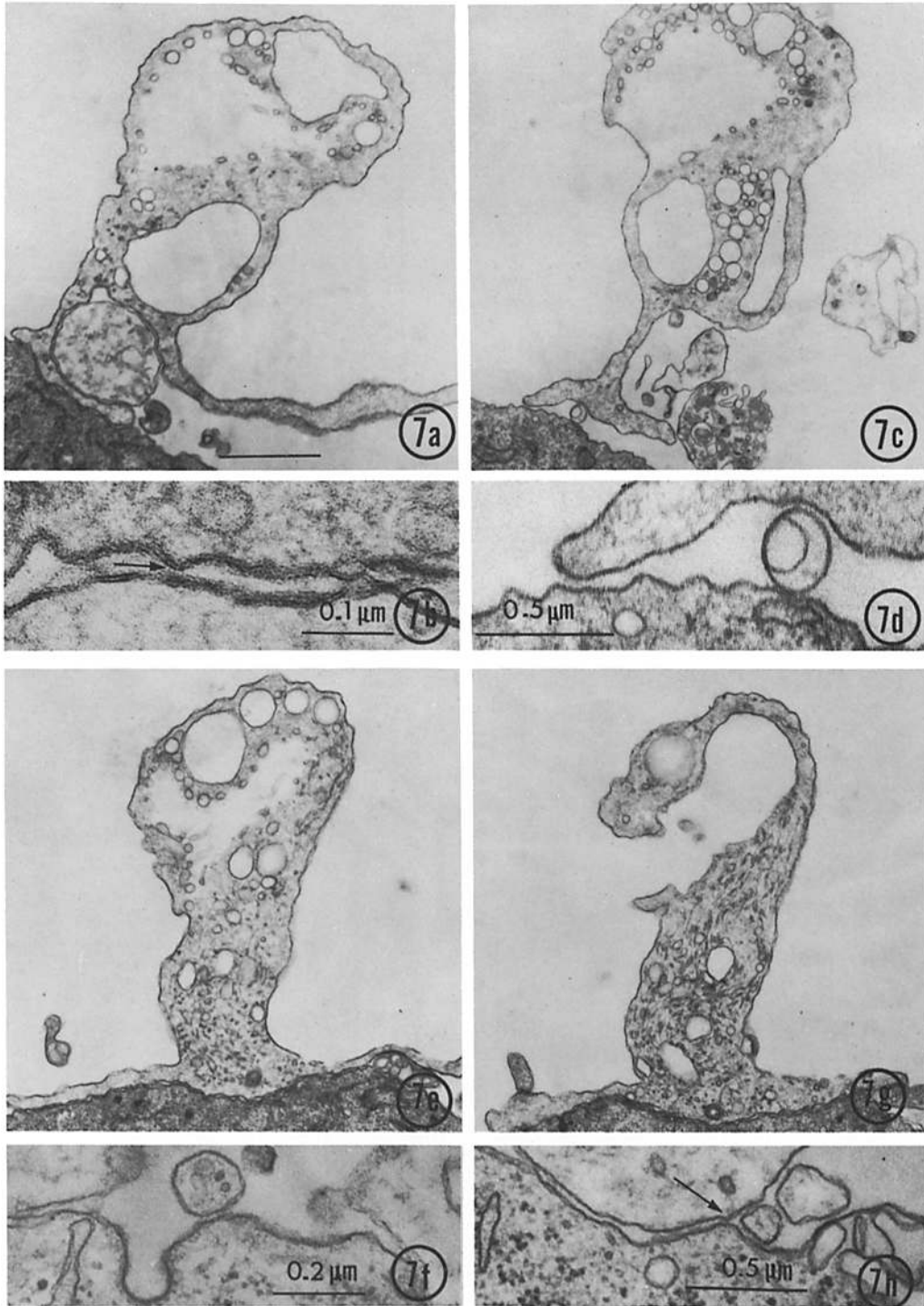


FIGURE 7 This plate illustrates four areas (*a, c, e, g*) from a set of serial sections through the distal part of a SC neuritic growth cone in early contact with the surface of a SCG neuronal soma, together with detail of contacted areas. Filopodia, now flattened and platelike in form, are applied to $\sim 20 \mu\text{m}^2$ of cell surface. Outer membrane leaflets of the apposing elements are separated by a minimum cleft width of $\sim 8 \text{ nm}$ at numerous points (*b, h*, arrow). Pre- or postsynaptic density is not present (*d*). Within 24 h of contact, coated vesicles, 100 nm in diameter, are commonly seen in the somal cytoplasm at or near contact areas; at (*f*) one is seen in continuity with the plasma membrane; (*a-e* and *g*) 5 h after initial contact; and (*f* and *h*) 12 h after initial contact. (*a, c, e, and g*) $\times 15,000$, bar, $1 \mu\text{m}$; (*b*) $\times 140,000$; (*d*) $\times 40,000$; (*f*) $\times 70,000$; and (*h*) $\times 36,000$.

TABLE I

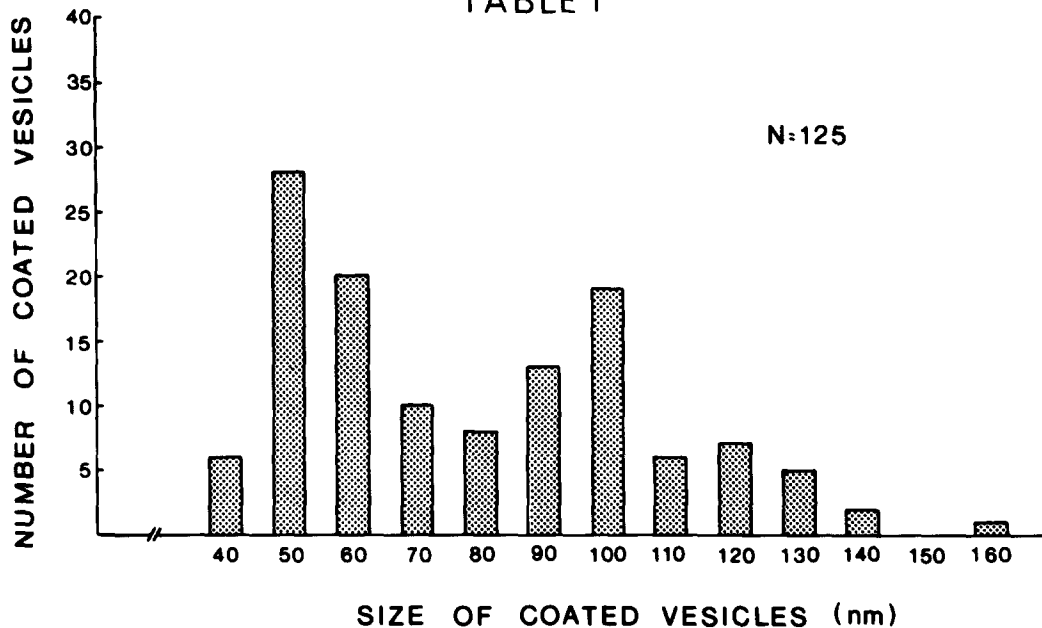


TABLE II

Incidence of Small (SCV) and Large (LCV) Coated Vesicles in Golgi Regions (and 0.5- μ m Surrounding Zones) in Superior Cervical Ganglion (SCG) Somata with or without Contact by Spinal Cord (SC) Neurites

| Cell preparation | No. samples analyzed | SCV (< 80 nm) per μ m ² | LCV (> 80 nm) per μ m ² |
|-------------------|----------------------|----------------------------------------|----------------------------------------|
| Uncontacted cells | | | |
| SCG alone, 1 div | 7 | 0.135 | 0.115 |
| SCG + SC, 1 div* | 7 | 0.190 | 0.154 |
| SCG + SC, 5 div* | 15 | 0.093† | 0.218† |
| Contacted cells§ | | | |
| SCG + SC, 5 div* | 12 | 0.333† | 0.449† |

* Days in vitro (div) refer to time in culture following the addition of SC to SCG cells.

† Based on the signed rank test derived from Wilcoxon, as well as Mann and Whitney (see reference 80), the null hypothesis that the large or small coated vesicle density is similar in the Golgi regions of uncontacted and contacted cells is rejected at the 0.01 level.

§ Cells sampled were in close proximity to SC where contact usually occurred by 2 div.

development. In cultures up to 3 wk old the 100-nm coated vesicles remain prominent in the Golgi area, cytoplasm, and at the surface of the SCG soma. Frequently, they are seen as coated

caveolae beside previously formed postsynaptic densities, as in Fig. 16.

Our impression that traffic of coated vesicles in the SCG neuronal soma is largely from Golgi region to somal surface derives from the above observations and also from the use of electron-dense tracers. In these tracer studies no distinction was made between large and small coated vesicles. After 4- or 6-min exposure to HRP, labeled coated vesicles are not seen within the soma interior. Some coated vesicles very near the somal plasmalemma appear to contain label. It can be shown by serial sectioning, however, that at least some of these are actually open to the exterior at another level and may be in the process of exocytosis rather than endocytosis. In contrast, some somal lysosomes are labeled and, in the SCG fiber and growth cone areas, coated vesicles as well as other structures are labeled (14). After a longer exposure (24 min) to a higher concentration of HRP (5 rather than 2 or 3 mg/ml), there is much more labeling throughout the SCG neuron; in the soma, lysosomes and tubular structures are heavily labeled (Fig. 10). Even at this longer interval, label was observed in only 20% of the 50 coated vesicles counted; some of these were very near the somal surface. Coated vesicles contiguous with Golgi cisternae are never labeled, either in contacted or in uncontacted cells.

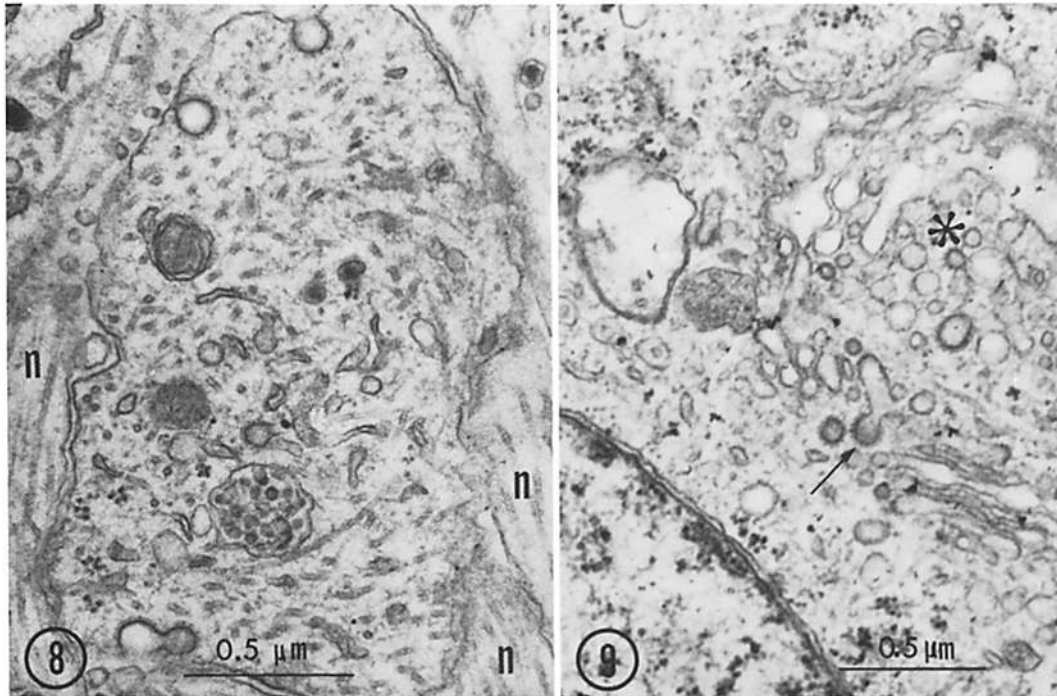


FIGURE 8 Large coated vesicles are seen in continuity with the plasma membrane of a portion of a SCG neuron which is surrounded by SC neurites (*n*). 47 h after initial contact. $\times 46,000$.

FIGURE 9 The Golgi apparatus of a 5-day contacted neuron is more extensive than that of an uncontacted neuron of the same age in culture. Cisternae may be dilated and large coated vesicles, 100 nm in diameter, are seen in continuity with them (arrow). This is the class of vesicle thought to be in continuity with the plasma membrane in the contact area. A smaller class of coated vesicle (*) also is contiguous with Golgi elements. $\times 39,000$.

In agreement with these experiments employing HRP, coated vesicles in the soma interior are very rarely labeled after 1- or 8-min exposure to ferritin (Fig. 12). A few at the surface are labeled, again perhaps because they are open to the cell exterior at some level; usually some ferritin adheres to the somal plasmalemma. At 8 min, ferritin is just beginning to appear in the immature multivesicular bodies (Fig. 12 *b*); by another 10 min, considerable amounts have begun to accumulate in these bodies (Fig. 12 *c*). In contrast, growth cone coated vesicles are clearly labeled after 1- or 8-min exposure to ferritin. Thus, in the case of both markers it appears that uptake occurs primarily at the growing tip. Many unlabeled coated vesicles seen in the somal area appear to be derived from the Golgi apparatus and to be destined for the cell surface. Those few labeled coated vesicles seen in the soma may have travelled retrogradely from the fiber or growth cone, as will be discussed below.

In osmicated (Figs. 11 *a* and *b*) or unsmicated (Figs. 11 *c* and *d*) material, coated vesicles seen in continuity with the Golgi cisternae, throughout the cytoplasm, or continuous with the somal plasma membrane are stained heavily with E-PTA, which is known to stain synaptic membrane specializations (9). In contrast to the more mature synapse, the only other putative synaptic-related structure stained by E-PTA at early stages is the large dense-corded vesicle.

Changes in SC Cones after Contact

Before the appearance of postsynaptic density, SC growth cones adjacent to the SCG somata and related to them by one or more close contacts show only those cytoplasmic features previously described. Where postsynaptic density is visible, however, the synaptic cleft is enlarged to 13 nm (Fig. 13) and clear round vesicles corresponding in

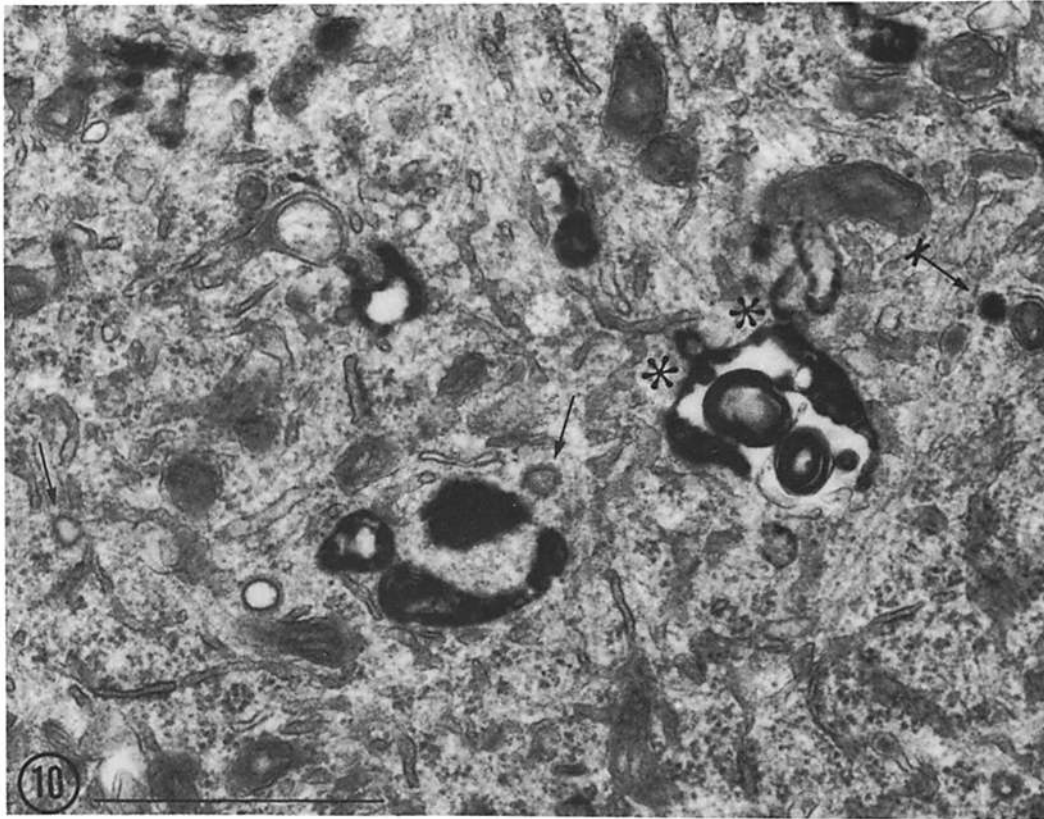


FIGURE 10 A portion of the soma of an uncontacted SCG neuron which had been exposed to horseradish peroxidase for 24 min. Reaction product is seen within lysosomes and structures fusing with them (*). Of the coated vesicles in the field, two are clearly unlabeled (arrows) and one is definitively labeled (x→). $\times 38,000$. Bar, $1 \mu\text{m}$.

size to cholinergic synaptic vesicles are frequently found in adjacent growth cone cytoplasm (Fig. 14). In rare instances, chains of similar vesicles give the impression of their origin from smooth reticulum (Fig. 15). In developing junctions approximately 24 h after first contact, vesicles are few in number and centrally placed within the cone (Fig. 14). As vesicles increase in number, some become clustered near the plasma membrane opposite the postsynaptic membrane specialization. Only after a considerable number of synaptic vesicles and some dense-cored vesicles have gathered at the presynaptic membrane is it possible to detect the appearance of small presynaptic dense projections (Fig. 17). The cleft width increases to $\sim 18 \text{ nm}$. It is estimated that in our culture system the time required between initial contact and this degree of development is $\sim 48 \text{ h}$.

As vesicles gather at the presynaptic membrane opposite postsynaptic density, presynaptic and

cleft material appears. With the E-PTA technique, presynaptic dense projections and synaptic cleft material are demonstrable only after the appearance of stained postsynaptic density. As already noted, the cores of large dense-cored vesicles are E-PTA positive.

With the appearance of a synaptic area, many growth cones no longer possess filament-containing filopodia. Branched membranous reticulum, mound areas, and an occasional mitochondrion lie immediately adjacent to the synaptic vesicles (Fig. 18). The growth cone lysosomal region with its autophagic vacuoles containing myelin figures and dense-cored vesicles is still present. As the number of synaptic vesicles and the length of synaptic membrane specialization increase, the growth cone reticulum and lysosomal elements become diminished (Fig. 19). Other cones retain filopodia together with reticulum and lysosomal elements in the presence of one or more synaptic areas. Such

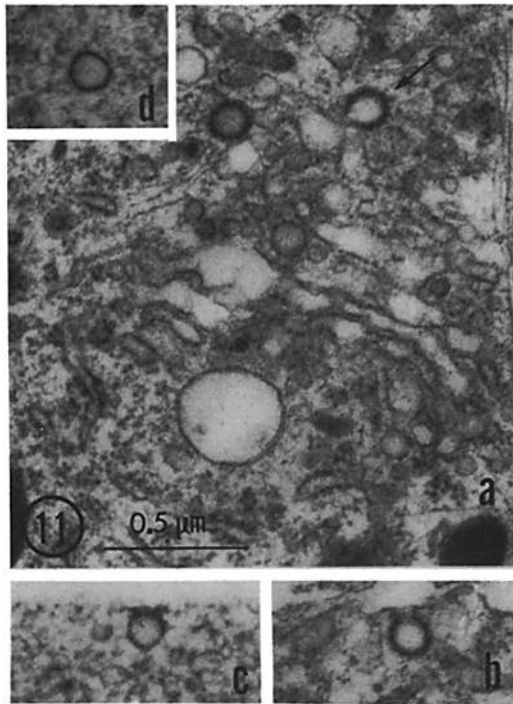


FIGURE 11 In material fixed with glutaraldehyde and then OsO_4 , the 100-nm coated vesicles continuous with the neuronal Golgi cisternae (*a*, arrow) or the neuronal plasmalemma in a contact area (*b*) show a marked affinity for E-PTA stain. (*c* and *d*) shows E-PTA staining of these structures in nonosmicated material. 48 h after initial contact. $\times 46,000$.

cones may be destined to make additional contacts which would in time develop into synapses of the "de passage" variety.

An analysis of the minimum time elapsing between initial growth cone-somal contact and the morphological events described in the foregoing sections permits the following summary: (*a*) an increase in number of large somal coated vesicles at the site of apposition between growth cone and SCG neuronal soma (Fig. 7 *f*), 6 h; (*b*) the presence of postsynaptic density without opposed synaptic vesicles (Fig. 13), 15 h; (*c*) the presence of a few synaptic vesicles opposite postsynaptic density (Fig. 14), 18 h; (*d*) ongoing reorganization of the interior of the growth cone to form a synaptic bouton (Fig. 18), 18 h; (*e*) formation of a synaptic profile without remnants of growth cone organelles (Fig. 19), 36 h.

Further Maturation of Synaptic Elements

During the remaining 2 wk of the culture period studied, the synapses mature further. Synaptic

vesicles increase markedly in number and are present in dense clusters near the presynaptic membrane (Fig. 20 *a*). Large dense-cored vesicles (~ 120 nm in diameter) also are frequently present in this area. E-PTA staining demonstrates an increase in height and width of presynaptic dense projections and the concomitant appearance and increase of cleft material (Fig. 20 *c*). Lysosomes and branched reticulum, typical of growth cones, are further reduced in the bouton interior and mound areas are absent from the plasma membrane. Most boutons contain a few mitochondria. Synaptic cleft widths achieve the maximum value of 22 nm. The larger coated vesicles still are frequently seen in continuity with the postsynaptic membrane or in the adjoining cytoplasm (Fig. 20 *b*).

After 2–3 days in culture, the network formed by the neurites of the SCG neurons expands and many contacts are made by SCG neurons with each other (72), these occurring adjacent to contacts by SC neurites (17). Maturing synaptic profiles of the SCG-SCG type, as seen in aldehyde-fixed material, are crowded with pleiomorphic vesicles 50–70 nm in largest diameter and thus can sometimes be distinguished from the cholinergic synapse formed with SC neurites. Preliminary observations without the benefit of timed intervals suggest that the synapse between SCG neurons is formed in the same manner as is the SC-SCG synapse. Where the two synaptic types are forming simultaneously, it is not possible to distinguish morphologically between 100-nm coated vesicles related to postsynaptic areas of the two different synaptic types. Both the accumulation of synaptic vesicles and the gradual replacement of growth cone organelles appear to follow the sequence already described for the SC-SCG synapse.

DISCUSSION

Our interpretation of the sequence of cellular interactions that occur during synapse formation in the culture system employed is summarized in Fig. 21. The organelles of the presynaptic element subserving motility and exploration are replaced or modified for the new requirements of chemical transmission. Of the components typically a part of the growth cone, the first to disappear are the filopodia and their distinguishing filamentous feltwork; they are present only before and immediately after contact. The characteristic branched membranous reticulum is retained in quantity in the differentiating terminal as the first synaptic

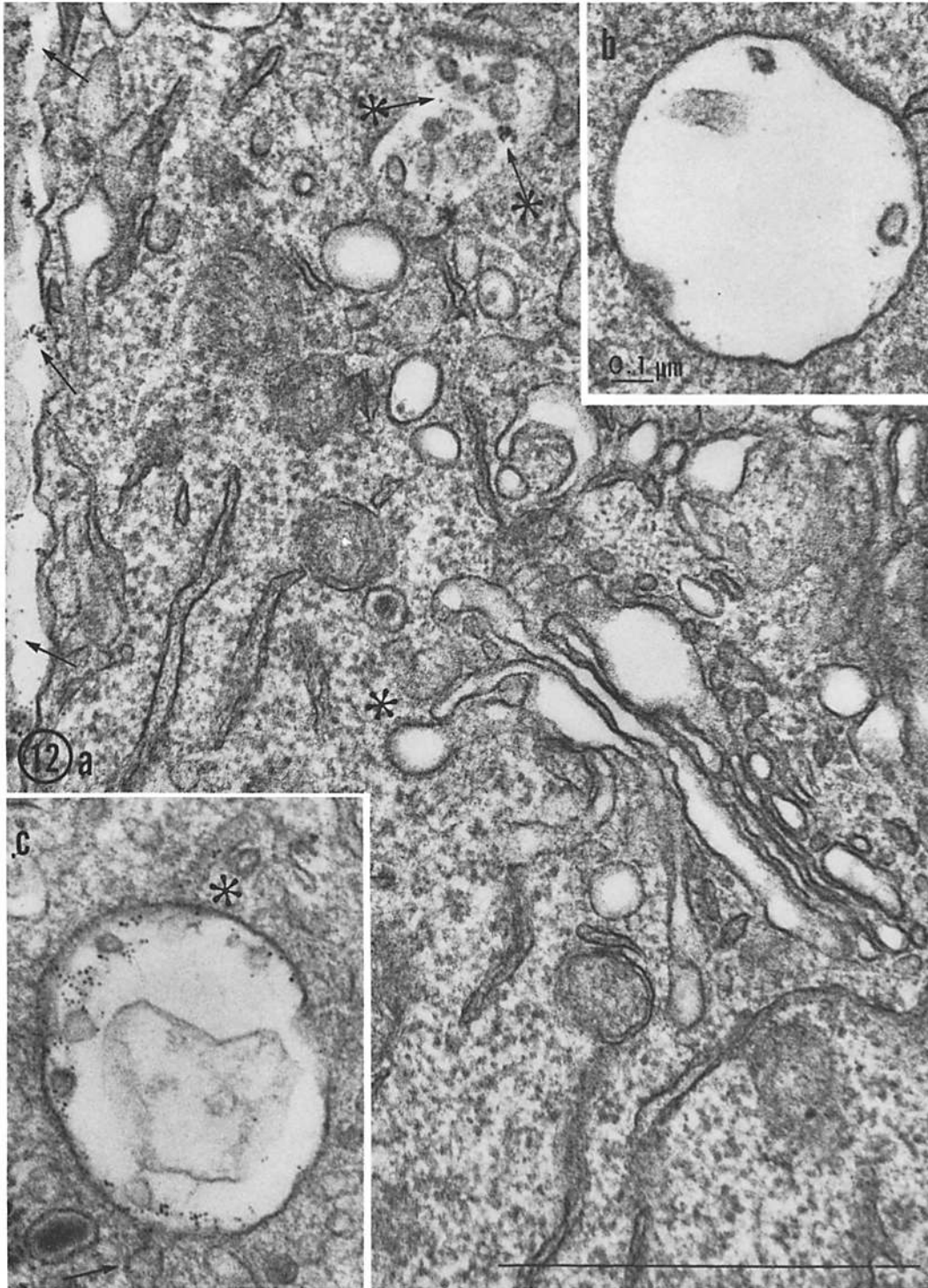


FIGURE 12 (a) Uncontacted SCG neuronal soma exposed to ferritin for 8 min. Ferritin is present only along the cell exterior (arrows) and within a multivesicular body (*-arrows); none is seen in the interior of the coated vesicle (*). (b) Another multivesicular body is seen to contain a few ferritin particles after 8-min exposure. (c) More ferritin particles are seen in multivesicular bodies after an additional 10-min period. A structure designated by an arrow and possibly a labeled tubular element (*) are fusing with this multivesicular body. (a) $\times 63,000$, bar $1 \mu\text{m}$; (b) $\times 86,000$; (c) $\times 65,000$.

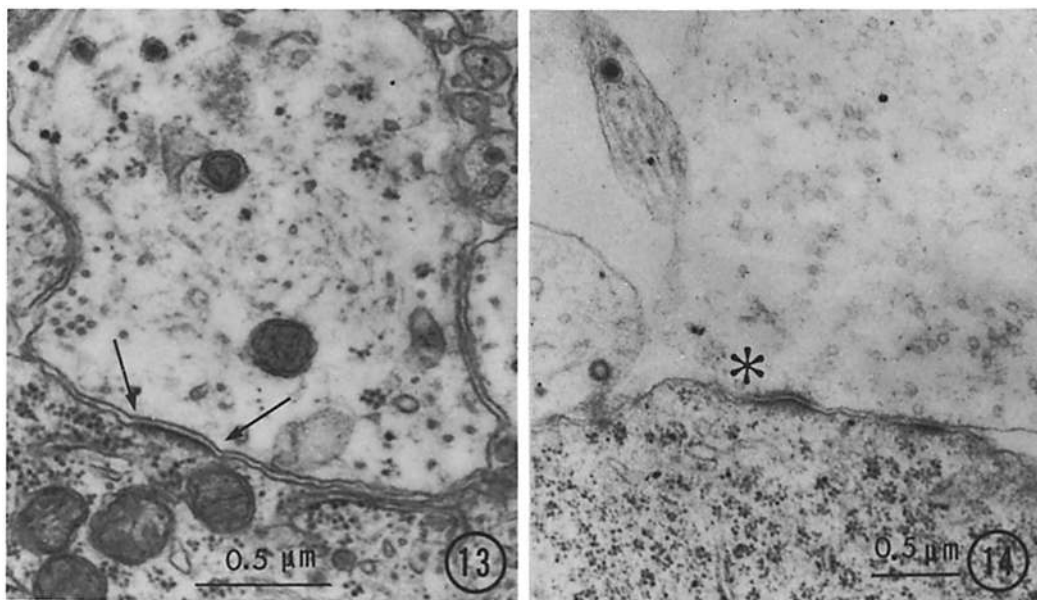


FIGURE 13 SC neuritic process in contact with a SCG neuronal soma. In the contact area a marked density (between arrows) is seen on the "postsynaptic" membrane. As yet, no synaptic organelles are present in the SC process. 28 h after initial contact. $\times 44,000$.

FIGURE 14 Portion of a SC neuritic growth cone in contact with a SCG neuronal soma. Two areas of postsynaptic density are present. Synaptic vesicles are appearing among the filamentous material of the cone near that area of presynaptic membrane opposite the postsynaptic density. At the left (*) there is a suggestion of presynaptic dense material as well. 36 h after initial contact. $\times 25,000$.

vesicles appear. This is noteworthy in view of the suggestion that synaptic vesicles may derive from agranular reticulum (4, 46, 82, 35). The prominence of this organelle in the active growth cone when synaptic vesicles are not present indicates that in this position it subserves some as yet unknown additional function unique to the growing tip. The prominent growth cone lysosomes presumably participate in these changes before receding to be represented by an occasional multivesicular body in the mature terminal (66).

Two aspects of the proposed developmental sequence will be discussed in some detail: (a) the membrane relationships established in the early phases of contact between pre- and postsynaptic elements, and (b) and the response that this early contact appears to induce in the coated vesicle population within the target cell.

A critical question regarding the nature of the initial contact is whether, at some early stage, a type of junction is formed to allow direct communication of small molecules between cells, i.e. a gap junction that would allow electrical and metabolic coupling between cells (26, 41). In our survey of

early contacts, no typical tight or gap junctions were found. The type of contact first seen consisted of a punctate region where the apposed outer membrane leaflets were separated by 7–10 nm. This type of contact partially resembles that described between fibroblasts in the initial phases of contact inhibition shortly after collision (33; see also 34). In both cases, there is a narrowing of the intercellular space at small focal regions to less than 100 nm. This somewhat irregular narrowing does not resemble the highly regular 2–3 nm spacing of a gap junction. Whereas the spacing of the fibroblast and SC-SCG contacts is similar, a major difference is that within the contacting fibroblasts there is a prompt appearance (within minutes) of cytoplasmic material subjacent to the contact; this is not observed in the early stages of contact between growth cone and nerve cell and, furthermore, when cytoplasmic dense material does appear it is asymmetrically disposed.

The absence of demonstrable gap junctions suggests that surface interaction and adhesivity are the critical early events rather than intercellular communication via smaller molecule exchange.

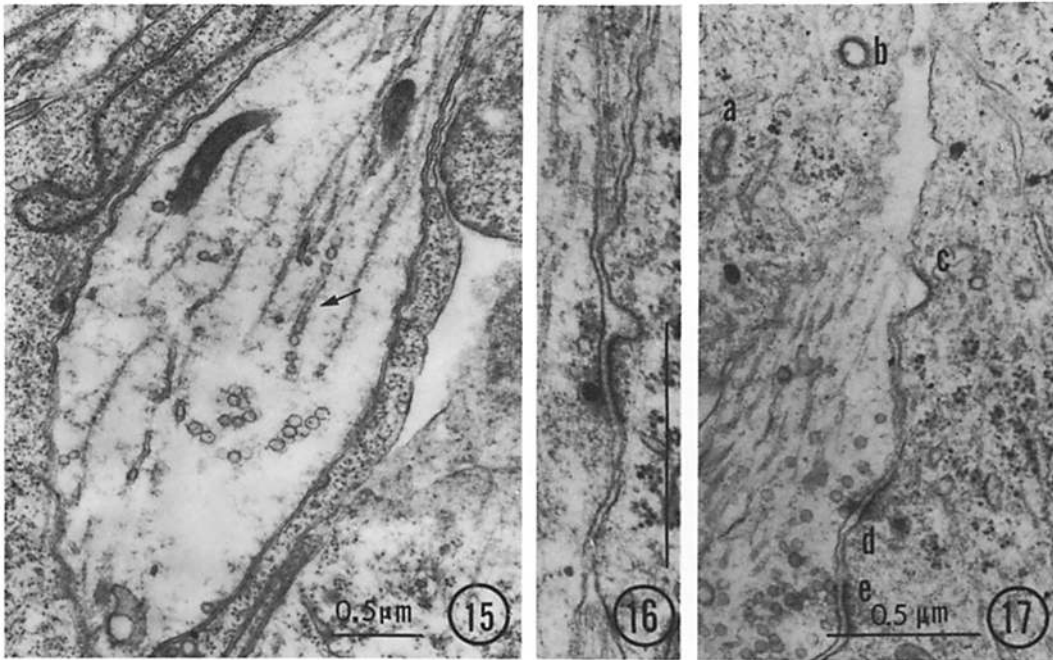


FIGURE 15 SC neuritic process. Vesicles with the approximate dimensions of synaptic vesicles are seen in relation to a short segment of smooth endoplasmic reticulum (arrow). 43 h after initial contact. $\times 26,000$.

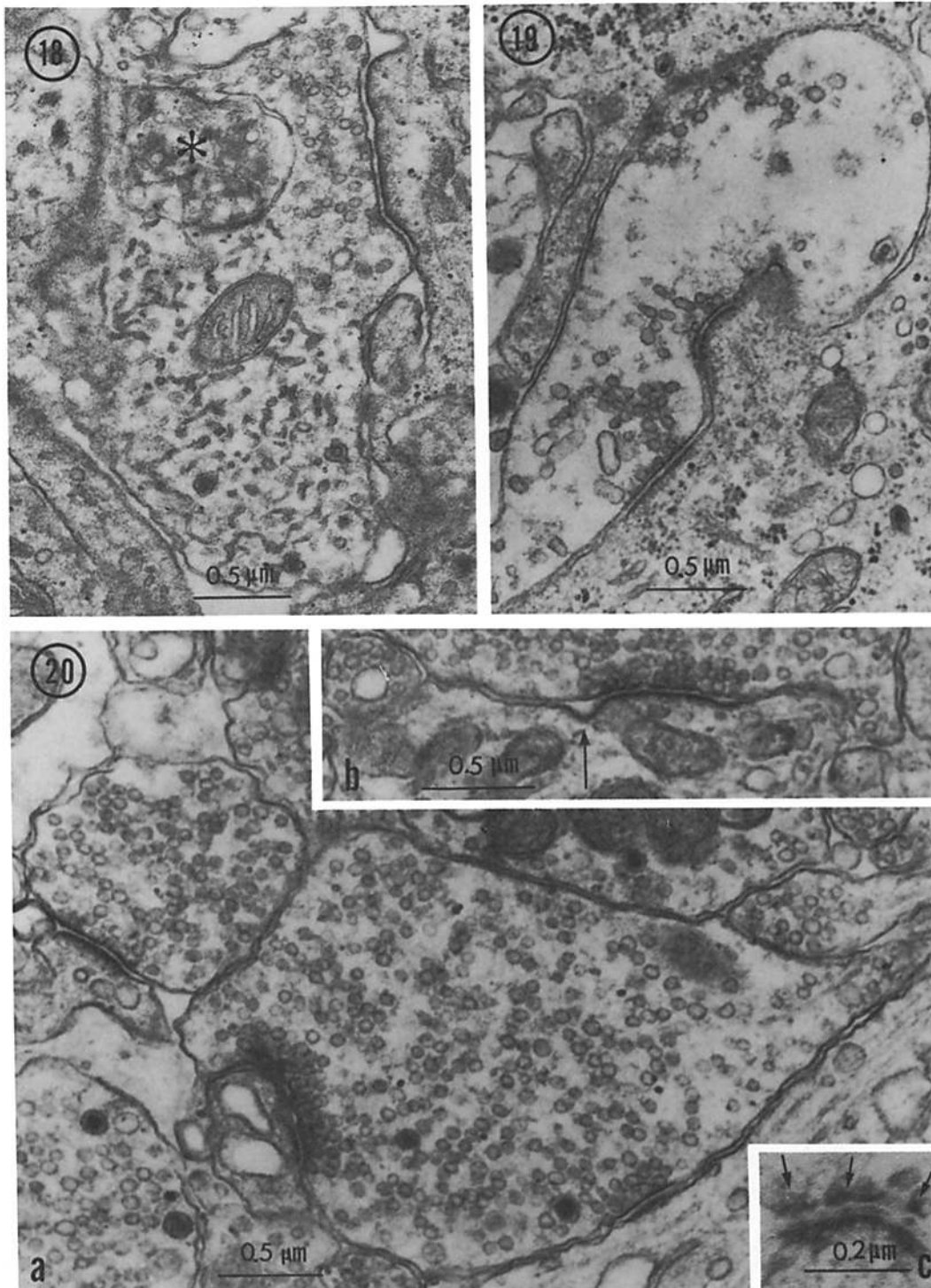
FIGURE 16 Coated caveola adjoining an already established postsynaptic density. A synaptic and a dense-cored vesicle are seen at the presynaptic membrane. 43 h after initial contact. $\times 43,000$. Bar, $1 \mu\text{m}$.

FIGURE 17 Portion of a SC neurite passing between two SCG neuronal somas. At *a*, *b*, *c*, *d*, and *e*, five of the postulated stages of synapse formation are seen. At *a* and *b* coated vesicles are seen near the neuronal plasmalemma and at *c*, a vesicle is continuous with it. At *d* and *e*, increasing numbers of synaptic vesicles are clustering at the presynaptic membrane. 43 h after initial contact. $\times 40,000$.

The results of the early contact between potentially synapsing neurons in our cultures may be considered to represent the early "paralysis" phase of contact inhibition (1) for, as described in Results, movement of the growth cone ceases within several minutes after contact. In later stages of contact between cells exhibiting contact inhibition, gap junctions are known to form (see review in 26); this was not seen in any stage of synapse development by the methods used in the present study. It should be noted, however, that the gap junctions which provide the apparent basis for electrical coupling between contact-inhibited cells *in vivo* and *in vitro* may have small dimensions and, thus, may be difficult to demonstrate in thin sections (73). A more definitive answer to the question of whether gap junctions are formed at some stage in synaptogenesis must await application of freeze-fraction or physiological techniques to early stages of synapse formation. We would note that signals

acting only at the cell surface and not (knowingly) involving cytoplasmic exchange of molecules may provide a potent influence on the cell interior, presumably by effecting a signal via protein molecules that traverse the cell membrane. An example is the signal for mitogenesis initiated by concanavalin A binding to the surface of lymphocytes (22).

Presumably, a signal of this type is involved in the stimulation of coated vesicle formation that we have observed in contacted neuronal somata. We propose that coated vesicles arise by budding from Golgi membrane and subsequently pass to the cell surface, particularly to that portion where contact has been made or a synapse has begun to form. This proposal is similar to that first advanced by Altman (5; in studies of developing rat cerebellum) and later by Stelzner et al. (81; in studies of embryonic chick spinal cord). In both cases, the frequent observation of large coated vesicles near



the forming synaptic site suggested that they were fusing with the plasma membrane, thus contributing coat material to the postsynaptic density. In the present paper, we emphasize (as in references 5 and 81) that this is the primary event occurring after substantial initial contact has occurred between pre- and postsynaptic elements. The presence of coated caveolae beside areas of postsynaptic density suggests to us a method for elongation of the junctional area during development; many coated vesicles, therefore, may be required to provide material for one fully formed postsynaptic dense region. Many investigators, however, have interpreted the coated vesicles near synaptic junctions to be pinocytotic (84; reviewed in 65), primarily because of comparison with work done with a variety of tissues implicating coated vesicles in endocytosis (reviewed in 44).

Are the coated vesicles seen to be contiguous with the somal plasmalemma fusing with or forming from the surface membrane? The difficulties in deciding this point are as follows. In general, coated vesicles involved in differing functions are not morphologically distinguishable; when demonstrable secretion product or pinocytosed tracer is lacking, the most apparent variation in coated vesicles is that of size. In a number of cell types there appear to be at least two size classes of coated vesicles, and these appear to differ in function. The generalization has been made (reviewed in 66) that a population of coated vesicles averaging 60 nm in diameter derives from the Golgi apparatus whereas those coated vesicles arising from the cell surface membrane are somewhat larger, averaging 100 nm in diameter. The population of coated vesicles seen at the plasmalemma of the cultured ganglion cells described in this paper is the larger one, comparable to those

known to be involved in uptake in other cell types; furthermore, in this same cell type, coated vesicles seen to take up exogenous tracer at the growth cone may be 100 nm in diameter (M. Bunge, unpublished observations).

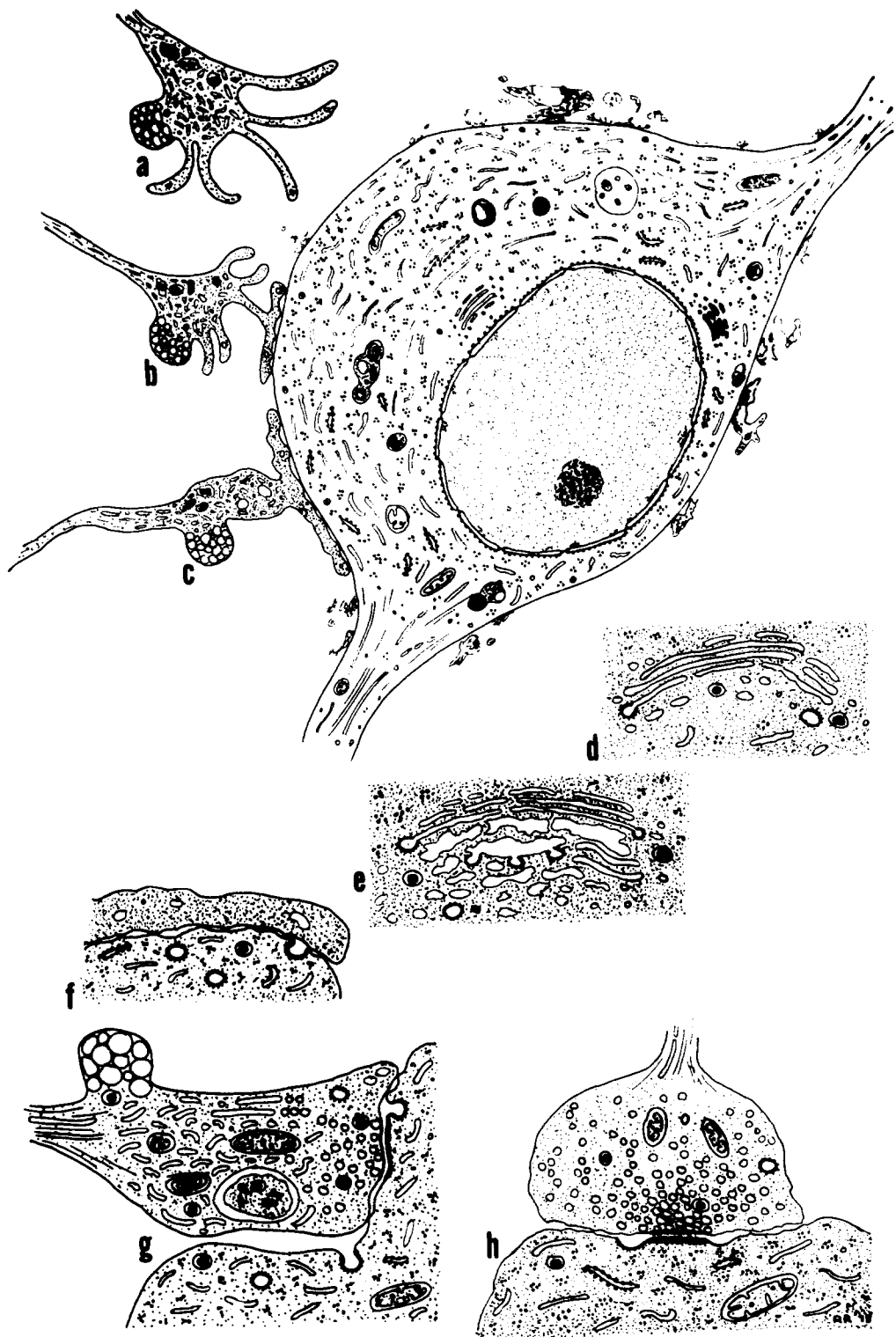
On the other hand, the following points argue in favor of our proposal that many coated vesicles travel centrifugally from the Golgi apparatus. It has been found that some of the small hydrolytic enzyme-laden vesicles known to be primary lysosomes are coated and that they arise from the Golgi apparatus (or the related GERL region; 39, 60) and disperse into the cytoplasm (25, 59). Also, coated vesicles may carry tyrosinase to premelanosomes within the cytoplasm (54) or may contain Golgi-processed zymogens destined for export from the cell (42).

Furthermore, several reports have suggested that coated vesicles from the cell interior fuse with the plasmalemma. (a) Friend and Farquhar (25) found that in rat vas deferens epithelial cells exposed to HRP for 10 min there was an increase in tracer-free, smaller coated vesicles in the Golgi region; 30 min later, their number had diminished in this area but had increased in the zone beneath the plasmalemma with which some appeared to be contiguous. These coated vesicles were considered to be in the process of joining the plasma membrane. (b) In the proximal intestinal epithelium of perinatal rat, Rodewald (74) found that certain antibodies were taken up into tubular invaginations of the apical membrane and transferred to interior coated vesicles which in turn dumped their content at the lateral cell border by exocytosis. (c) Ehrenreich et al. (23) observed low density lipoprotein destined for discharge in coated vesicles near the cell surface or in partly coated large vacuoles open to the exterior of ethanol-stimulated

FIGURE 18 This figure shows a SC growth cone undergoing reorganization to become a synaptic ending. Part of the lysosomal area (*), branched membranous reticulum, and synaptic vesicles are present, 46 h after initial contact. $\times 30,000$.

FIGURE 19 SC neuritic profile, lying between two SCG neuronal somata, shows the typical morphology of the "immature" synaptic ending. Synaptic vesicles are few in number and are clustered at the presynaptic membrane. Other areas of the ending appear empty, 48 h after initial contact. $\times 32,000$.

FIGURE 20 Synaptic endings in a mature culture, 3 wk after contact. The endings are crowded with synaptic vesicles. Pre- and postsynaptic thickenings are present (a). In (b) a coated vesicle (arrow) is seen adjoining an established postsynaptic density. The appearance of a nearly mature synapse in nonosmicated, E-PTA-stained tissue is illustrated in (c). In addition to postsynaptic and cleft material, presynaptic dense projections (arrows) are stained. (a and b) $\times 32,000$; (c) $\times 85,000$.



hepatocytes. (d) Coated areas also exist on the membrane enclosing discharging secretory granules of nonciliated bronchiolar cells (Kuhn et al., reference 48). (e) There is increasing evidence that lysosomal enzymes are secreted by cells (e.g., 21), suggesting that primary lysosomes, some of which may be coated, approach and fuse with the plasmalemma. There are many examples of vesicle transport, albeit in the form of noncoated membrane, in the direction of the cell surface from the interior, notably the Golgi region. In addition to the well-known delivery of membrane-bounded secretion droplets, this is considered to be a mechanism by which plasma membrane and/or adherent coat material is added (e.g., 36, 75, 7, 11, 19, 6, 8). It has been demonstrated that certain of the surface membrane moieties are added in the Golgi region, indicating a flow of packages of membrane from Golgi region to surface (reviewed in 87).

In the cultured SCG neurons described here, unlabeled coated vesicles are often seen in the region of the maturing rather than the forming face of the Golgi complex. The forming Golgi face receives product from the granular endoplasmic reticulum (31, 85); here, contiguous coated vesicles are considered to be transferring newly made material to the Golgi cisternae. These vesicles belong to the smaller class (31). That the region of

the maturing face is the site of egress of secretory product and vesicles including coated vesicles is well documented (e.g., 58, 79, 25, 39, 30, 31, 42, 85, 86). Our observations, then, would be consistent with the proposal that the SCG neuronal coated vesicles are arising from rather than fusing with the Golgi apparatus.

Finally, the work with exogenous tracers for short time-periods indicates a paucity of somal endocytosis via coated vesicles compared to the substantial amount of uptake at the fiber tip. After 1-, 4-, 6- or 8-min exposure to ferritin or HRP, labeled coated vesicles are rarely seen in the soma interior although multivesicular bodies have begun to be labeled. This appears to be the first lysosomal structure labeled, as Rosenbluth and Wissig (76) demonstrated. Some coated vesicles near the plasmalemma appear labeled, but they may be open to the exterior at another level (as seen in serial sections) and thus be exocytotic rather than endocytotic in nature. By these time-periods, however, certain growth cone organelles including coated vesicles are labeled with exogenous tracers (14). After 24-min exposure to HRP, the soma is much more heavily labeled, with marker present in some (but not more than 20% of) coated vesicles.

In those samples in which uptake was assessed in

FIGURE 21 In the upper part of the diagram is an isolated SCG neuronal soma as it appears after 2-3 days in culture. Some cellular debris resulting from the mechanical dissociation process clings to the plasmalemma. The nucleus is eccentric, and the cytoplasm is characterized mainly by polysomes, smooth and rough endoplasmic reticulum, Golgi apparatus, mitochondria, multivesicular bodies, and other lysosomal structures. At *a*, the soma is approached by a SC neuritic growth cone exhibiting filopodia, a mound area, branched membranous reticulum and several large lysosomes. At *b*, a single filopodium of this growth cone is depicted as having contacted the neuronal surface. Other filopodia are withdrawing. At *c*, only the highly flattened, contacting process remains, and its surface membrane has developed close contacts with the somal plasmalemma at multiple points. *Inset d* depicts the typical Golgi apparatus of a cultured SCG neuron before contact. A few coated vesicles are in continuity with cisternae or present in the adjacent cytoplasm together with an occasional large dense-cored vesicle. Changes occurring in the Golgi complex of a contacted neuron are shown in *e*. A greater number of coated vesicles are present, some being contiguous with the region of the maturing face of the Golgi complex. These coated vesicles are considered to migrate to the neuronal surface in the area of filopodial contact and, there, fuse with the plasmalemma (as at *f*), thereby contributing membrane with undercoating (postsynaptic density). This is considered to be the first definitive sign of synaptic specialization. A more advanced stage of synaptogenesis is diagrammed at *g*. On the presynaptic side, synaptic vesicles and large dense-cored vesicles are appearing among the growth cone organelles and are clustering at that part of the membrane apposed to the postsynaptic density. In *h*, the large amount of membranous reticulum, lysosomes and mound area typical of the growth cone are no longer present. A few mitochondria, some reticulum, occasional large dense-cored vesicles and numerous synaptic vesicles now characterize the ending. Presynaptic dense material is gradually appearing, some cleft material is seen, and the cleft widens. Postsynaptic membrane density increases in length as the addition of large coated vesicles continues.

all areas of the same nerve cell, the growth cones were 250 μm or less from the soma. If a retrograde movement of at least 70 mm/day or $\sim 50 \mu\text{m}/\text{min}$ (51, 47) is assumed, tracer taken up initially at the tip could reach the soma within 5 min or less. We submit, therefore, that the bulk of tracer could be taken up at the fiber tip and transported back to the soma. The label may be carried in tubular structures and coated vesicles to the soma where they fuse with lysosomal structures; many configurations suggestive of uniting lysosomal elements and labeled tubular structures have been seen in an unpublished tracer uptake study by M. Bunge and have been published by others (38, 51, 57).

Two of the papers most often quoted in support of uptake by the neuronal soma are those by Rosenbluth and Wissig (76) and Holtzman and Peterson (40). In the former paper (76), somata of extirpated "soaked" ganglia contained ferritin at 1 h; in the second paper (40), cultured ganglion somata contained HRP at 4 h. It seems possible to us that these time-periods were long enough to allow transport of the tracer (over a distance of 3–12 mm) from the cut ends or terminals or other areas of the fibers to the somata.

Undoubtedly, some uptake occurs at or near the neuronal soma. When HRP is injected into areas of the brain containing cell bodies, and then when their terminal areas in another part of the brain are examined, there is evidence that anterograde passage of the tracer has occurred. In these situations, however, the HRP reaction product is so little that it is not visible at the light microscope level as in retrograde transport and examination must be done at the electron microscope level to detect any evidence of product (51, 57). This suggests that some uptake takes place at or near the soma but that it is far less than that at the terminals. The large amount of uptake of HRP at terminals (not growing, but established) and subsequent retrograde transport are underscored by the fact that administration of HRP has become a new tool in neuroanatomy for tracing fiber pathways in the brain by light microscopy.

Our discussion above centers primarily on directions of vesicle traffic, for we consider these observations to be among the most important in the present study. In a recent review of acetylcholine (ACh) receptor properties in developing muscle, Fambrough et al. (24) present evidence that ACh receptors in maturing skeletal muscle fibers appear first *within* the developing cells and then are transferred *to* the surface. Their statement

that "presumably the internal material is preformed membrane, and the insertion of this membrane into the surface involves an exocytotic mechanism" fits well with our concept of coated vesicle function during synapse formation. It would appear judicious, however, to avoid suggesting a direct correlation between receptor appearance and development of synaptic adhesivity. The coated vesicles delivered to the postsynaptic region of the developing synapse appear to contribute directly to the postsynaptic density; they may also be instrumental in contributing to the substantial adhesion of the synaptic membranes as was first suggested by Altman (5). The dramatic loosening of synaptic endings on mature neurons undergoing chromatolysis (e.g., see footnote 2) may result from interference, during the chromatolytic period, with the ongoing production and delivery of these vesicles to the postsynaptic sites. The occurrence of coated caveolae adjoining mature postsynaptic areas in electron micrographs published by many investigators suggests ongoing replenishment throughout life.

Because synapses are known to form in the presence of neurotransmitter receptor blocking agents (20), it seems likely that the processes of recognition and adhesion are independent of the mechanisms involved in receptor and neurotransmitter concentration at the synaptic region. A subsequent function of the synaptic adhesion may be to immobilize receptor molecules at the most appropriate site for their response to transmitter release. This suggestion may be testable with the use of a marking ligand for the receptor, but unfortunately the most useful of these, α -bungarotoxin, does not bind to the ACh receptor sites on the autonomic ganglion cells employed in the present study (61; also, Ko, Burton and Bunge, unpublished observations). The application of culture techniques, similar to those employed in the present study, to a species known to be susceptible to autonomic ganglion blockade by α -bungarotoxin would appear worthwhile.

The authors gratefully acknowledge the skill of Dr. Patrick Wood in assisting in the preparation of the cultures. We thank Prof. Ralph Bradshaw for supplying NGF, Dr. Marie-France Maylié-Pfenninger for contributing the purified ferritin, Dr. Harold Burton for help with statistical analysis, and Mrs. Ann K. Williams for measurements of coated vesicles. Dr. Karl Pfenninger offered suggestions for improving the manuscript.

² Purves, D. 1975. Submitted for publication.

The research was supported by Grant NS 09923 from the National Institute of Neurological Diseases and Stroke, U.S. Public Health Service. R. P. Bunge is a Beaumont-May Institute of Neurology Scholar in Anatomy.

Portions of this study have appeared in abstract form (70, 71).

Received for publication 7 April 1975, and in revised form 29 September 1975.

REFERENCES

1. ABERCROMBIE, M. 1970. Contact inhibition in tissue culture. *In Vitro (Rockville)*. **6**:128-142.
2. ADINOLFI, A. M. 1972. Morphogenesis of synaptic junctions in layers I and II of the somatic sensory cortex. *Exp. Neurol.* **34**:372-382.
3. AINSWORTH, S. K., and M. J. KARNOVSKY. 1972. An ultrastructural staining method for enhancing the size and electron opacity of ferritin in thin sections. *J. Histochem. Cytochem.* **20**:225-229.
4. AKERT, K., E. KAWANA, and C. SANDRI. 1971. ZIO-positive and ZIO-negative vesicles in nerve terminals. *Progr. Brain Res.* **34**:305-317.
5. ALTMAN, J. 1971. Coated vesicles and synaptogenesis. A developmental study in the cerebellar cortex of the rat. *Brain Res.* **30**:311-322.
6. BENES, F., J. A. HIGGINS, and R. J. BARNETT. 1973. Ultrastructural localization of phospholipid synthesis in the rat trigeminal nerve during myelination. *J. Cell Biol.* **57**:613-629.
7. BENNETT, G., and C. P. LEBLOND. 1970. Formation of cell coat material for the whole surface of columnar cells in the rat small intestine, as visualized by radioautography with L-fucose-³H. *J. Cell Biol.* **46**:409-416.
8. BENNETT, G., C. P. LEBLOND, and A. HADDAD. 1974. Migration of glycoprotein from the Golgi apparatus to the surface of various cell types as shown by radioautography after labeled fucose injection into rats. *J. Cell Biol.* **60**:258-284.
9. BLOOM, F. E., and G. K. AGHAJANIAN. 1966. Cytochemistry of synapses; selective staining method for electron microscopy. *Science (Wash. D.C.)* **154**:1575-1577.
10. BODIAN, D. 1968. Development of fine structure of spinal cord in monkey fetuses. II. Pre-reflex period to period of long intersegmental reflexes. *J. Comp. Neurol.* **133**:113-165.
11. BONNEVILLE, M. A., and M. WEINSTOCK. 1970. Brush border development in the intestinal absorptive cells of *Xenopus* during metamorphosis. *J. Cell Biol.* **44**:151-171.
12. BRAY, D. 1970. Surface movements during the growth of single explanted neurons. *Proc. Natl. Acad. Sci. U. S. A.* **65**:905-910.
13. BRAY, D. 1973. Branching patterns of individual sympathetic neurons in culture. *J. Cell Biol.* **56**:702-712.
14. BUNGE, M. B. 1973. Uptake of peroxidase by growth cones of cultured neurons. *Anat. Rec.* **175**:280.
15. BUNGE, M. B. 1973. Fine structure of nerve fibers and growth cones of isolated sympathetic neurons in culture. *J. Cell Biol.* **56**:713-735.
16. BUNGE, M. B., R. P. BUNGE, and E. R. PETERSON. 1967. The onset of synapse formation in spinal cord cultures as studied by electron microscopy. *Brain Res.* **6**:728-749.
17. BUNGE, R. P., R. REES, P. WOOD, H. BURTON, and C.-P. KO. 1974. Anatomical and physiological observations on synapses formed on isolated autonomic neurons in tissue culture. *Brain Res.* **66**:401-412.
18. BUNGE, R. P., and P. WOOD. 1973. Studies on the transplantation of spinal cord tissue in the rat. I. The development of a culture system for the hemisections of embryonic spinal cord. *Brain Res.* **57**:261-276.
19. CHLAPOWSKI, F. J., and R. N. BAND. 1971. Assembly of lipids into membranes in *Acanthamoeba palestinensis*. II. The origin and fate of glycerol-³H-labeled phospholipids of cellular membranes. *J. Cell Biol.* **50**:634-651.
20. COHEN, M. W. 1972. The development of neuromuscular connexions in the presence of d-tubocurarine. *Brain Res.* **41**:457-463.
21. DINGLE, J. T. 1969. The extra-cellular secretion of lysosomal enzymes. *In Lysosomes in Biology and Pathology*. J. T. Dingle and H. B. Fell, editors. Noord-Hollandsche Uitg. Mij., Amsterdam. **2**:421-436.
22. EDELMAN, G. M. 1974. Surface alterations and mitogenesis in lymphocytes. *In Control of Proliferation in Animal Cells*. B. Clarkson and R. Baserga, editors. Cold Spring Harbor Conf. Cell Proliferation. **1**:357-377.
23. EHRENREICH, J. H., J. J. M. BERGERM, P. SIEKEVITZ, and G. E. PALADE. 1973. Golgi fractions prepared from rat liver homogenates. I. Isolation procedure and morphological characterization. *J. Cell Biol.* **59**:45-72.
24. FAMBROUGH, D., H. C. HARTZELL, J. E. RASH, and A. K. RITCHIE. 1974. Receptor properties of developing muscle. *Trophic Functions of the Neuron*. *Ann. N. Y. Acad. Sci.* **228**:47-61.
25. FRIEND, D. S., and M. G. FARQUHAR. 1967. Functions of coated vesicles during protein absorption in the rat vas deferens. *J. Cell Biol.* **35**:357-376.
26. GILULA, N. B., O. R. REEVES, and A. STEINBACH. 1972. Metabolic coupling, ionic coupling and cell contacts. *Nature (Lond.)*. **235**:262-265.
27. GLEES, P., and B. L. SHEPPARD. 1964. Electron microscopical studies of the synapse in the developing chick spinal cord. *Z. Zellforsch. Mikrosk. Anat.* **62**:356-362.
28. GRAHAM, R. C., JR., and M. J. KARNOVSKY. 1966. The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse

- kidney: Ultrastructural cytochemistry by a new technique. *J. Histochem. Cytochem.* **14**:291-302.
29. GRAINGER, F., and D. W. JAMES. 1970. Association of glial cells with the terminal parts of neurite bundles extending from chick spinal cord in vitro. *Z. Zellforsch. Mikrosk. Anat.* **108**:93-104.
 30. GROVE, S. N., C. E. BRACKER, and D. J. MORRÉ. 1968. Cytochrome differentiation in the endoplasmic reticulum—Golgi apparatus—vesicle complex. *Science (Wash. D. C.)* **161**:171-173.
 31. HADDAD, A., M. D. SMITH, A. HERSCOVICS, N. J. NADLER, and C. P. LEBLOND. 1971. Radioautographic study of in vivo and in vitro migration of fucose-³H into thyroglobulin by rat thyroid follicular cells. *J. Cell Biol.* **49**:856-882.
 32. HAYES, B. P., and A. ROBERTS. 1974. The distribution of synapses along the spinal cord of an amphibian embryo: An electron microscope study of junction development. *Cell Tiss. Res.* **153**:227-244.
 33. HEAYSMAN, J. E. M., and S. M. PEGRUM. 1973. Early contacts between fibroblasts. An ultrastructural study. *Exp. Cell Res.* **78**:71-78.
 34. HEAYSMAN, J. E. M., and S. M. PEGRUM. 1973. Early contacts between normal fibroblasts and mouse sarcoma cells. An ultrastructural study. *Exp. Cell Res.* **78**:479-481.
 35. HEUSER, J. E., and T. S. REESE. 1973. Evidence for recycling of synaptic vesicle membrane during transmitter release at the frog neuromuscular junction. *J. Cell Biol.* **57**:315-344.
 36. HICKS, R. M. 1966. The function of the Golgi complex in transitional epithelium: synthesis of the thick cell membrane. *J. Cell Biol.* **30**:623-643.
 37. HIRANO, A., and H. M. DEMBITZER. 1974. Observations on the development of the weaver mouse cerebellum. *J. Neuropathol. Exp. Neurol.* **33**:354-364.
 38. HOLTZMAN, E. 1969. Lysosomes in the physiology and pathology of neurons. In *Lysosomes in Biology and Pathology*. J. T. Dingle and H. B. Fell, editors. Noord-Hollandsche Uitg. Mij., Amsterdam. **1**:192-216.
 39. HOLTZMAN, E., A. B. NOVIKOFF, and H. VILLAVARDE. 1967. Lysosomes and GERL in normal and chromatolytic neurons of the rat ganglion nodosum. *J. Cell Biol.* **33**:419-435.
 40. HOLTZMAN, E., and E. R. PETERSON. 1969. Uptake of protein by mammalian neurons. *J. Cell Biol.* **40**:863-869.
 41. HÜLSER, D. F., and A. DEMSEY. 1973. Gap and low-resistance junctions between cells in culture. *Z. Naturforsch. Teil B Anorg. Chem. Org. Chem. Biochem. Biophys. Biol.* **28**:603-606.
 42. JAMESON, J. D., and G. E. PALADE. 1971. Synthesis, intracellular transport, and discharge of secreting proteins in stimulated pancreatic exocrine cells. *J. Cell Biol.* **50**:135-158.
 43. JONES, E. G., and T. P. S. POWELL. 1970. An electron microscopic study of terminal degeneration in the neocortex of the cat. *Philos. Trans. Roy. Soc. Lond. B. Biol. Sci.* **257**:29-43.
 44. KANASEKI, T., and K. KADOTA. 1969. The "vesicle in a basket." A morphological study of the coated vesicle isolated from the nerve endings of the guinea pig brain with special reference to the mechanism of membrane movements. *J. Cell Biol.* **42**:202-220.
 45. KARNOVSKY, M. J. 1967. The ultrastructural basis of capillary permeability studied with peroxidase as a tracer. *J. Cell Biol.* **35**:2-13.
 46. KAWANA, E., C. SANDRI, and K. AKERT. 1971. Ultrastructure of growth cones in the cerebellar cortex of the neonatal rat and cat. *Z. Zellforsch. Mikrosk. Anat.* **115**:284-298.
 47. KRISTENSSON, K. 1975. Retrograde axonal transport of protein tracers. In *The Use of Axonal Transport for Studies of Neuronal Connectivity*. W. M. Cowan and M. Cuénod, editors. American Elsevier Publishing Co., New York. 69-82.
 48. KUHN, C., L. A. CALLAWAY, and F. B. ASKIN. 1974. The formation of granules in the bronchiolar Clara cells of the rat. I. Electron microscopy. *J. Ultrastruct. Res.* **49**:387-400.
 49. LANDMESSER, L., and G. PILAR. 1972. The onset and development of transmission in the chick ciliary ganglion. *J. Physiol. (Lond.)* **222**:691-713.
 50. LARRAMENDI, L. M. H. 1969. Analysis of synaptogenesis in the cerebellum of the mouse. In *Neurobiology of Cerebellar Evolution and Development*. R. Llinas, editor. Institute for Biomedical Research, American Medical Association, Chicago, Ill. 803-843.
 51. LAVAIL, J. H., and M. M. LAVAIL. 1974. The retrograde intraaxonal transport of horseradish peroxidase in the chick visual system: A light and electron microscope study. *J. Comp. Neurol.* **157**:303-358.
 52. LEIBOVITZ, A. 1963. The growth and maintenance of tissue-cell cultures in free gas exchange with the atmosphere. *Am. J. Hyg.* **78**:173-180.
 53. LUDUENA, M. A. 1973. The growth of spinal ganglion neurons in serum-free medium. *Dev. Biol.* **33**:470-476.
 54. MAUL, G. G., and J. A. BRUMBAUGH. 1971. On the possible function of coated vesicles in melanogenesis of the regenerating fowl feather. *J. Cell Biol.* **48**:41-48.
 55. MOLLENHAUER, H. H. 1964. Plastic embedding mixtures for use in electron microscopy. *Stain Technol.* **39**:111-115.
 56. NAKAI, J., and Y. KAWASAKI. 1959. Studies on the mechanism determining the course of nerve fibers in tissue culture. I. The reaction of the growth cone to various obstructions. *Z. Zellforsch. Mikrosk. Anat.* **51**:108-122.
 57. NAUTA, H. J. W., I. R. KAISERMAN-ABRAMOF, and R. J. LASEK. 1975. Electron microscopic observa-

- tions of horseradish peroxidase transported from the caudoputamen to the substantia nigra in the rat: possible involvement of the agranular reticulum. *Brain Res.* **85**:373-384.
58. NEUTRA, M., and C. P. LEBLOND. 1966. Synthesis of the carbohydrate of mucus in the Golgi complex as shown by electron microscope radioautography of goblet cells from rats injected with glucose-H³. *J. Cell Biol.* **30**:119-136.
 59. NICHOLS, B. A., D. F. BAINTON, and M. G. FARQUHAR. 1971. Differentiation of monocytes. Origin, nature, and fate of their azurophil granules. *J. Cell Biol.* **50**:498-515.
 60. NOVIKOFF, P. M., A. B. NOVIKOFF, N. QUINTANA, and J.-J. HAUW. 1971. Golgi apparatus, GERL and lysosomes of neurons in rat dorsal root ganglia, studied by thick section and thin section cytochemistry. *J. Cell Biol.* **50**:859-886.
 61. OBATA, K. 1974. Transmitter sensitivities of some nerve and muscle cells in culture. *Brain Res.* **73**:71-88.
 62. O'LAGUE, P. H., K. OBATA, P. CLAUDE, E. J. FURSPAN, and D. D. POTTER. 1974. Evidence for cholinergic synapses between dissociated rat sympathetic neurons in cell culture. *Proc. Natl. Acad. Sci. U. S. A.* **71**:3602-3606.
 63. OLSON, M., and R. P. BUNGE. 1973. Anatomical observations on the specificity of synapse formation in tissue culture. *Brain Res.* **59**:19-33.
 64. OPPENHEIM, R. W., and R. F. FOELIX. 1972. Synaptogenesis in the chick embryo spinal cord. *Nature (Lond.)*. **235**:126-128.
 65. PAPPAS, G. D., and S. G. WAXMAN. 1972. Synaptic fine structure-morphological correlates of chemical and electrotonic transmission. In *Structure and Function of Synapses*. G. D. Pappas and D. P. Purpura, editors. Raven Press, New York. 1-43.
 66. PETERS, A., S. L. PALAY, and H. DEF. WEBSTER. 1970. *The Fine Structure of the Nervous System. The Cells and Their Processes*. Hoeber Medical Division of Harper & Row, Publishers, New York.
 67. PFENNINGER, K. H., and M. B. BUNGE. 1973. Observations on plasmalemmal growth zones in developing neural tissue. *J. Cell Biol.* **59** (2, Pt. 2):264 a (Abstr.).
 68. PFENNINGER, K. H., and R. P. BUNGE. 1974. Freeze-fracturing of nerve growth cones and young fibers. A study of developing plasma membrane. *J. Cell Biol.* **63**:180-196.
 69. RAISMAN, G., P. M. FIELD, A. J. OSTBERG, L. L. IVERSEN, and R. E. ZIGMOND. 1974. A quantitative ultrastructural and biochemical analysis of the process of reinnervation of the superior cervical ganglia in the adult rat. *Brain Res.* **71**:1-16.
 70. REES, R. 1974. Observations on the mechanism of synaptogenesis on isolated neurons in tissue culture. *Anat. Rec.* **178**:445.
 71. REES, R. P. 1975. Morphological changes occurring in the neuritic growth cone and target neuron from the time of initial contact to the completion of a synaptic profile. *Anat. Rec.* **181**:458.
 72. REES, R. P., and R. P. BUNGE. 1974. Morphological and cytochemical studies of synapses formed in culture between isolated rat superior cervical ganglion neurons. *J. Comp. Neurol.* **157**:1-12.
 73. REVEL, J. P., A. G. YEE, and A. J. HUDSPETH. 1971. Gap junctions between electrotonically coupled cells in tissue culture and in brown fat. *Proc. Natl. Acad. Sci. U. S. A.* **68**:2924-2927.
 74. RODEWALD, R. 1973. Intestinal transport of antibodies in the newborn rat. *J. Cell Biol.* **58**:189-211.
 75. ROSEN, W. G. 1968. Ultrastructure and physiology of pollen. *Annu. Rev. Plant. Physiol.* **19**:435-462.
 76. ROSENBLUTH, J., and S. L. WISSIG. 1964. The distribution of exogenous ferritin in toad spinal ganglia and the mechanism of its uptake by neurons. *J. Cell Biol.* **23**:307-325.
 77. SCOTT, B. S. 1971. Effect of potassium on neuron survival in cultures of dissociated human nervous tissue. *Exp. Neurol.* **30**:297-308.
 78. SIMIONESCU, N., M. SIMIONESCU, and G. E. PALADE. 1973. Permeability of muscle capillaries to exogenous myoglobin. *J. Cell Biol.* **57**:424-452.
 79. SMITH, R. E., and M. G. FARQUHAR. 1966. Lysosome function in the regulation of the secretory process in cells of the anterior pituitary gland. *J. Cell Biol.* **31**:319-347.
 80. SNEDECOR, G. W., and W. G. COCHRAN. 1967. *Statistical Methods*. The Iowa State University Press, Ames, Iowa. 6th ed.
 81. STELZNER, D. J., A. H. MARTIN, and G. L. SCOTT. 1973. Early stages of synaptogenesis in the cervical spinal cord of the chick embryo. *Z. Zellforsch. mikrosk. Anat.* **138**:475-488.
 82. TEICHBERG, S., and E. HOLTZMAN. 1973. Axonal agranular reticulum and synaptic vesicles in cultured embryonic chick sympathetic neurons. *J. Cell Biol.* **57**:88-108.
 83. VENABLE, J. H., and R. COGGESHALL. 1965. A simplified lead citrate stain for use in electron microscopy. *J. Cell Biol.* **25**:407-408.
 84. WAXMAN, S. G., and G. D. PAPPAS. 1969. Pinocytosis at postsynaptic membranes: electron microscopic evidence. *Brain Res.* **14**:240-244.
 85. WEINSTOCK, A., and C. P. LEBLOND. 1971. Elaboration of the matrix glycoprotein of enamel by the secretory ameloblasts of the rat incisor as revealed by radioautography after galactose-³H injection. *J. Cell Biol.* **51**:26-51.
 86. WEINSTOCK, M., and C. P. LEBLOND. 1974. Synthesis, migration, and release of precursor collagen by odontoblasts as visualized by radioautography after [³H] proline administration. *J. Cell Biol.* **60**:92-127.
 87. WHALEY, W. G., M. DAUWALDER, and J. E. KEPHART. 1972. Golgi apparatus: influence on cell surfaces. *Science (Wash. D. C.)* **175**:596-599.