SURFACE STRUCTURE OF ISOLATED NEURONS

Detachment of Nerve Terminals

during Axon Regeneration

ANDERS HAMBERGER, HANS-ARNE HANSSON,
and JOHAN SJÖSTRAND

From the Institute of Neurobiology, University of Göteborg, Göteborg, Sweden

ABSTRACT

Freehand, isolated neuronal perikarya from the hypoglossal nucleus of the rabbit have been examined with light-and electron-microscopy (transmission and scanning). The surface of the cell bodies was largely covered with spherical particles which were 0.5–2 μ in diameter. Transmission electron microscopy proved that the spherical particles were synaptic nerve terminals. Crush of the hypoglossal nerve which leads to chromatolysis and swelling of the neuronal cell bodies results in a conspicuous reduction in the number of terminals attached to the surface of hypoglossal neurons. This effect was observed both for isolated neurons and in tissue sections. The effect is considered in relation to earlier reported variations in the adherence of neuropil to isolated neuronal perikarya. The functional importance of nerve ending detachment in connection with nerve injury is discussed.

The connection of synaptic nerve endings to nerve cell perikarya and proximal dendrites is ordinarily considered very stable, and the synaptosome fraction isolated by homogenization and gradient centrifugation of nervous tissue contains particles with presynaptic and postsynaptic membranes in close attachment (De Robertis et al., 1961, Gray and Whittaker, 1962).

During freehand dissection of fresh nerve-cell bodies from the hypoglossal nucleus, it was apparent that the isolation of axotomized neurons was significantly easier than for control cells (Watson, 1966; Hamberger and Sjöstrand, 1966). Consequently, a change in the mechanical forces which keep a nerve cell body attached to the surrounding neuropil seems to occur during nerve regeneration. The significant reduction in size of the monosynaptic excitatory postsynaptic potential (Eccles et al., 1958) in motoneurons after their ventral roots had been severed would also suggest

a change in the connections between nerve terminals and perikarya. Recent ultrastructural studies show a detachment of presynaptic membranes from the chromatolytic nerve cell bodies of the facial nucleus simultaneously with a proliferation of perineuronal microglial cells (Blinzinger and Kreutzberg, 1968). The glial cells seem to displace apparently intact nerve terminals over large areas of the motoneuron perikarya. Since it has been suggested that the chromatolytic reaction during nerve regeneration causes a change towards an embryonic stage (Eccles, 1964), it is possible that the detachment of synaptic terminals is an expression of the plasticity normally found during development (Conradi and Skoglund, 1969).

We have used the hypoglossal nucleus of the rabbit in a number of studies for the investigation of nerve- and glia-cell reactions during nerve regeneration (Sjöstrand, 1965, Hamberger and Sjöstrand, 1966; Hamberger and Sjöstrand, in

preparation). Am important problem is the interaction between the nerve cell body and the surrounding structures during the phase of axonal outgrowth.

Scanning electron microscopy makes it possible to analyze the surface of hand-dissected single neurons and to visualize at a high resolution the structures attached to it. By the use of this method along with light microscopy, we have shown that the nerve terminals over large areas of the perikaryal surface are detached in the chromatolytic phase. Transmission electron microscopy confirmed the identity of the synaptic terminals which had no observable signs of degeneration.

MATERIALS AND METHODS

Albino rabbits weighing 1.5–2.0 kg were used. The right hypoglossal nerve was exposed under pentobarbital anaesthesia and crushed with a forceps cooled to −70°C in order to ensure complete destruction of the nerve. The animals used for isolation of single nerve cells were killed by an air embolus 4, 6, and 14 days after nerve crush.

Isolation of Neurons

The hypoglossal region was identified in the freshly cut slices and the isolation procedure was carried out under a stereomicroscope at 50 times magnification (Hydén, 1959; Hamberger and Sjöstrand, 1966). The hypoglossal nucleus was cut out as a thin flake of tissue with iris scissors and transferred in the cold to a drop of dissecting medium (10% Ficoll [Pharmacia Fine Chemicals, Inc., Uppsala, Sweden], 100 mM NaCl, 30 mM tris-chloride pH 7.4). The Ficoll medium was superior although attempts were made with other media including 0.3 M sucrose, and Krebs-Ringer phosphate medium. By gently teasing the tissue on a cover glass with stainless steel needles, 30 μ in diameter, neuronal perikarya with proximal dendrites and axons were isolated within 30 min after the death of the animal. Deiters giant nerve cells from the lateral vestibular nucleus and spinal ganglion cells isolated from their satellite cells were also examined.

Transmission Electron Microscopy

Isolation of single neurons for transmission electron microscopy was carried out in either of the following two ways: (a) Small flakes of nervous tissue with neurons hanging almost free attached to the tissue pieces were fixed in 2.5% glutaraldehyde in the Ficoll solution; (b) single cells were isolated by freehand dissection and placed on the bottom of a plastic Petri dish and fixed as described above.

All specimens were postfixed in 2% osmium tetrox-

ide, dehydrated in ethanol and hydroxypropyl methacrylate, and embedded in Epon 812. The specimens were trimmed by freehand and in an LKB Pyramitome (LKB Instruments, Inc., Stockholm, Sweden).

For electron microscopy of tissue sections, rabbits were perfused via the ascending aorta with glutaraldehyde-formaldehyde (Karnovsky, 1965). After 10 min the brain stem was removed and fixed for an additional 60 min. The hypoglossal nuclei were dissected out under a stereomicroscope and postfixed in 2% osmium tetroxide in 0.1 mM cacodylate buffer, pH 7.4, for 90 min at 4°C. The tissue was dehydrated in a graded series of ethanol and embedded in Epon 812 according to Luft (1961).

Thin sections were cut with an LKB Ultratom III, and were examined in a Siemens Elmiskop 1A after staining with uranyl acetate and lead citrate.

Light Microscopy

Freshly isolated, unfixed neurons suspended in the dissection medium were examined in a phase-contrast microscope with Nomarski optical equipment (Zeiss, Oberkochen, Germany).

Scanning Electron Microscopy

The isolated nerve cells were fixed for 30 min at 4°C in a small amount of dissecting medium containing 2% purified glutaraldehyde, and dehydrated in a graded series of acetone at room temperature. The air-dried cells were coated with a thin layer of carbon and a layer of copper or gold in a high vacuum evaporator. The material was examined in a Stereoscan scanning electron microscope (Cambridge Instrument Co., Ltd, Cambridge, England) operated at an accelerating voltage of up to 20 kv.

RESULTS

Neuronal Surface Structures in Control Material

TRANSMISSION ELECTRON MICROSCOPY OF ISOLATED HYPOGLOSSAL NERVE CELLS showed that these cells were more shrunken than in tissue sections. The characteristic features of nerve cells, the large nucleus with nucleolus and the cell organelles including clusters of ribosomes and profiles of rough endoplasmic reticulum, were observed. The cell structures, particularly in the vicinity of the plasma membrane, were badly damaged during the dehydration and embedding procedures. The cells were, however, to a large extent seen surrounded by the plasma membrane. Electron-opaque bodies surrounded by membrane

were attached to the outer surface of the plasma membrane (Fig. 1). They were characterized by a high electron opacity of the membrane in contact with the nerve cell and contained vesicles and mitochondria. By these criteria they could be identified as synaptic nerve terminals. One or two terminals could be observed in most of the thin sections of isolated nerve cells. Glial processes were not seen.

TRANSMISSION ELECTRON MICROSCOPY OF HYPOGLOSSAL SECTIONS showed the presence of numerous synaptic terminals around the neuronal perikarya and dendrites (Fig. 2). These terminals were mostly round to oval, 0.5–2 μ in diameter. Increased electron opacity of the presynaptic membranes, large aggregates of synaptic vesicles, and a few mitochondria were observed. Other structures in contact with the cell soma were glial cell processes and myelinated and unmyelinated axons.

LIGHT MICROSCOPY OF THE ISOLATED HYPOGLOSSAL NERVE CELLS with the aid of phase-contrast and Nomarski optical equipment revealed the presence of a large number of spherical to ovoid particles along the surface of the perikarya and the processes (Fig. 3). Their number decreased rapidly after about 30 min in the solution, and a reduced number was observed on cells not isolated immediately after the death of the animal.

SCANNING ELECTRON MICROSCOPY of isolated, freehand dissected neurons enabled us to study large parts of the surface of a single cell as well as the structures which remained attached after the gentle isolation procedure. The gross morphology of the hypoglossal neurons was similar to that observed in light microscopy, but the cells, particularly the surface structures, were shrunken due to the fixation and dehydration procedures. Fig. 4 shows a cell at low magnification. By varying the angle of evaporation for the deposition of the copper and carbon layers and by varying the angle of the incident electron beam, we were able to visualize surface structures with a higher resolution and depth of focus than in the light microscope. The surface of the perikarya, the dendritic tree, and proximal part of the axon were to a large extent covered by small particles (Figs. 5 and 6).

The surface of the cell consisted of a continuous membrane which at higher magnification showed numerous ridges probably due to shrinkage during

the preparation. Almost hemispherical depressions with a diameter of 0.5–1 μ (Fig. 7) were observed on the surface of the perikarya, which was not covered with particles. The surface particles were spherical or ovoid, 0.5–2 μ in diameter. A thin fiber was occasionally seen to emerge from the particles.

Structures of the same type were observed on the surface of the giant nerve cells of Deiters but not on the surface of the spinal ganglion cells.

The Perikaryal Surface during Nerve Regeneration

TRANSMISSION ELECTRON MICROSCOPY of tissue sections showed a detachment of synaptic terminals from the plasma membrane over large areas of the perikarya on the 4th, 6th, and 14th day after nerve crush (Figs. 8–10). The terminals and the neuronal plasma membranes were largely separated by interposed glial cell processes. No certain signs of degeneration could be observed in the displaced synaptic terminals in any stage of chromatolysis. Reactive glial cells were observed surrounding the neurons even 4 days after nerve crush (Fig. 8). The cytoplasm of the glial cell was of intermediate opacity and contained round to oval dense bodies resembling lysosomes. Occasional microtubular and filamentous structures were seen along with mitochondria and rough and smooth endoplasmic reticulum. During the later phase of chromatolysis of the neurons an increased number of astrocytic processes filled with filamentous structures were seen enveloping the neuronal perikarya (Figs. 9 and 10). Reactive glial cells as described 4 days after nerve crush could still be seen in the vicinity of the nerve cell bodies, and their lysosomes contained occasionally electron-opaque crystal structures (Fig. 9).

LIGHT MICROSCOPY of isolated cells revealed that most of the spherical particles observed on the surface of control nerve cells were absent on the surface of the regenerating nerve cells in different stages of chromatolysis (Fig. 11). The manual isolation of the nerve cells was considerably easier than in the control material, which made it possible to obtain longer cell processes.

SCANNING ELECTRON MICROSCOPY: At low magnification it could be observed that the perikaryal surface was smoother than in the controls (Fig. 12). The number of spherical particles per unit area was greatly reduced (Figs. 13 and
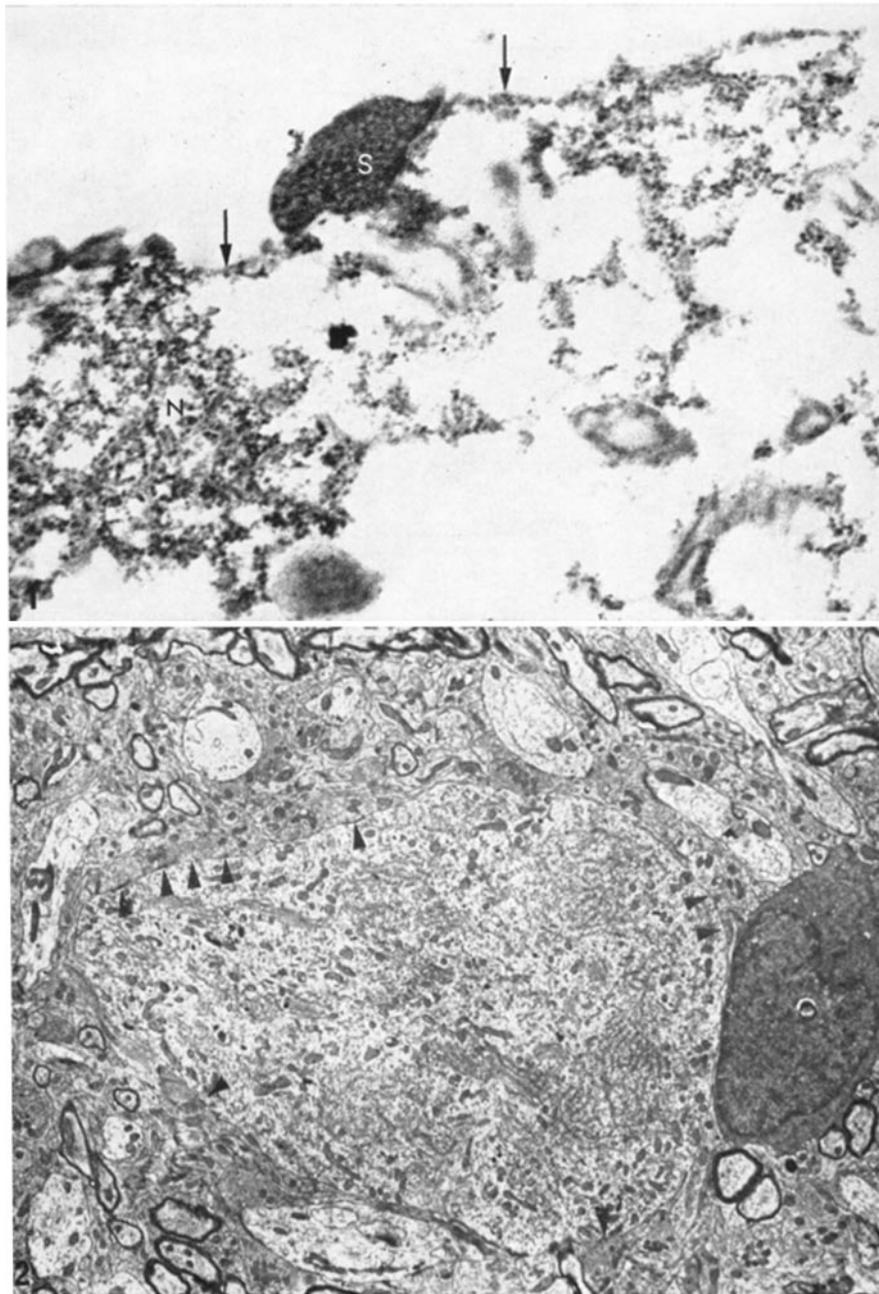
FIGURE 1 Transmission electron micrograph of an isolated hypoglossal nerve cell. A synaptic nerve terminal (s) is attached to the plasma membrane (arrows). (N) Nissl body. × 47,000.

FIGURE 2 Cell body of a hypoglossal neuron from a nonoperated animal. An oligodendrocyte (o) is observed to the right. The nerve cell plasma membrane is covered by a large number of synaptic terminals (arrows). × 3650.
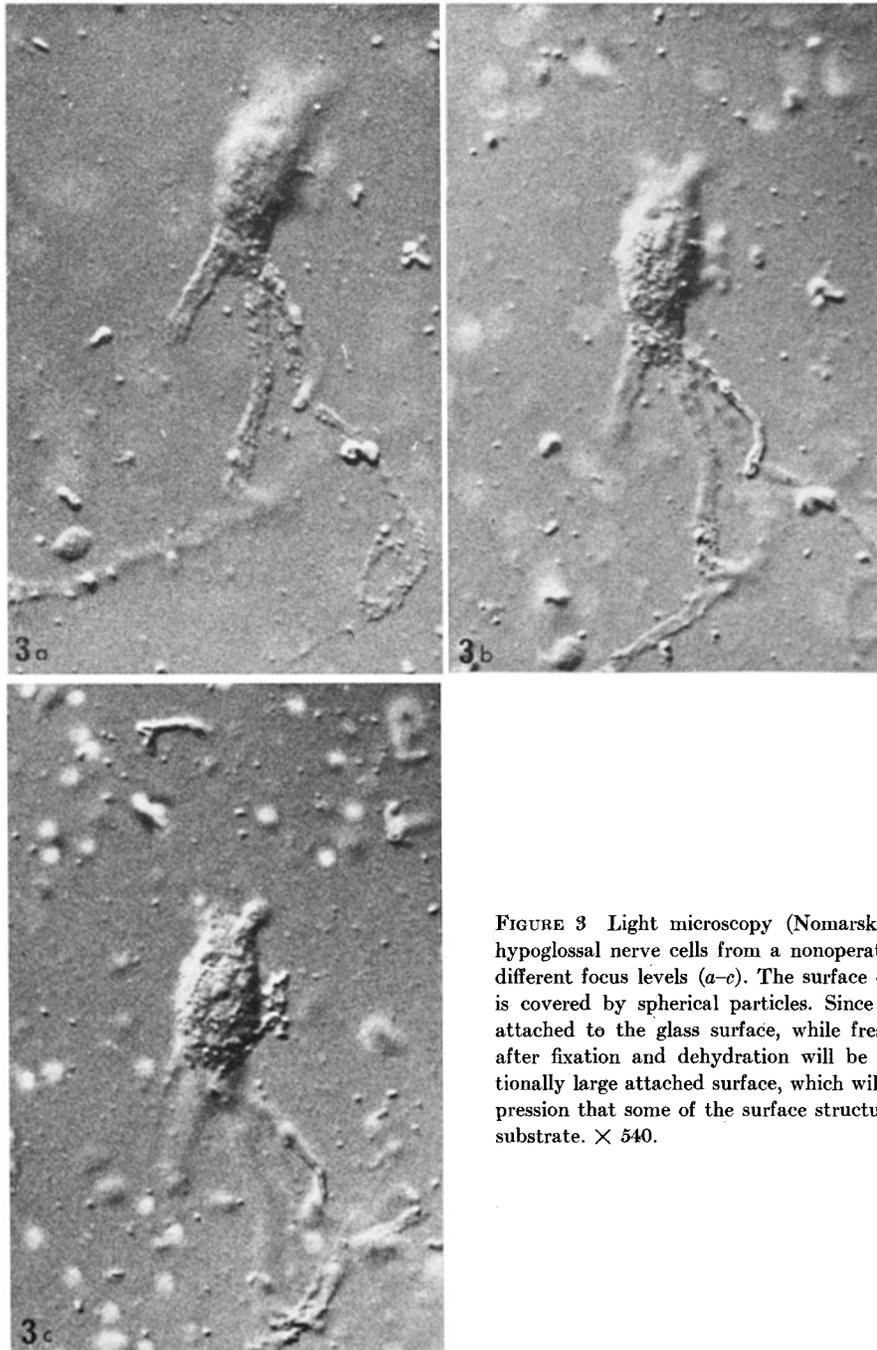
FIGURE 3 Light microscopy (Nomarski) of isolated hypoglossal nerve cells from a nonoperated animal at different focus levels (a–c). The surface of the neuron is covered by spherical particles. Since the cells are attached to the glass surface, while fresh, the result after fixation and dehydration will be an unproportionally large attached surface, which will give the impression that some of the surface structures lie on the substrate. × 540.
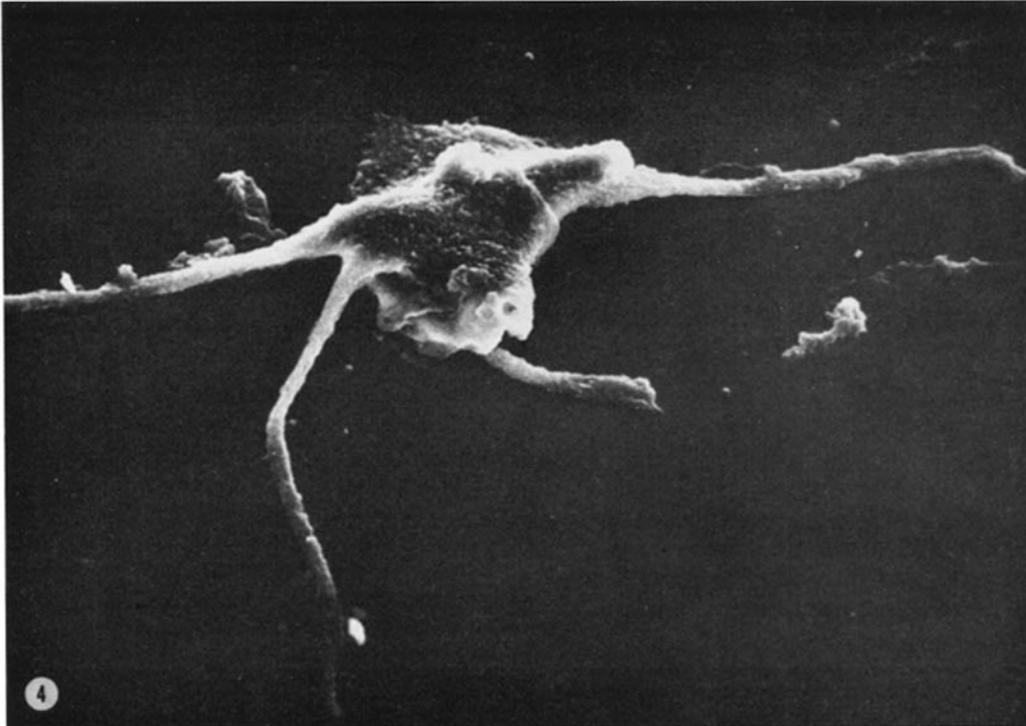
FIGURE 4   Scanning electron micrograph of isolated hypoglossal neuron from control animal. The perikaryal surface is covered by spherical particles. × 1350.
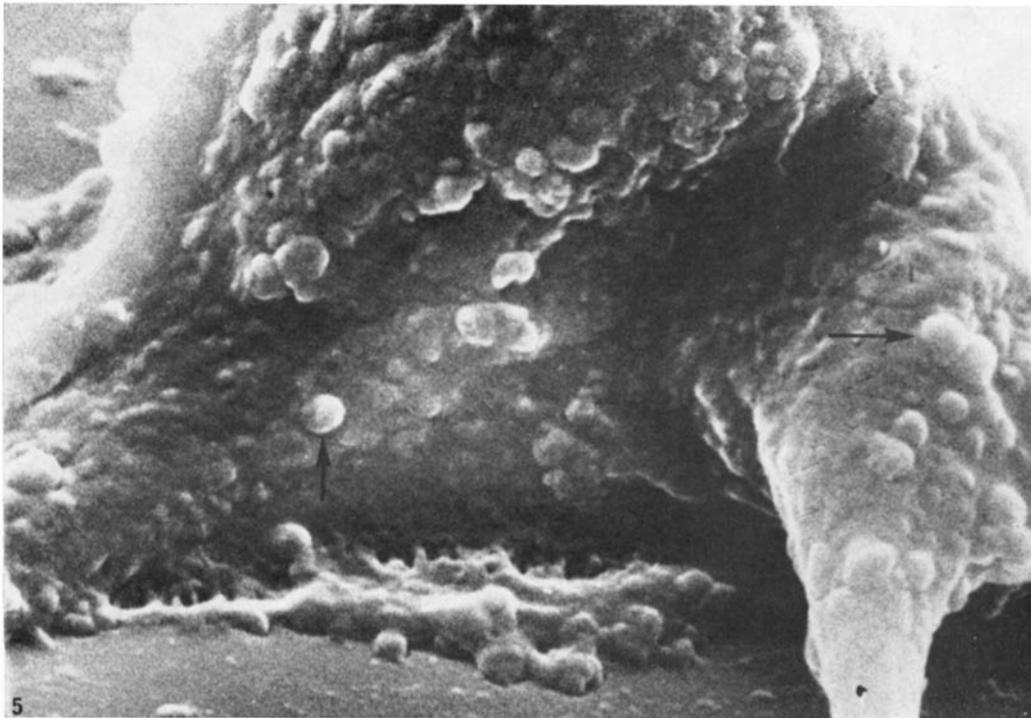
FIGURE 5   Scanning electron micrograph showing part of hypoglossal perikaryon and base of dendrites in a control animal. The surface is largely covered with spherical to ovoid particles (arrows). × 7900.
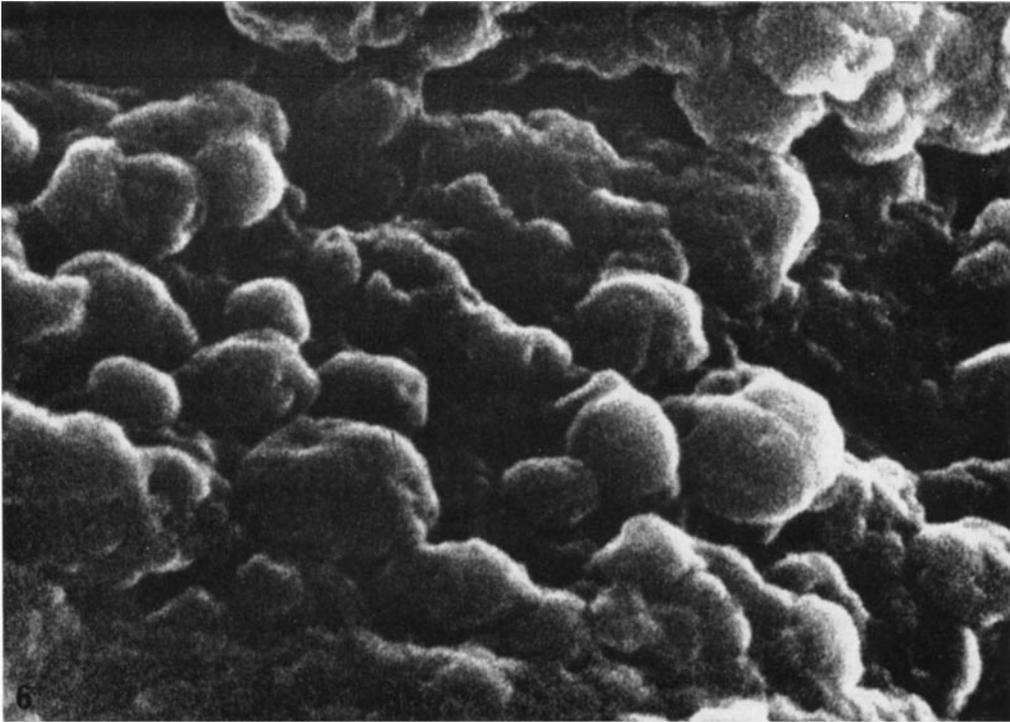
FIGURE 6   Detail of perikaryal surface of control neuron. A variation in particle size is observed. Scanning electron micrograph. × 23,300.
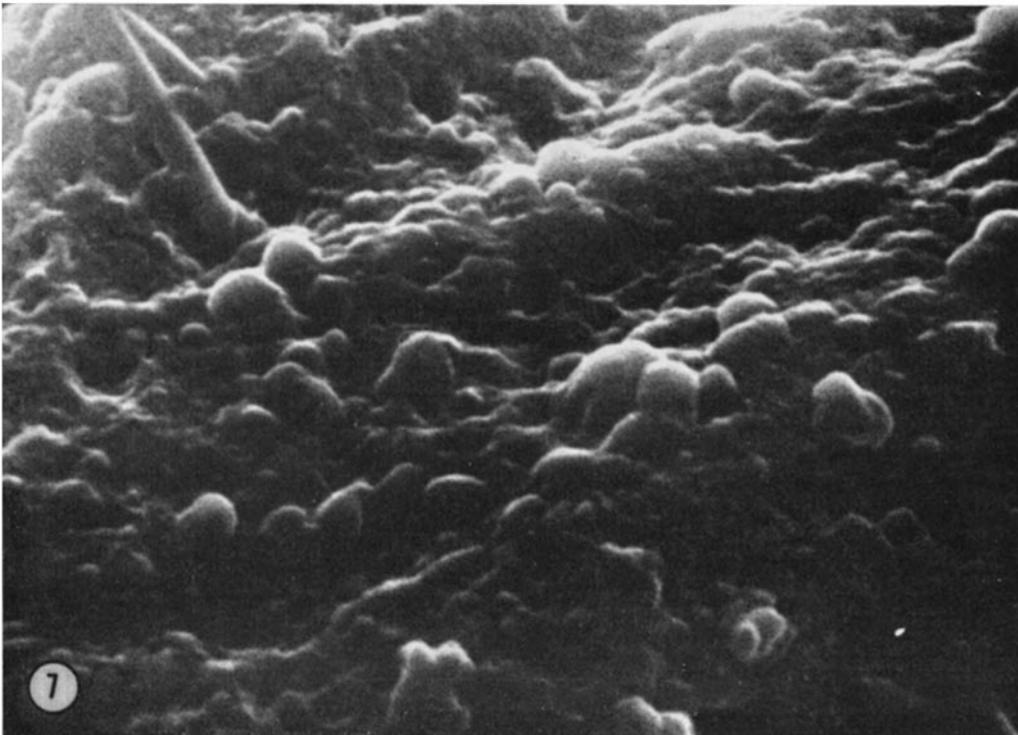
FIGURE 7   Perikaryal surface of control animal exhibiting particles, depressions, and ridges. Scanning electron micrograph. × 10,200.
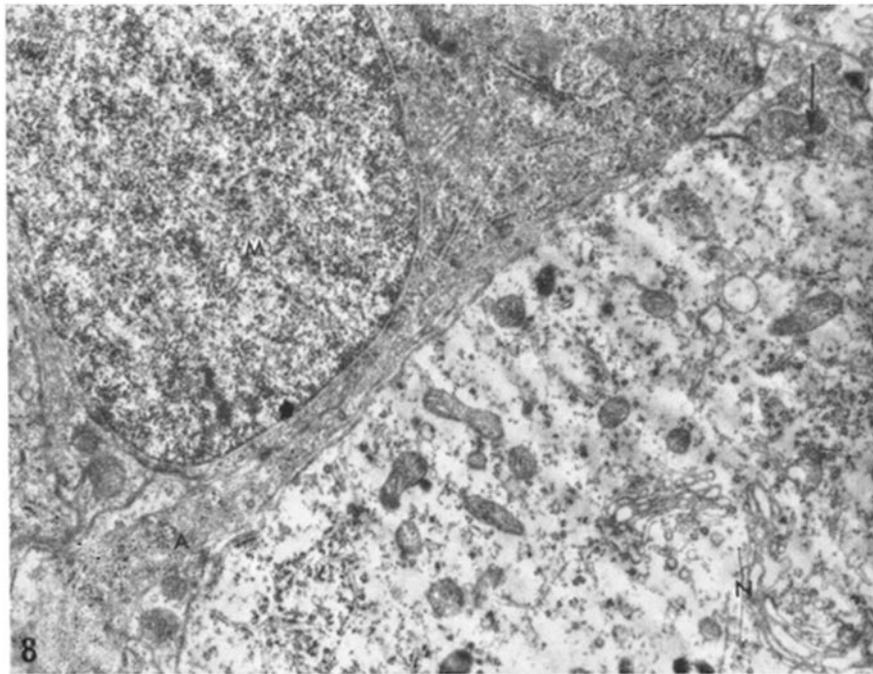
FIGURE 8 Portion of a chromatolyzed neuron (N) 4 days after nerve crush. The reactive glial cell (M) is to a large extent separated from the neuron by a thin sheet of glial cytoplasm (A). One synaptic terminal (arrow) is still attached to the plasma membrane. × 16,800.

14). The difference between control and experimental material was seen within 4 days after nerve crush. The most marked changes, however, were found from the 6th to the 14th postoperative day. The surface proper was almost devoid of the 0.5–1 μ depressions observed in the control cells but had a moderately granular appearance.

DISCUSSION

The present results, based on investigations with both light- and electron-microscopy, demonstrate that the spherical particles observed on the surface of isolated hypoglossal nerve cells are synaptic terminals. The surface structures on the nerve cells fulfill the criteria for synaptic bodies as defined by Peters, Palay, and Webster (1970). The terminals were also observed on the surface of isolated giant nerve cells of Deiters. The retinal ganglion cells and the spinal ganglion cells, known to lack synaptic terminals on their cell bodies (Polyak, 1941; Andres, 1961; Dowling and Boycott, 1969) consequently do not show any surface particles when observed with light- or scanning electron-microscopy (Hansson 1970 a,b). Satake et al. (1968) have

shown nerve terminals on neurons prepared from a cell suspension of brain tissue.

A most interesting finding in this study is the conspicuous reduction in the number of attached synaptic nerve terminals during the retrograde reaction of the hypoglossal nerve cell body after axonal injury. In accordance with our findings, Blinzinger and Kreutzberg (1968) demonstrated a displacement of synaptic boutons around regenerating motor neurons after sectioning of the facial nerve in rats. There was a removal of apparently intact synaptic terminals from the perikaryal surface. In the early phase of chromatolysis, reactive glial cells were seen in juxtaposition to the nerve cell bodies. A similar cell type observed by Blinzinger and Kreutzberg (1968) and Cammermeyer (1970) in the regenerating facial nucleus has been interpreted as a microglial cell. These cells may, however, represent a third type of glial cell (Peters, Palay, and Webster, 1970).

In contrast to the presence of reactive glial cell nuclei close to the chromatolytic neuron in the rat (Blinzinger and Kreutzberg, 1968), the nuclei of the reactive glial cells in the present study were
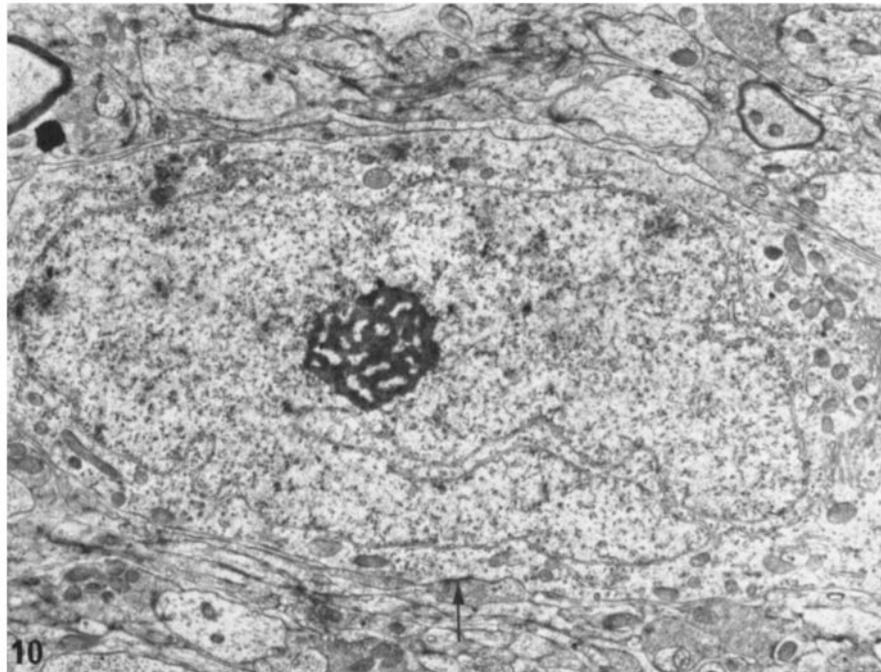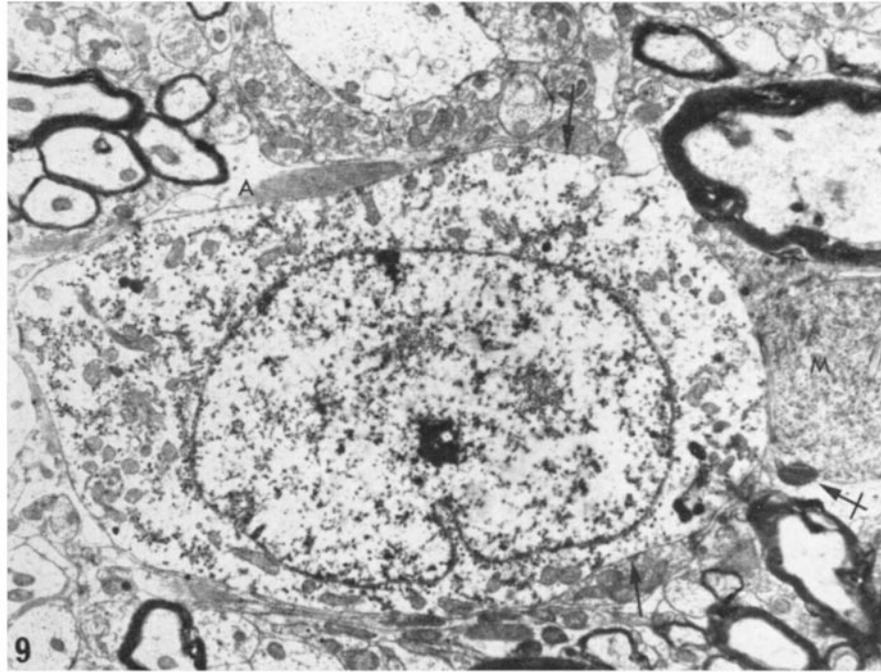
FIGURE 9   Chromatolyzed neuron 14 days after nerve crush. Several astrocytic cell processes (A) envelop the perikaryon. A reactive glial cell (M) containing a dense body (†) is seen in direct contact with the plasma membrane of the nerve cell. A few synaptic terminals (arrows) remain. × 4500.

FIGURE 10   A part of a chromatolyzed neuron 14 days after nerve crush. Most of the cell surface is enveloped by astrocytic processes. One synaptic terminal (arrow) attached to the plasma membrane can be identified. × 3500.
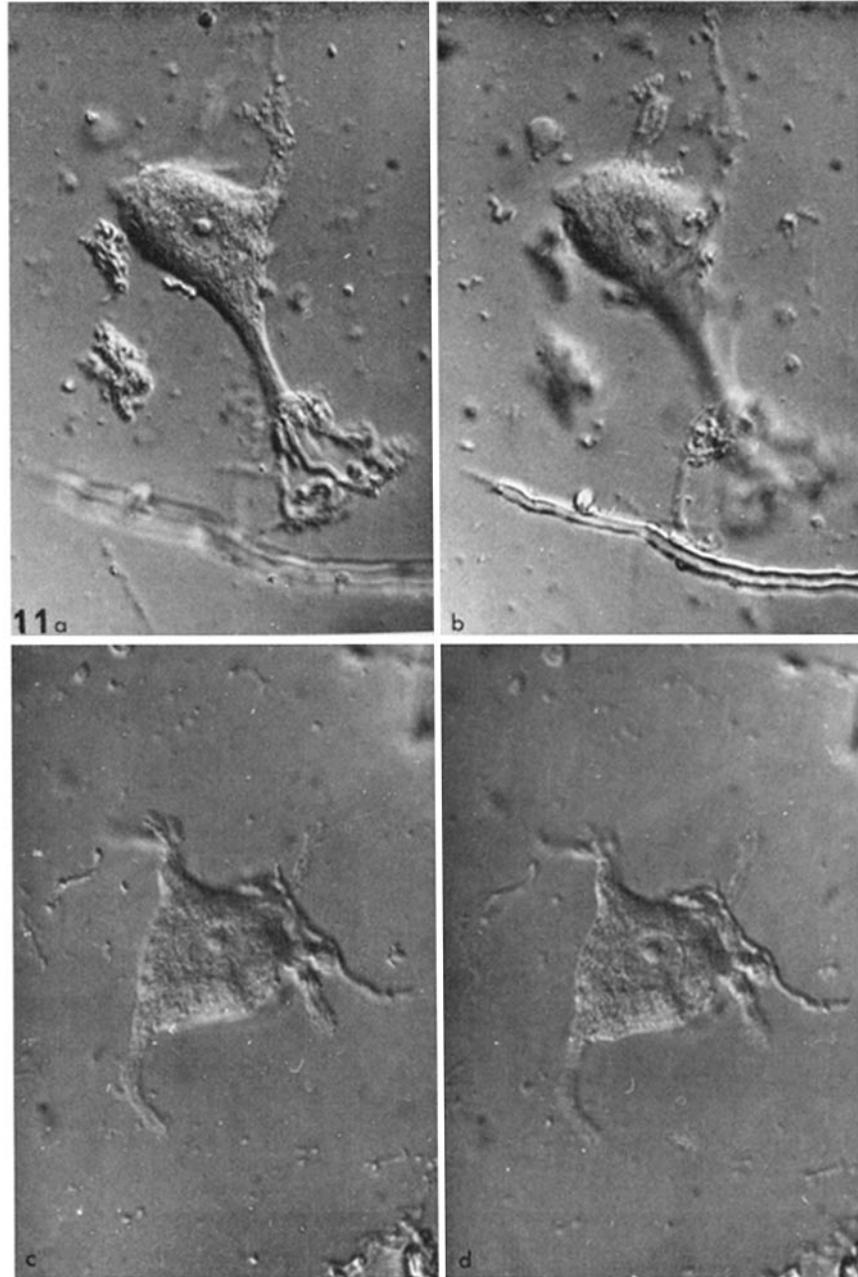
FIGURE 11 Light microscopy (Nomarski) of two isolated hypoglossal nerve cells 4 days after nerve crush. The cells are shown at different focus levels. The surface of the neuron is almost devoid of the spherical particles shown in Fig. 3. × 780.
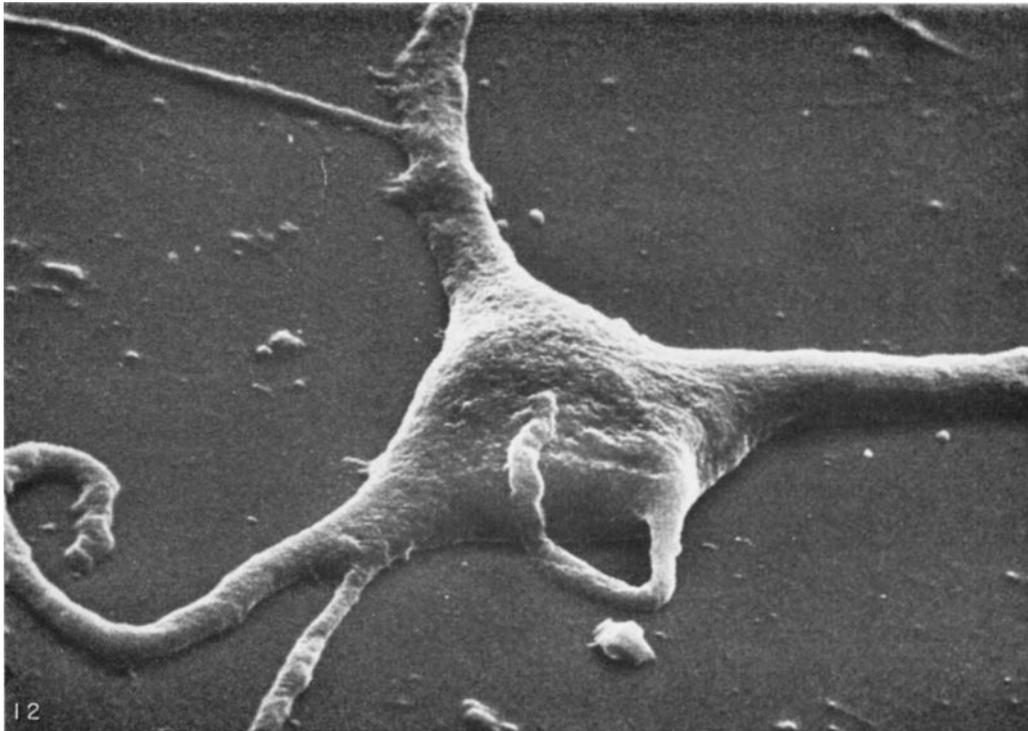
FIGURE 12 Scanning electron micrograph of hypoglossal neuron 6 days after nerve crush. The perikaryal surface is smoother than in control cells (cf. Fig. 4). × 1550.
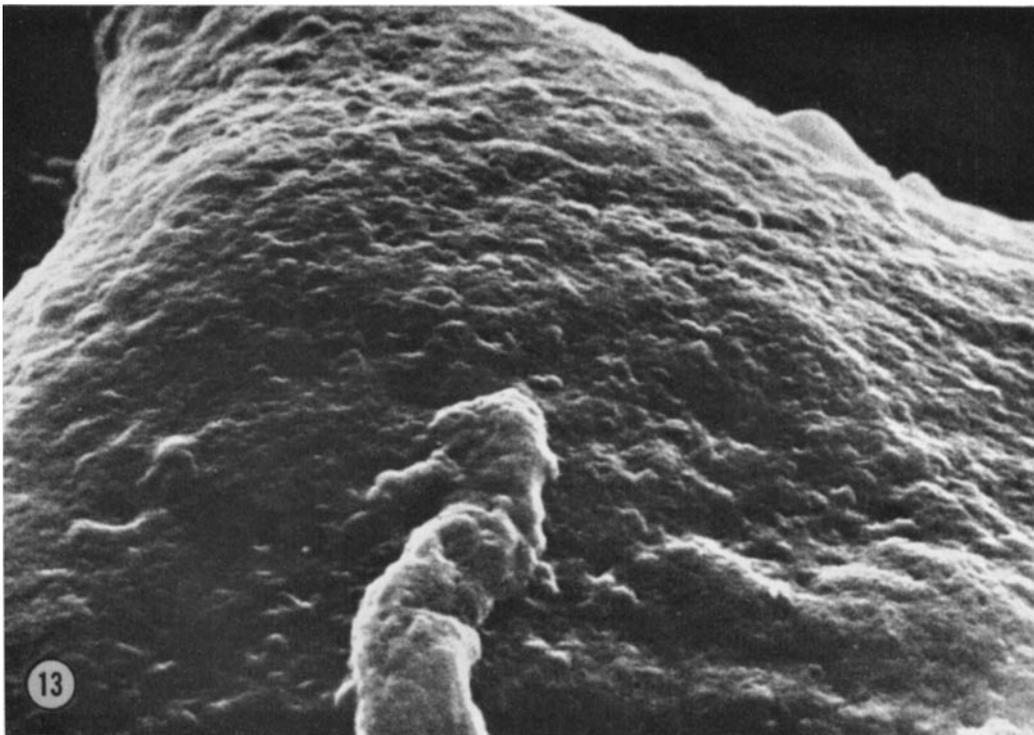
FIGURE 13 Perikaryal surface of the same cell as in Fig. 12. Note the virtual absence of spherical particles on the relatively smooth surface. × 6000.
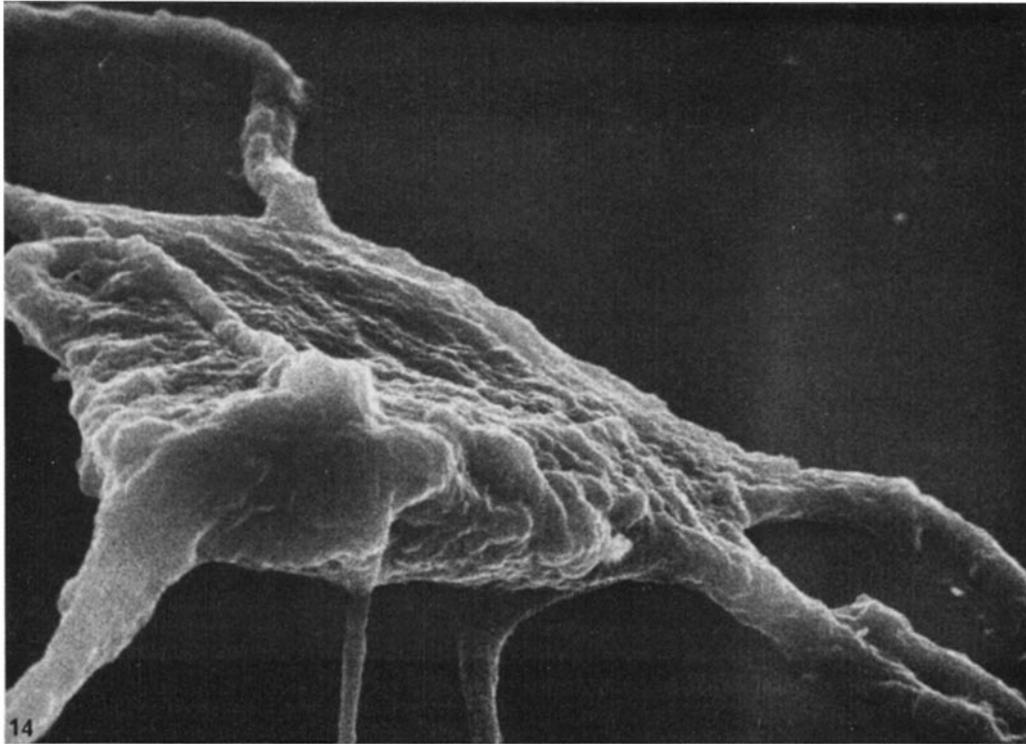
Figure 14   Hypoglossal neuron 14 days after nerve crush. The perikaryal surface is free of spherical particles (cf. Fig. 7). No regular surface impressions are seen. Scanning electron micrograph. × 3100.

more remote. This may indicate a true species difference, since few proliferating glial cell nuclei were seen in the vicinity of chromatolytic neurons in the rabbit (Sjöstrand, unpublished observations) compared to the abundance of glial cell nuclei labeled with thymidine-^{3}H surrounding neurons in the rat (Kreutzberg, 1966).

The hemispherical depressions on the nerve cell surface in the control material may indicate previous sites of synaptic contracts, since it is probable that a certain number of terminals are removed also from the control neurons during cell isolation. The virtual absence of synaptic terminals on the neurons during chromatolysis is, however, conspicuous. No glial cell processes were found attached to the surface of the isolated neurons in either control or regenerating material. This indicates that there is a definite difference in the strength of the mechanical attachment of, on one hand, neuron-nerve terminal and, on the other hand, neuron-glia, the former being much stronger.

The low adherence of neuropil to nerve cell bodies during chromatolysis (Watson, 1966; Hamberger and Sjöstrand, 1966) may therefore be explained by the less tight attachment of glial cells than by axons terminating on the nerve cell body.

A large number of studies on the comparative metabolic properties of mammalian nerve cell bodies and neuropil have been performed on freehand isolated single neuronal perikarya (Giacobini, 1957; Hydén, Løvtrup and Pigon, 1958; Hydén, 1959; Hamberger, 1963; Epstein and O'Connor, 1965; Hamberger and Sjöstrand, 1966; Watson, 1966).

The loss of synaptic terminals during nerve regeneration is of practical importance for the interpretation of results obtained by micromethods on the constituents and activities of single cells (Hamberger and Sjöstrand, 1966).

The intactness of isolated nerve cell bodies, and particularly of their plasma membrane, has been a matter of discussion during recent years (Roots and Johnston, 1964; Bondareff and Hydén, 1969).

The presence of neuronal surface structures, presumably synaptic terminals, has been demonstrated by light microscopy after supravital staining of single cells with methylene blue (Hydén, 1961). With the aid of Nomarski optics, a granular surface could be demonstrated for isolated neurons (Wolfe and Derry, 1968). However, the power of resolution of the light microscope does not allow a conclusive identification of the surface structures or evaluation of the state of the plasma membrane. Recently, relatively well preserved neuronal plasma membranes have been demonstrated by electron microscopy of isolated cells (Satake et al., 1968; Bondareff and Hydén, 1969), which is in agreement with the results obtained in the present study.

A question of great interest is whether the observed phenomenon of synapse detachment constitutes a more generalized cell reaction during nerve injury. It may be an expression of synaptic plasticity, as indicated by morphological and neurophysiological studies on injured neurons (Eccles, 1964; Raisman, 1969). The reaction would facilitate the establishment of new synaptic connections as observed during development (Conradi and Skoglund, 1969). Whether the synaptic contacts are detached primarily due to a change in the perikaryal plasma membrane—the volume of the hypoglossal neuron increases by at least 200% during chromatolysis (Brattgård et al., 1957)—or because of synapse displacement by perineuronal glial cells is still unknown.

REFERENCES

ANDRES, K. H. 1961. *Z. Zellforsch. Mikrosk. Anat.* **55**:1.

BLINZINGER, K., and G. KREUTZBERG. 1968. *Z. Zellforsch. Mikrosk. Anat.* **85**:440.

BONDAREFF, W., and H. HYDÉN. 1969. *J. Ultrastruct. Res.* **26**:399.

BRATTGÅRD, S.-O., T.-E. EDSTRÖM, and H. HYDÉN. 1957. *J. Neurochem.* **1**:316.

CAMMERMEYER, J. 1970. *Neurosci. Res. Program Bull. Res.* **3**:43.

CONRADI, S., and S. SKOGLUND. 1969. *Acta Physiol. Scand. Suppl.* **333**:1.

DE ROBERTIS, E., A. P. DE IRALDI, G. RODRIGUEZ, and J. GOMEZ. 1961. *J. Biophys. Biochem. Cytol.* **9**:229.

DOWLING, J. E., and B. B. BOYCOTT. 1969. *In* The Retina. B. R. Straatsma, M. O. Hall, R. A. Allen, and F. Crescitelli, editors. University of California Press, Los Angeles. 145.

ECCLES, J. C. 1964. The Physiology of Synapses. Springer-Verlag KG, Berlin.

ECCLES, J. C., B. LIBET, and R. R. YOUNG. 1958. *J. Physiol. (London).* **143**:11.

EPSTEIN, M. H., and J. S. O'CONNOR. 1965. *J. Neurochem.* **12**:389.

GIACOBINI, E. 1957. *J. Neurochem.* **1**:234.

GRAY, E. G., and V. P. WHITTAKER. 1962. *J. Anat.* **96**:79.

HAMBERGER, A. 1963. *Acta Physiol. Scand.* **58**:203.

HAMBERGER, A., and J. SJÖSTRAND. 1966. *Acta Physiol. Scand.* **67**:76.

HANSSON, H.-A. 1970 a,b. *Z. Zellforsch. Mikrosk. Anat.* **107**:23 and 45.

HYDÉN, H. 1959. *Nature (London).* **184**:433.

HYDÉN, H. 1961. The Neuron. *In* The Cell. J. Brachet and A. E. Mirsky, editors. Academic Press Inc., New York. **4**:215.

HYDÉN, H., S. LØVTRUP and A. PIGON. 1958. *J. Neurochem.* **2**:304.

KARNOVSKY, M. J. 1965. *J. Cell Biol.* **27**:137 A.

KREUTZBERG, G. W. 1966. *Acta Neuropathol.* **7**:149.

LUFT, J. H. 1961. *J. Biophys. Biochem. Cytol.* **9**:409.

PETERS, A., S. L. PALAY, and H. DE F. WEBSTER. 1970. The Fine Structure of the Nervous System. Hoeber Medical Division, Harper and Row Publishers, New York.

POLYAK, S. L. 1941. The retina. Chicago University Press, Chicago.

RAISMAN, G. 1969. *Brain Res.* **14**:25.

ROOTS, B. I., and P. V. JOHNSTON. 1964. *J. Ultrastruct. Res.* **10**:350.

SATAKE, M., S. I. HASEGAWA, S. AKE, and R. TANAKA. 1968. *Brain Res.* **11**:246.

SJÖSTRAND, J. 1965. *Z. Zellforsch Mikrosk. Anat.* **68**:481.

WATSON, W. E. 1966. *J. Neurochem.* **13**:536.

WOLFE, M., and D. M. DERRY. 1968. *In* Applied Neurochemistry. A. N. Davison and J. Dobbing, editors. Blackwell Scientific Publications Ltd., Oxford. Cover.