SELECTIVE RETROGRADE TRANSSYNAPTIC TRANSFER OF A PROTEIN, TETANUS TOXIN, SUBSEQUENT TO ITS RETROGRADE AXONAL TRANSPORT

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ABSTRACT

The fate of tetanus toxin (mol wt 150,000) subsequent to its retrograde axonal transport in peripheral sympathetic neurons of the rat was studied by both electron microscope autoradiography and cytochemistry using toxin-horseradish peroxidase (HRP) coupling products, and compared to that of nerve growth factor (NGF), cholera toxin, and the lectins wheat germ agglutinin (WGA), phytohaemagglutinin (PHA), and ricin. All these macromolecules are taken up by adrenergic nerve terminals and transported retrogradely in a selective, highly efficient manner. This selective uptake and transport is a consequence of the binding of these macromolecules to specific receptive sites on the nerve terminal membrane. All these ligands are transported in the axons within smooth vesicles, cisternae, and tubules. In the cell bodies these membrane compartments fuse and most of the transported macromolecules are finally incorporated into lysosomes. The cell nuclei, the parallel Golgi cisternae, and the extracellular space always remain unlabeled. In the case of tetanus toxin, however, a substantial fraction of the labeled material appears in presynaptic cholinergic nerve terminals which innervate the labeled ganglion cells. In these terminals tetanus toxin-HRP is localized in 500–1,000 Å Diam vesicles. In contrast, such a retrograde transsynaptic transfer is not at all or only very rarely detectable after retrograde transport of cholera toxin, NGF, WGA, PHA, or ricin. An atoxic fragment of the tetanus toxin, which contains the ganglioside-binding site, behaves like intact toxin. With all these macromolecules, the extracellular space and the glial cells in the ganglion remain unlabeled. We conclude that the selectivity of this transsynaptic transfer of tetanus toxin is due to a selective release of the toxin from the postsynaptic dendrites. This release is immediately followed by an uptake into the presynaptic terminals.

KEY WORDS retrograde transport · transsynaptic transfer · uptake and release of macromolecules · tetanus toxin · lectins

The uptake of tetanus toxin (mol wt 150,000) (30) by nerve terminals and the retrograde axonal transport within a given neuron (16, 39, 53) are known to occur with high selectivity if compared to uptake and transport of horseradish peroxidase (HRP) or albumin (14, 49, 53). A similar selective uptake and retrograde transport has been shown for cholera toxin, wheat germ agglutinin (WGA) (14, 55), ricin, and phytohaemagglutinin (PHA) (14) as well as for nerve growth factor (NGF) (14,

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52, 53) and antibodies against dopamine β-hydroxylase, an enzyme selectively located in adrenergic neurons (17, 63). The uptake and transport of these toxins and lectins seem to depend on properties common to all peripheral and central neurons (48, 50, 51, 53, 55). In contrast, the uptake and retrograde axonal transport of NGF is confined to peripheral adrenergic and sensory neurons (53) and that of antibodies against dopamine β-hydroxylase exclusively to adrenergic neurons (17). Biochemical and morphological studies have shown that the binding of these particular macromolecules to specific components of the nerve terminal membrane is a necessary prerequisite for this selective, highly efficient uptake (14, 49, 55). In the case of retrogradely transported tetanus toxin, a substantial fraction of the labeled material is released after arrival at the cell body by postsynaptic dendrites and is then taken up by presynaptic nerve terminals innervating the corresponding adrenergic or spinal neurons (46, 47). Again, this transsynaptic transfer shows a high selectivity. Although ^{125}I-tetanus toxin and ^{125}I-NGF are transported retrogradely by sympathetic neurons in similarly large amounts, only tetanus toxin but not NGF is transferred transsynaptically (47). However, since NGF may not be taken up by the preganglionic cholinergic nerve terminals (53), it was of importance to study the possibility of retrograde transsynaptic transfer of additional macromolecules such as lectins or cholera toxin, which are taken up and transported by all peripheral and central neurons investigated so far.

With the use of electron microscope, autoradiography and coupling products of tetanus toxin, NGF, or WGA with HRP, the present study demonstrated that transsynaptic transfer is limited to tetanus toxin. This specificity seems to be due to a selective release of tetanus toxin from the dendrites. This finding points to distinct differences in the intracellular processing of transport organelles containing lectins, cholera toxin, or NGF on the one hand and tetanus toxin on the other.

MATERIALS AND METHODS

Labeling of Proteins with ^{125}I

Highly purified tetanus toxin was prepared by Dr. B. Bizzini, Pasteur Institute, Paris, France (4). An atoxic fragment of tetanus toxin, prepared by mild proteolysis (5), was a gift from the same laboratory. Cholera toxin was purchased from Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y., and was dialysed extensively against phosphate buffer before use. Choleragenoid, the nontoxic binding subunit of cholera toxin, was prepared by Dr. R. Germanier, Swiss Institute for Sera and Vaccines, Bern, Switzerland (18). NGF was prepared from adult male mouse salivary glands by a modified procedure (56) of Bocchini and Angeletti (6). WGA was purchased from Senn Chemicals, Dielsdorf, Switzerland, and PHA from Grand Island Biological Co., Grand Island, N. Y. Ricin (RCA II) was a gift from Dr. M. Burger, Biocenter, University of Basle, Switzerland. All the proteins were checked for purity by gel electrophoresis with or without SDS before use. HRP (type VI) was obtained from Sigma Chemical Co., St. Louis, Mo.

Carrier-free Na-^{125}I was purchased from EIR, Würenlingen, Switzerland, and the proteins were labeled by the chloramine T method (14, 52). This labeling procedure has been shown to interfere only slightly, if at all, with the biological activities of the proteins used (14). The resulting specific activities of the ^{125}I-labeled proteins ranged between 10 and 40 μCi/μg, and the protein concentrations between 0.1 and 0.5 μg/μl. The retrograde transport of all iodinated proteins was checked biochemically, as previously described (14, 53), before they were used for electron microscope autoradiograpy.

Preparation of HRP-Coupling Products

Different coupling procedures had to be used for each protein because of the very labile biological activities of both NGF and tetanus toxin. WGA was coupled to HRP by the two-step glutaraldehyde method (1). An NGF-HRP coupling product was synthesized using the coupling procedure described by Nakane and Kawaoi (34, 45). This coupling product retained its biological activity as shown by fiber outgrowth from embryonic chicken dorsal root ganglia, by an induction of tyrosine hydroxylase in rat sympathetic ganglia in organ culture and by selective retrograde transport (45).

Tetanus toxin was coupled to HRP using carbodiimide. Briefly, 5 mg of highly purified tetanus toxin was added to 6 mg of HRP in a final volume of 0.8 ml of isotonic saline solution. The reaction was started by the addition of 50 mg of 1-ethyl-3 (3-dimethylaminopropyl) carbodiimide-HCl (Sigma). The reaction mixture was kept at room temperature for 2 h, and then loaded onto a Sepharose 6B column (0.85 × 30 cm), previously equilibrated with 5 mM phosphate buffer. The optical densities of each fraction were measured at 280 nm for protein content and at 403 nm for the HRP chromophore. No free tetanus toxin could be detected. The fraction containing coupled HRP-tetanus toxin was collected and concentrated by ammonium sulfate precipitation (75% saturated ammonium sulfate solution). After centrifugation, the pellet was redissolved in 100 μl of isotonic saline, and dialysed for 3–4 h against 500 ml of cold 0.15 M NaCl.

The final protein concentrations were in the range of 1–5 mg/ml for WGA-, NGF-, and toxin-HRP. All the HRP coupling products showed highly efficient retro-

grade transport, demonstrated by the accumulation of ^{125}I-labeled coupling products in superior cervical ganglia 14 h after injection into the anterior eye chamber. In contrast, the toxicity of the tetanus toxin-HRP coupling products was remarkably reduced as can be deduced from the reduced tetanic symptoms in the injected animals.

Control Experiments for HRP-Coupling Products

Bovine serum albumin (BSA, Sigma) was coupled to HRP either by the two-step glutaraldehyde procedure or by the use of carbodiimide as previously described for WGA and tetanus toxin, respectively. Retrograde transport of these BSA-coupling products and of free HRP (type VI, 80 μg injected in 10 μl) was tested histochemically (see below), or by labeling the proteins with ^{125}I (specific activity: 20–30 μCi/μg) and counting the superior cervical ganglia 14 h after injection into the anterior eye chamber. No retrograde transport of either BSA-HRP or free HRP could be detected under the present experimental conditions.

Animals

For all the experiments, female Sprague-Dawley rats weighing 150–200 g were used. The animals were kept at a constant temperature of 23°C and supplied with the usual lab chow diet (Nafag AG, Gossau SG, Switzerland) and water ad libitum.

Injection Procedure

Ten μl of either ^{125}I-labeled tetanus toxin, tetanus toxin fragment, cholera toxin, choleragenoid, NGF, WGA, PHA, or ricin (for autoradiography) or of WGA-, NGF-, or tetanus toxin-HRP coupling product were injected unilaterally into the anterior eye chamber and 20 μl unilaterally into the submandibular gland of the same animals. From previous studies on the retrograde transport of tetanus toxin and NGF in the peripheral sympathetic nervous system, it is known that the peak of radioactivity in the superior cervical ganglion occurs 12–14 h after injection (53). Accordingly, the animals were fixed 14 h after injection. After injection of tetanus toxin, tetanic symptoms appeared at ~12 h and were followed by the death of the animals between 15 and 20 h after injection (mainly due to tetanus toxin escaping from the site of injection into the circulation). In the case of WGA-HRP, an additional group of animals was killed 8 h after injection and a further group 24 h after injection. For each ligand and experimental setup, a group of 4–6 animals was used.

Fixation

The animals were anesthetized with ether and perfused through the heart with a short prerinse of Ringer solution containing 1,000 U-USP of heparin (Liquemin, Roche Products Ltd., London) and 0.1% procaine, and for 10 min with a mixture of 2.5% glutaraldehyde and 1% paraformaldehyde (freshly generated from paraformaldehyde powder) in 0.1 M phosphate buffer, pH 7.4. After careful dissection of the superior cervical ganglion of the injected side, the ganglia were immersed for an additional 2 h (autoradiography) or 45 min (histochemistry) in the same fixative. All the ganglia were counted in a Packard γ-counter (Packard Instrument Co., Downers Grove, Ill.) and the values were compared to the ones obtained from the ganglion of the non-injected side dissected simultaneously to determine the total amount of radioactivity accumulated in the ganglion by retrograde transport (14, 53).

Autoradiography

After a wash overnight in 0.1 M phosphate buffer containing 5% sucrose, the ganglia were postfixed for 2 h in 1.33% OsO_4 in cacodylate buffer, washed in the same buffer, dehydrated and embedded in Epon 812. No loss of radioactivity occurred from the tissues during fixation and embedding.

For light microscope autoradiography semithin sections were coated with Ilford L_4 emulsion diluted 1:1 with distilled water. The exposure time was 30 d at 4°C in a dry atmosphere. Autoradiograms were developed with Kodak D19 developer at 20°C for 6 min.

For electron microscope autoradiography, 50-nm sections were cut and mounted by means of a hair loop on objective slides previously coated with celloidin. The sections were coated with a monolayer of Ilford L_4 emulsion by the dipping technique following the procedure described in detail by Kopriwa (26). The exposure time was 90 d at 4°C in a dry atmosphere. Autoradiograms were developed with Microdol-X at 20°C for 3 min, fixed in 24% sodium thiosulfate for 2 min, washed 3–5 times in distilled water, and transferred to 100-mesh copper grids as described by Kopriwa (26). Unstained sections or sections stained with uranyl acetate alone or with uranyl acetate (2 min) followed by lead citrate according to Reynolds (20 min) were examined in a Zeiss EM 10. Before staining, the celloidin film overlying the emulsion was removed by dipping the grids briefly into isoamylacetate. This treatment improved the access of the staining solutions to the sections and reduced unspecific lead precipitates.

One single section was selected for each ganglion (animal) and systematically screened for silver grains. All the silver grains encountered were photographed at a primary magnification of ×5,400 in a Zeiss 10 electron microscope. Analysis of labeling and silver grain distribution was performed by the method described by Salpeter and McHenry (44) as described earlier (47).

Morphometry

The relative volume occupied by the various components of the neuropil in the superior cervical ganglion was determined by a stereological procedure (point-counting) using sets of random pictures taken at a pri-

mary magnification of ×3,400 on the same sections used for the autoradiographic analysis (47).

Histochemistry

The fixed ganglia were washed for 2 h in 0.1 M phosphate buffer, pH 7.4, containing 5% sucrose (three changes of buffer at 20°C with continuous rotation of the samples). The ganglia were dehydrated in an ascending series of polyethyleneglycol water mixtures (PEG A 1,000, melting point 37°–40°C, Fluka, Buchs, Switzerland) at 40°C and under reduced pressure (100–200 Torr) within 5 h and embedded in PEG (32, 45). The blocks were kept at 4°C overnight. 10 μm sections were cut, floated for 15 min on phosphate-buffered saline (PBS) containing 5% glycerol to remove the PEG, and mounted on gelatin-coated objective slides. HRP activity was visualized by incubation with diaminobenzidine (0.03%) and H_2O_2 (0.01%) in Tris-buffer, pH 7.6 (21, 32) for 90 min. The reaction was monitored under the light microscope, and appropriate sections containing labeled cells were selected for electron microscopy. These sections were incubated with 1.33% OsO_4 for 2 h, washed, dehydrated, and embedded by inverting a gelatin capsule filled with Epon over the section. After polymerization, the glass slide was removed by dipping into liquid nitrogen (32). No reaction product was observed when diaminobenzidine was omitted from the reaction mixture.

RESULTS

Autoradiography

The light microscope autoradiograms of superior cervical ganglia showed the same proportion of labeled neurons—~20% of the total cell population—after injection of either ^{125}I-labeled tetanus toxin, toxin fragment, NGF, cholera toxin, choleragenoid, WGA, ricin, or PHA. These labeled cells represent the neurons projecting to the injected target organs, i.e., to the iris and the submandibular gland. The overall labeling intensity, however, varied remarkably: WGA produced by far the strongest labeling. A very pronounced labeling was also seen after retrograde transport of tetanus and cholera toxins and NGF, but only a moderate labeling after injection of ricin and PHA. These autoradiographic results correspond to the levels of radioactivity determined in the same ganglia by γ-counting immediately after fixation (data not shown). From the specific activities of the labeled proteins, the total amount of retrogradely transported material could be estimated to be 20–30 fmol for ^{125}I-WGA and 10–20 fmol for ^{125}I-tetanus toxin and the other ligands (for detailed analysis, see reference 14).

Electron microscope autoradiography showed that labeled axons, cell bodies, and dendrites were a consistent finding for all the ligands injected (Table I). The silver grain density (% silver grain/% section surface or tissue volume occupied by the labeled structure) was high for dendrites and axons whereas it was at background levels for glial cells and extracellular space (Fig. 1). However, a most significant difference between the various macromolecules became apparent with respect to the labeling of preganglionic cholinergic nerve terminals: they were heavily labeled after retrograde transport of ^{125}I-tetanus toxin and tetanus toxin fragment showing the highest labeling density of all the structures in the ganglion (Table I, Figs. 1 and 2). This highest labeling density also excludes the possibility that the silver grains overlying presynaptic terminals have been generated by "cross-

TABLE I

Structures in the Superior Cervical Ganglion Labeled by Retrograde Transport of Proteins

	(a) Tet. tox.	Tet. tox fragm.	NGF	WGA	PHA	Ricin	Cholera toxin	Cholera genoid	(b) Volume density
Axons	30.7 ± 1.9	31.3 ± 4.7	26.1 ± 2.9	26.3 ± 3.3	32.2 ± 1.4	22.0 ± 0.6	38.9 ± 3.4	36.7 ± 1.9	11.8 ± 1.3
Dendrites	28.5 ± 3.4	24.5 ± 1.6	42.1 ± 0.5	27.3 ± 3.6	34.1 ± 3.4	24.2 ± 2.0	43.1 ± 2.9	44.5 ± 3.9	21.4 ± 4.5
Presynaptic terminals	12.8 ± 1.1	17.7 ± 5.9	0.5 ± 0.5	0.4 ± 0.2	0.4 ± 0.4	0.4 ± 0.3	0.1 ± 0.1	1.1 ± 0.5	2.3 ± 0.3
Glial cell cytoplasm	19.2 ± 3.8	17.7 ± 0.4	18.1 ± 0.7	32.3 ± 1.5	22.0 ± 1.4	29.3 ± 0.4	8.3 ± 0.9	8.6 ± 1.5	37.6 ± 2.2
Glial cell nuclei	2.8 ± 1.4	0.6 ± 0.4	2.1 ± 0.7	1.2 ± 0.1	2.0 ± 0.9	2.4 ± 0.6	3.1 ± 0.6	2.6 ± 1.8	7.3 ± 2.5
Extracellular space	5.5 ± 1.3	6.5 ± 0.1	9.5 ± 3.5	10.0 ± 1.0	5.7 ± 1.7	16.0 ± 4.5	6.2 ± 0.4	5.4 ± 1.3	14.5 ± 2.1
Blood vessels	0.4 ± 0.4	0.3 ± 0.2	0.9 ± 0.9	2.1 ± 1.6	1.6 ± 0.9	4.7 ± 1.4	0.4 ± 0.2	0.7 ± 0.4	4.5 ± 2.9
Total number of silver grains	204	508	197	1,605	287	484	895	331	

(*a*) Percentage distribution of autoradiographic silver grains over various structures of the neuropil* in the superior cervical ganglion 14 h after injection of ^{125}I-labeled ligands into the anterior eye chamber and the submandibular gland. The values represent the means ± SEM for each experimental group (3–4 animals). (*b*) Percentage of total tissue volume occupied by the corresponding structures.

* Since there was a significant variability in the number of labeled cell bodies found in a given random section and in their labeling intensity, nerve cell bodies were excluded from the present analysis. Their average labeling density at 14 h after injection is comparable to that of axons and dendrites. For a detailed analysis of cell somas after retrograde transport of tetanus toxin and NGF, see references 45, 47.

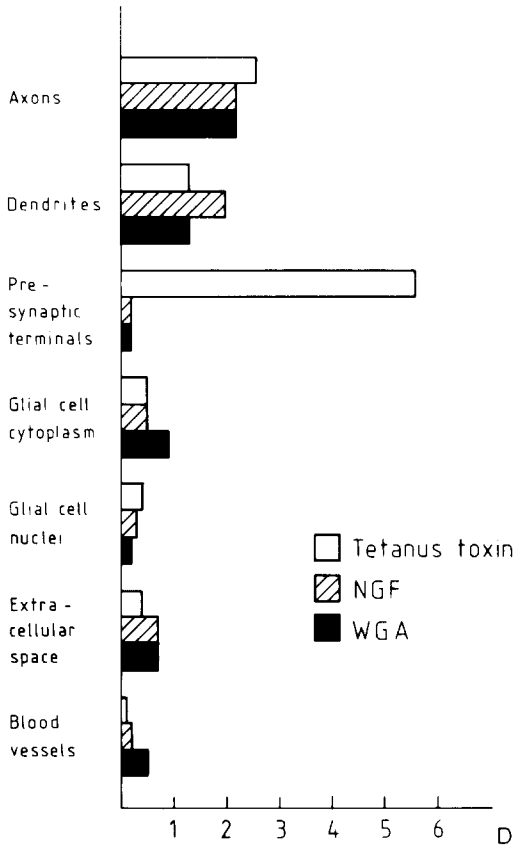


FIGURE 1 Density of autoradiographic silver grains over the various components of the neuropil (see footnote to Table I) of the superior cervical ganglion 14 h after injection of ^{125}I-labeled tetanus toxin, NGF, or WGA into the anterior eye chamber and the submandibular gland (D = % of silver grains/% of section surface occupied by the corresponding structure).

firing" from an adjacent labeled compartment. In sharp contrast to tetanus toxin, the nerve terminals remained unlabeled after retrograde transport of NGF, ricin, PHA, cholera toxin, and cholerageneoid (Table I, Fig. 1). In the case of ^{125}I-WGA, which is transported retrogradely most efficiently, a few labeled presynaptic nerve terminals have occasionally been observed (0.4% of the total neuropil label, compared with 12.8% in the case of tetanus toxin; Table I).

HRP-Coupling Products

Tetanus toxin, NGF, and WGA were covalently coupled to HRP. All these coupling products showed highly efficient retrograde transport. NGF-HRP has been shown to possess the biological activity of native NGF (fiber outgrowth in embryonic chicken dorsal root ganglia in culture, induction of tyrosine hydroxylase in sympathetic ganglia [45]). As in the autoradiographic experiments the intensity of the label in the ganglion cells and their axons was higher after retrograde transport of WGA-HRP than after tetanus toxin-HRP or NGF-HRP (Fig. 4).

No obvious qualitative differences in the pattern of labeled organelles in the postganglionic neurons could be observed between tetanus toxin-, WGA-, or NGF-HRP. The reaction product was consistently located in membrane compartments and was never observed free in the cytoplasm or within the nucleus. In the axons, sER-like tubules and cisternae, and vesicles with smooth surface seemed to represent the transport compartment (Fig. 3). In the cell body and in dendrites, secondary lysosomes (dense bodies and multivesicular bodies) were labeled in addition to short sER-like profiles and vesicles (Fig. 4). Labeled organelles were distributed all over the cells, but their highest density was found in the perinuclear region and in the vicinity of the Golgi fields.

Since labeled secondary lysosomes were only rarely found in axons, they presumably take up the tracer by fusion in the cell body. This interpretation was supported by the observation that, 8 h after injection of WGA-HRP into the anterior eye chamber, only a part of the total number of secondary lysosomes in the adrenergic ganglion cells contained reaction product, with the majority of the tracer still localized in sER-like compartments. However, 24 h after injection, the total population of dense bodies and multivesicular bodies appeared heavily labeled.

No labeling could be observed in the parallel cisternae of the Golgi complex after retrograde transport of toxin- or NGF-HRP. The same was true for WGA-HRP, regardless of the time of injection (8 h, 14 h, or 24 h after injection) (Fig. 4). Labeled smooth vesicles, sER-like tubules, and lysosomes were very common in the periphery of the Golgi field (Fig. 5).

Reaction product in the extracellular space has virtually never been observed after retrograde transport of tetanus toxin-HRP or NGF-HRP (Fig. 4). In the case of a few neurons labeled extremely heavily by retrogradely transported WGA-HRP, short segments of labeled extracellular space adjacent to a labeled cell body or dendrite could sometimes be observed.

For presynaptic cholinergic nerve terminals im-

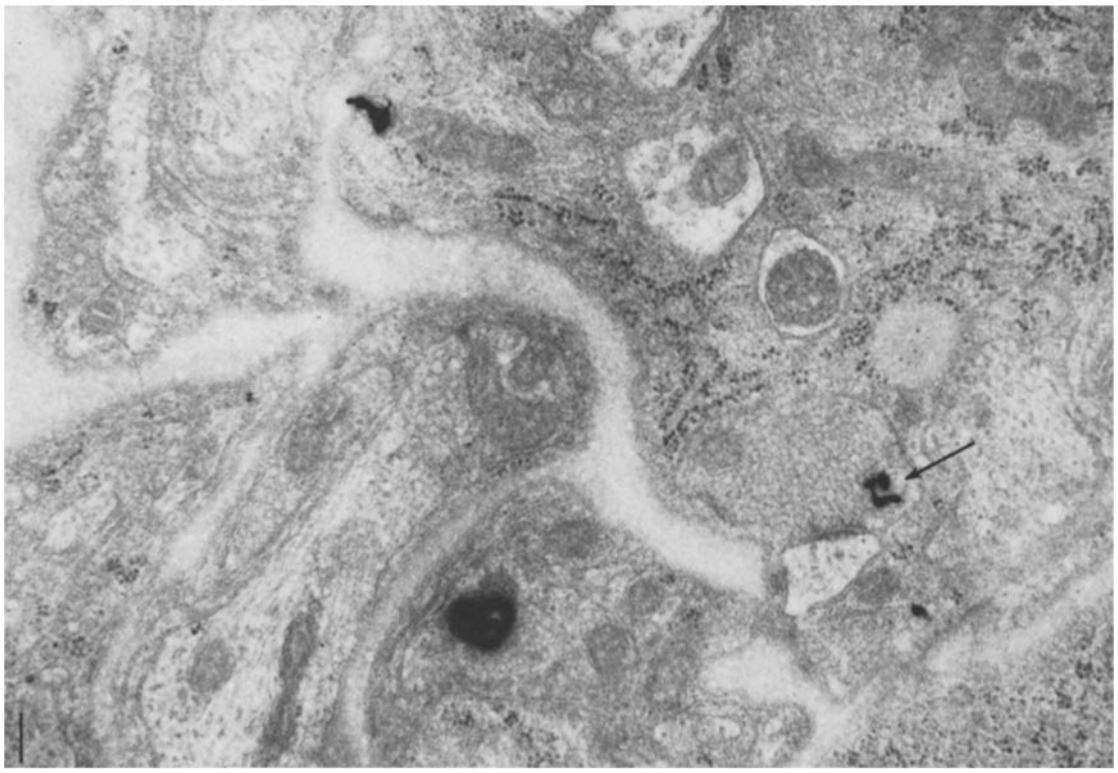


FIGURE 2 Labeled presynaptic cholinergic nerve terminal (arrow) in the superior cervical ganglion 14 h after injection of ^{125}I-tetanus toxin into the anterior eye chamber and the submandibular gland. Bar, 0.2 μm. × 30,000.

pinging on the adrenergic ganglion cells, the pattern of labeling was very similar to that observed with electron microscope autoradiography. After retrograde transport of tetanus toxin-HRP, nerve terminals containing reaction product were frequently observed (Fig. 6). Their postsynaptic counterpart was usually a labeled dendrite. The extracellular space and the synaptic cleft remained unlabeled in most cases, suggesting that the release of the tracer by the postsynaptic dendrite is immediately followed by uptake into the presynaptic terminal. Within the terminals tetanus toxin-HRP was localized in both smooth and coated vesicles of 500–1,000 Å Diam (Fig. 6). Since only a few pinocytotic profiles could be observed, we could not decide whether exclusively coated vesicles are involved in the initial uptake of the toxin, with a subsequent transformation into smooth vesicles.

In contrast to tetanus toxin, no labeled presynaptic nerve terminals could be found after retrograde transport of NGF-HRP, in spite of an overall labeling density very similar to that of tetanus toxin-HRP. With WGA-HRP, a cholinergic terminal containing reaction product was occasionally observed which is in agreement with the autoradiographic data, but the relative labeling of nerve terminals in comparison to dendrites or cell bodies did not compare with that of tetanus toxin.

DISCUSSION

Selective and highly efficient uptake and subsequent retrograde transport of tetanus toxin is initiated by binding of the toxin to specific binding sites on nerve terminal membranes (14, 49). These binding sites, probably di- and trisialogangliosides (2, 22, 23, 55), seem to be common constituents of nerve terminals (29), since uptake and retrograde transport of tetanus toxin is detectable in all peripheral and central neurons studied so far (48, 55). Subsequent to its retrograde transport, the toxin is transferred transsynaptically to the nerve terminals impinging on the dendrites of the neurons which were previously reached by retrograde axonal transport (46, 47). This retrograde trans-

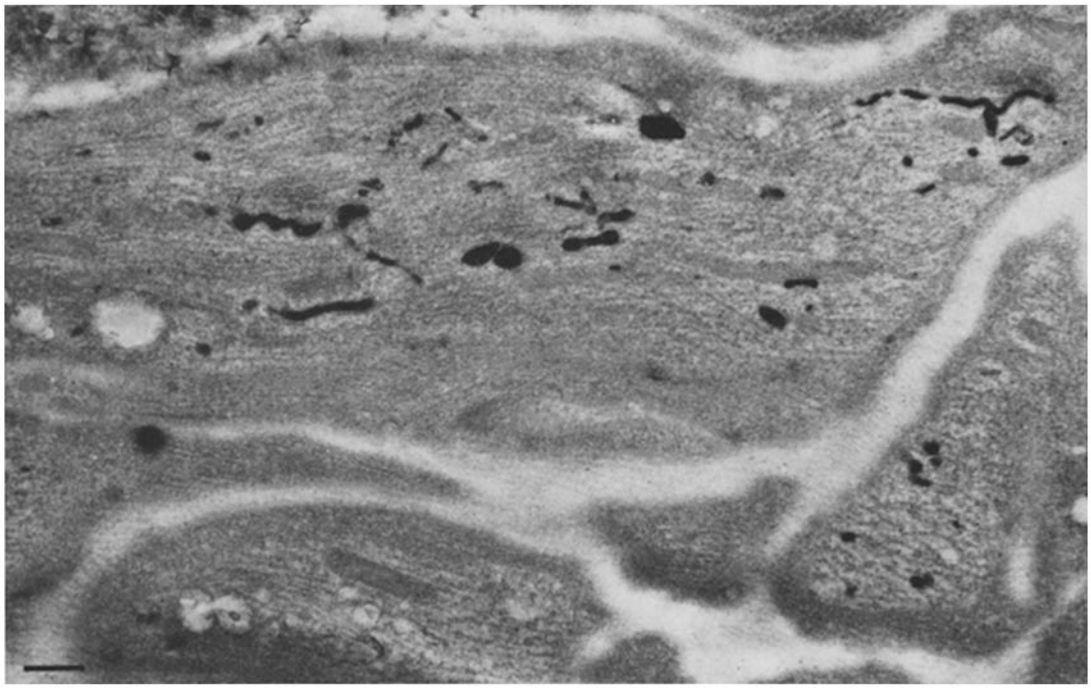


FIGURE 3 Longitudinal section through a postganglionic sympathetic axon. Retrogradely transported WGA-HRP is localized in vesicles and tubular structures. Bar, 0.2 μm. × 36,000.

synaptic transfer explains how tetanus toxin reaches its site of action in the spinal cord, i.e., the nerve terminals synapsing with the spinal motoneurons, where it selectively blocks the release of the inhibitory transmitters glycine and gamma-aminobutyric acid (GABA) (11, 12, 36). Such a blockade deprives the motoneurons of their inhibitory input and causes hyperactivity of the motoneurons and, consequently, tetanic muscle contraction. Thus, this electrophysiological effect suggests that the radioactivity observed by electron microscope autoradiography in presynaptic terminals represents either intact toxin or a specific active toxic subunit. Recent immunohistochemical and biochemical experiments have confirmed that the radioactivity transported retrogradely (9) and subsequently across the synapse represents intact tetanus toxin (15). So far, nothing is known, however, on the biochemical mechanism by which tetanus toxin inhibits the release of glycine and GABA in the spinal cord (11, 12, 36) and of acetylcholine at some types of neuromuscular junctions (13) and in central nervous system primary cultures (3). Nor is it known whether the toxin present in presynaptic nerve terminals in the superior cervical ganglion has an effect on the acetylcholine release from these synapses. The present study shows that there is a distinct difference between tetanus toxin on the one hand and NGF, cholera toxin, WGA, ricin, and PHA on the other hand after their retrograde axonal transport to the sympathetic ganglion: transsynaptic transfer of significant amounts of label occurs only in the case of tetanus toxin, whereas for cholera toxin, NGF, PHA, and ricin no transsynaptic transfer could be observed. After injection of WGA a few presynaptic terminals were labeled; their relative number and labeling density, however, was much smaller than after injection of tetanus toxin. In agreement with the autoradiographic results, no extracellular or glial accumulation of coupling products between NGF or WGA and HRP could be observed in the vinicity of the labeled neurons after retrograde transport. Such an extracellular accumulation of NGF- or WGA-HRP should be expected if these macromolecules would be released in the same way as tetanus toxin-HRP but not taken up by the presynaptic terminals. Moreover, such a failure of uptake seems very improbable since WGA has been shown to be taken up and transported retrogradely by all peripheral and central neurons studied so far (50, 51, 55). The

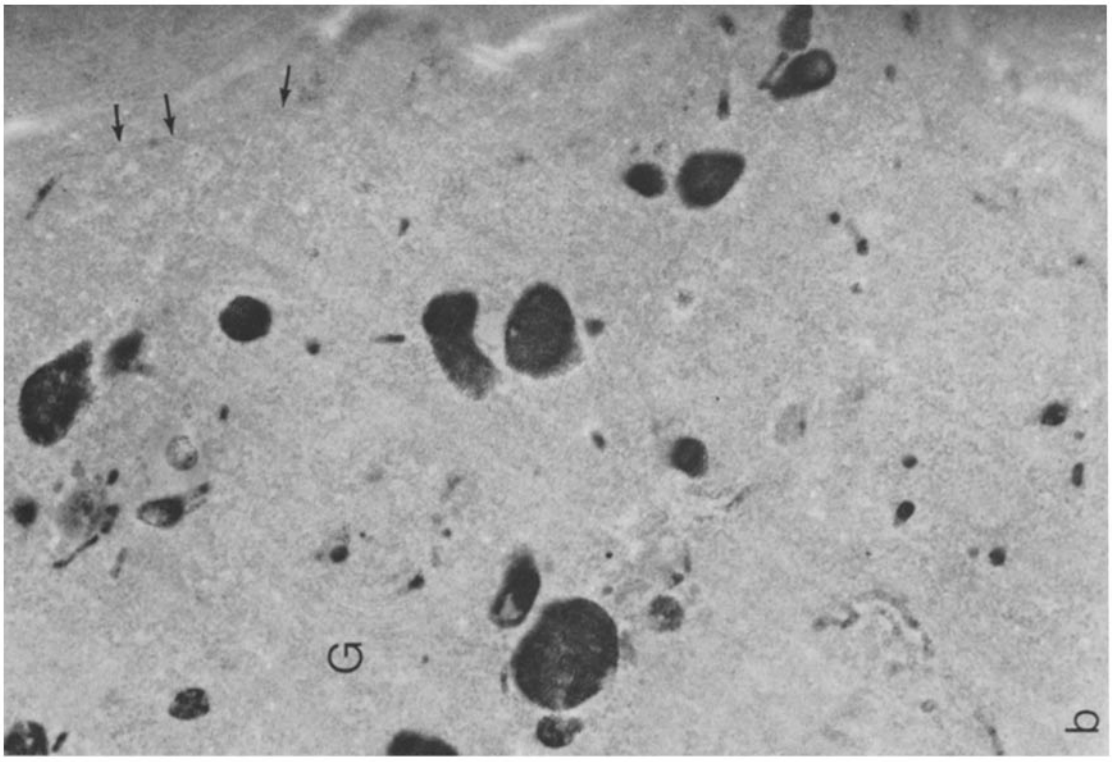
same is true also for cholera toxin (55) and PHA and ricin (unpublished results) which are transported by peripheral adrenergic, sensory, and motor neurons. Interestingly, the extracellular space and the synaptic cleft were free of reaction product also in the case of tetanus toxin-HRP, in spite of the pronounced labeling of presynaptic terminals. Therefore, a very rapid and efficient uptake by the synaptic terminals after the release of the toxin by the dendrites must be assumed. In addition, since only a very small percentage of the surface of the adrenergic ganglion cell is in contact with presynaptic terminals (62), the transfer of tetanus toxin most probably takes place preferentially in the region of the synapse, i.e., it is a truly transsynaptic transfer. The transfer itself seems to include an initial release by fusion of a vesicle or sER-like compartment with the surface membrane and a subsequent binding to the cell membrane of the presynaptic terminals followed by endocytosis, as it has been shown for the initial uptake by the adrenergic terminals (49) and for ^{125}I-tetanus toxin injected directly into the spinal cord (40). Release of an endogenous macromolecule, acetylcholinesterase, has been demonstrated from dendrites of central motoneurons as well as from sympathetic ganglion cells (25, 27).

The most intriguing question which remains is that concerning the signal responsible for the differential intracellular processing of the vesicles and sER-like compartments arriving by retrograde transport. Whereas some of those organelles containing tetanus toxin fuse with the cell membrane in the postsynaptic regions and release their content, the organelles containing cholera toxin, lectins, or NGF seem to deliver their contents exclusively to lysosomes. A simple distinction on the basis of the receptor—glycolipids versus glycoproteins—responsible for the initial binding and uptake at the adrenergic nerve terminal seems not to be possible, since both tetanus and cholera toxin seem to bind to gangliosides, albeit to different ones (2, 23, 55).

The statistical distribution of label in electron microscope autoradiograms and the HRP cytochemistry showed that there were no gross differences in the intracellular localization of the ligands (44, 47). Smooth vesicles and sER-like tubules and cisternae contain the tracers during the initial retrograde transport and may function as transport vehicles. Whether the compartments are true sER membranes or whether they are derived from the surface membrane and contain the ligand still bound to its membrane receptor has not been decided yet, although the latter possibility seems to be more probable (14, 24). All the ligands reach the cell bodies as chemically intact molecules (9, 14, 15, 54), and for NGF it has been shown that specific induction of tyrosine hydroxylase, an enzyme catalysing the rate-limiting step in the synthesis of the adrenergic transmitter noradrenaline, can be produced by the retrogradely transported NGF (37, 38). Fusion with lysosomes and progressive degradation seems to be the fate of NGF, cholera toxin, the lectins, and also of those tetanus toxin molecules which are not released by the dendrites.

Regardless of the different mechanisms of initial uptake, retrograde transport in sER-like organelles and subsequent incorporation into lysosomes seems to be a common mechanism also for HRP, ferritin, and colloidal thorium dioxide (8, 24, 28, 35, 41 58, 60, 61). Interestingly, the parallel Golgi cisternae never became labeled by retrogradely transported WGA-, NGF-, or tetanus toxin-HRP. This is in contrast to the observations reported for ricin- or PHA-coupling products taken up by dissociated sympathetic or sensory ganglion cells in vitro (19, 20). Although the uptake of these tracers seems to occur initially into smooth membrane compartments they subsequently are transferred to the GERL region and the innermost Golgi cisterna. If fusions with GERL elements occur at all after retrograde transport of tetanus toxin, WGA, or NGF, they seem much less pronounced than after direct uptake of lectins by cell bodies of dissociated neurons under cell culture conditions.

Retrograde transneuronal changes observable in the cell bodies of neurons projecting to nerve cells with axon lesions have been described by neuroanatomists following the application of degeneration methods for the tracing of fiber pathways (for review see reference 10). Detachment and retraction of presynaptic nerve terminals following lesions of the axon of the postsynaptic cell are well documented in superior cervical ganglion (31, 42), ciliary ganglion (7), and spinal and cranial motor nuclei (33, 57). In the superior cervical ganglion, these retrograde transsynaptic effects could also be elicited by a blockade of the axonal transport in the postsynaptic cells by colchicine (43). On the other hand, anterograde and retrograde exchange of specific signals between neurons and neurons and their target organs possibly transduced in part by macromolecules has been postulated on the basis of a large variety of develop-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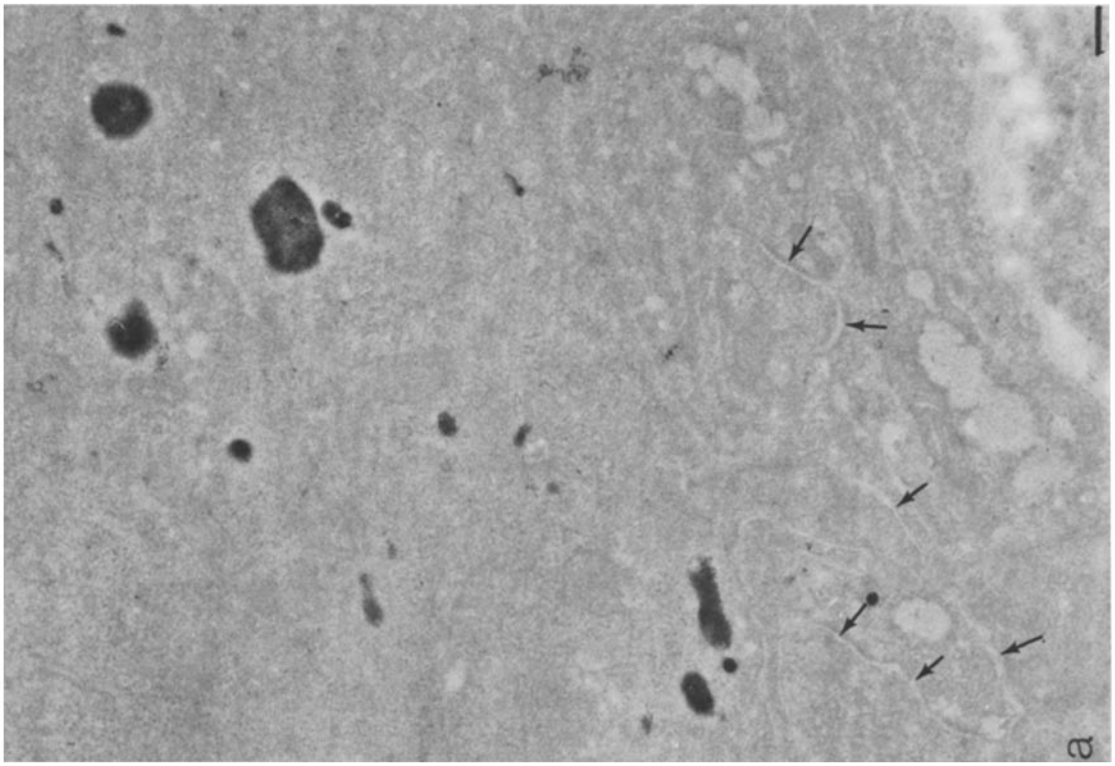
a

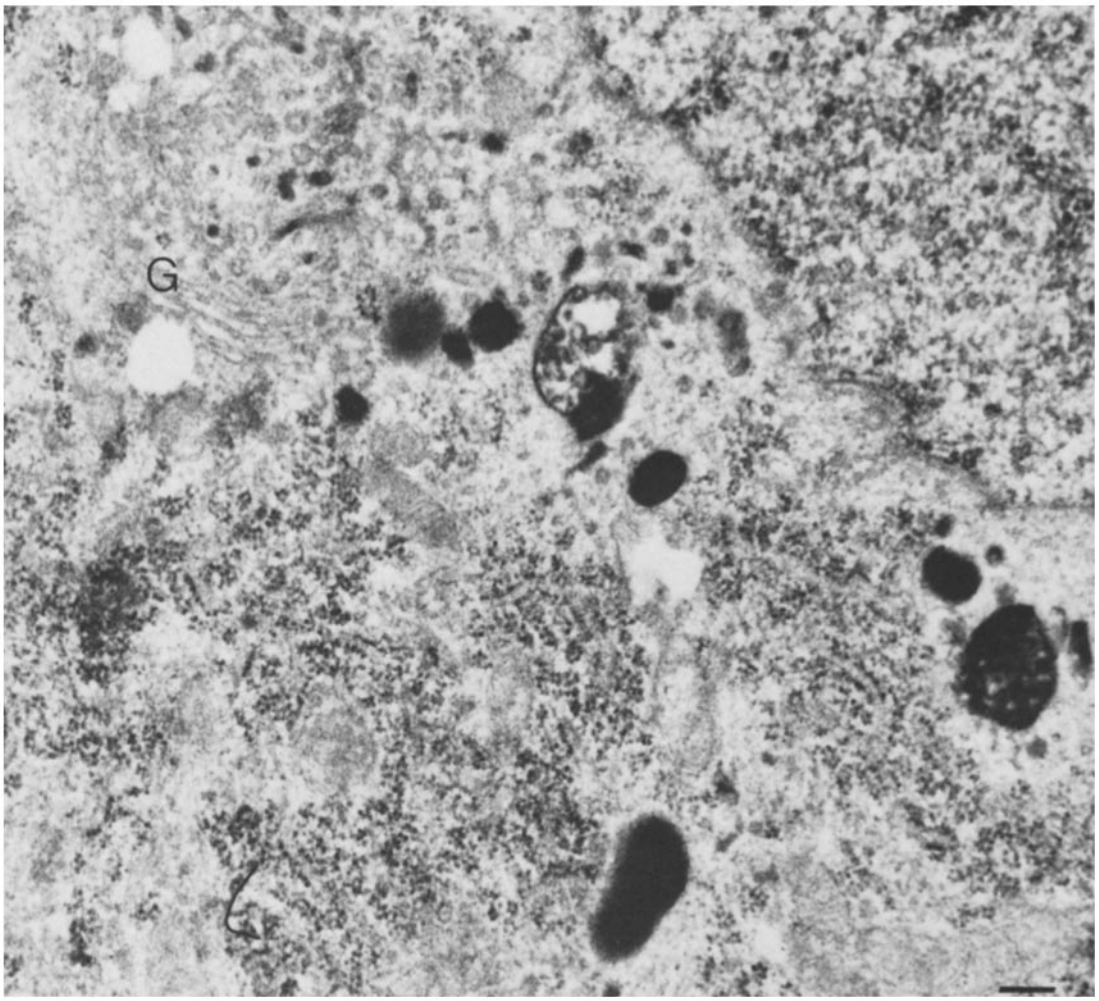

FIGURE 5 Perinuclear region of a sympathetic ganglion cell labeled by retrogradely transported WGA-HRP. Reaction product is present in secondary lysosomes and in vesicles and tubular elements especially in the vicinity of the Golgi complex (*G*). The parallel Golgi cisternae, however, are unlabeled. Bar, 0.2 μm. × 36,000.

mental studies performed in vivo and in vitro. Such "trophic" signals could be involved in the regulation of neuroblast survival and differentiation and in the development of specific synaptic interactions. While selective uptake and retrograde axonal transport of NGF may represent an example of a direct neuron-target organ interaction (59), the present results show the existence of a pathway for a high molecular weight macromolecule which moves retrogradely through a chain of two neurons. Tetanus toxin, an exogenous macromolecule which produces pathological effects in its host, makes use of a pre-existing mechanism which may serve for the transfer of endogenous

FIGURE 4 Cytoplasm of sympathetic ganglion cells after retrograde transport of tetanus toxin-HRP (*a*) and WGA-HRP (*b*). Cytochemical reaction product is present in secondary lysosomes and in small vesicular and tubular compartments. The Golgi cisternae (*G*), the cell membrane and the extracellular space (arrows) are free of reaction product. Unstained sections. Bar, 0.2 μm. × 30,000.

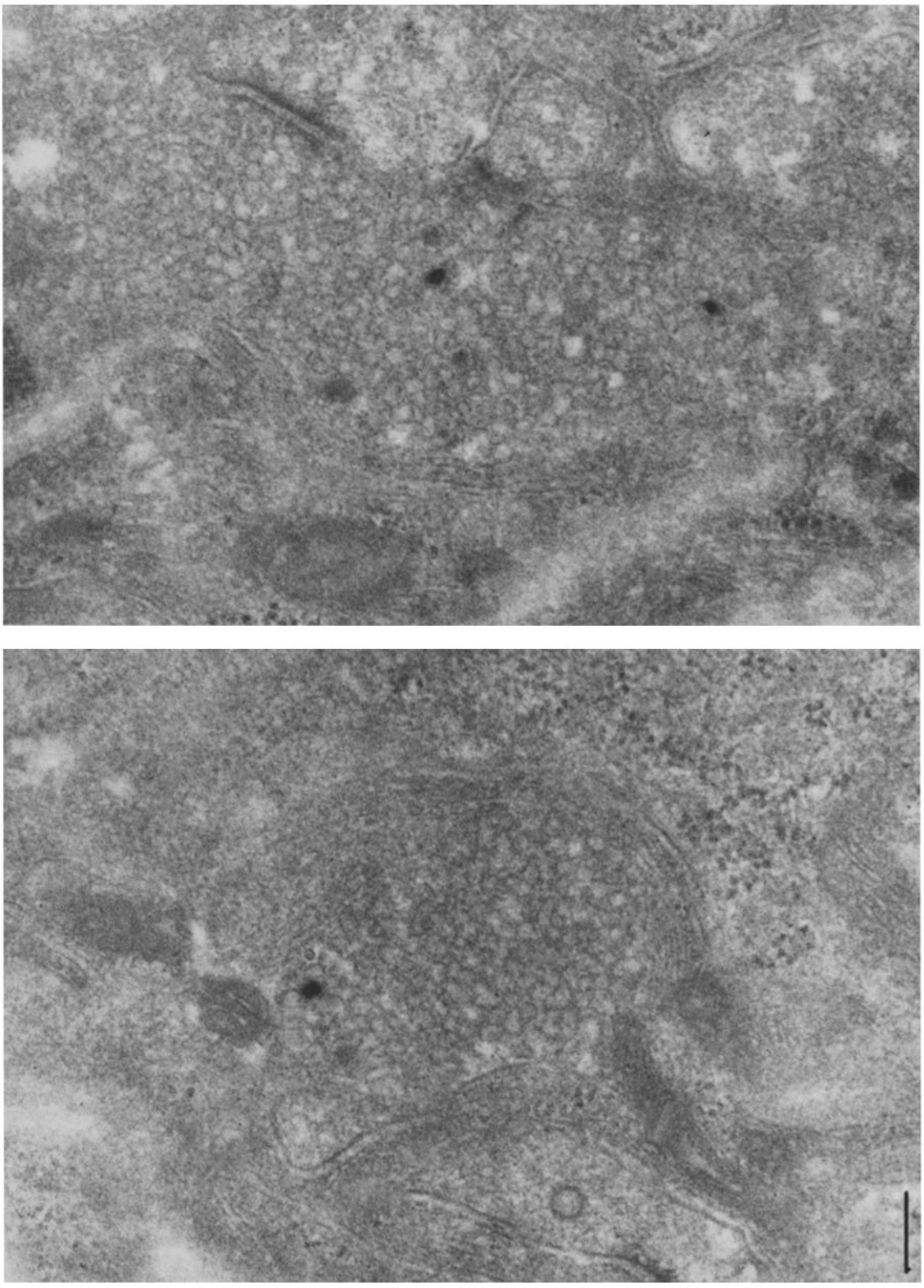

FIGURE 6 Cholinergic nerve terminals in the superior cervical ganglion containing cytochemical reaction product which represents tetanus toxin-HRP transferred transsynaptically subsequent to its retrograde axonal transport in the postganglionic cells. The labeled material is present in vesicles. Bar, 0.2 μm. $\times$ 68,000.

macromolecules carrying specific information retrogradely through chains of neurons. The high selectivity of this retrograde transsynaptic transfer which depends on a selective intracellular processing and postsynaptic release may further substantiate the physiological importance of this mechanism.

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