Mechanism of Uptake and Retrograde Axonal Transport of Noradrenaline in Sympathetic Neurons in Culture: Reserpine-resistant Large Dense-core Vesicles as Transport Vehicles

MARTIN E. SCHWAB and HANS THOENEN, *with the technical assistance of*
CHRISTIANE MULLER.
*Department of Neurochemistry, Max-Planck-Institute for Psychiatry, D-8033 Martinsried, Federal
Republic of Germany*

ABSTRACT The uptake and retrograde transport of noradrenaline (NA) within the axons of sympathetic neurons was investigated in an in vitro system. Dissociated neurons from the sympathetic ganglia of newborn rats were cultured for 3-6 wk in the absence of non-neuronal cells in a culture dish divided into three chambers. These allowed separate access to the axonal networks and to their cell bodies of origin. [^{3}H]NA (0.5 × 10^{-6} M), added to the axon chambers, was taken up by the desmethylimipramine- and cocaine-sensitive neuronal amine uptake mechanisms, and a substantial part was rapidly transported retrogradely along the axons to the nerve cell bodies. This transport was blocked by vinblastine or colchicine. In contrast with the storage of [^{3}H]NA in the axonal varicosities, which was totally prevented by reserpine (a drug that selectively inactivates the uptake of NA into adrenergic storage vesicles), the retrograde transport of [^{3}H]NA was only slightly diminished by reserpine pretreatment. Electron microscopic localization of the NA analogue 5-hydroxydopamine (5-OHDA) indicated that mainly large dense-core vesicles (700-1,200-Å diam) are the transport compartment involved. Whereas the majority of small and large vesicles lost their amine dense-core and were unable to take up 5-OHDA after reserpine pretreatment, the population of large dense-core vesicles and some small dense-core vesicles involved in the retrograde transport were resistant to this drug. It, therefore, seems that these vesicles maintained the amine uptake and storage mechanisms characteristic for adrenergic vesicles, but have lost the sensitivity of their amine carrier for reserpine. The retrograde transport of NA and 5-OHDA probably reflects the return of used synaptic vesicle membrane to the cell body in a form that is distinct from the membranous cisternae and prelysosomal structures involved in the retrograde axonal transport of extracellular tracers.

In general, neither the synthesis nor the degradation of macromolecular constituents of membranes in particular, occurs in the axons and nerve terminals of neurons (5, 16, 27, 28). Efficient transport systems are therefore needed to carry newly synthesized material from the cell body to the nerve terminals and back to the cell body. These transport functions are served by fast and slow anterograde transport, and by retrograde transport, respectively (23, 47, 58). Rapidly transported material in the anterograde direction is always present inside, or as a constituent of membranous organelles, mostly vesicles or organelles morphologically resembling the smooth endoplasmic reticulum (sER; [17, 23, 50, 63]). Organelles involved in the retrograde transport are tubular and vesicular structures, large cisternae, and prelysosomal organelles (multivesicular bodies, cup-shaped bodies; [46, 47, 50, 63]). Although the direction of transport (anterograde or retrograde) for a given organelle is certainly strictly regulated by the cell, the mechanisms responsible for the direction taken are unknown.

Membrane transport from the perikaryon to the nerve terminal serves two main functions: (*a*) renewal of the plasma membrane, involving the supply, e.g., of general structural constituents, and of specific receptors for extracellular signals, e.g., macromolecular factors, and neurotransmitters; and (*b*) renewal of synaptic vesicles (23, 28). Biochemical evidence

clearly indicates major compositional differences between synaptic vesicle membranes and plasma membranes (15, 34, 65, 68). Although temporary fusions take place between the two types of membrane during exocytotic transmitter release, vesicle membrane seems to be retrieved selectively (25, 26, 38, 67). Less clear is the origin of synaptic vesicles. Vesicles, especially large dense-core vesicles in adrenergic or serotoninergic neurons, are rapidly transported from the cell body to the terminals or varicosities (14, 21, 55, 59), and it has been suggested that, after an initial exocytosis, their membranes contribute to the formation of small dense-core vesicles, the predominant type of vesicle in most adrenergic nerve endings (49). However, in adrenergic neurons, budding of small and large catecholamine-storing vesicles from the axonal and terminal ER-like tubular network has also been observed (56), and a catecholamine-containing reticular network has been observed in axons and terminals (56, 61). In neuromuscular junctions, synaptic vesicles can bud from larger membrane cisternae during recovery after massive stimulation (25). It is not known whether the membrane components of synaptic vesicles and plasma membranes are part of the same organelle during anterograde transport, or whether they are always separated. The same is true for their retrograde transport. In adrenergic nerves, a retrograde transport of synaptic vesicle membrane is indicated by the accumulation of dopamine-β-hydroxylase (an enzyme involved in the synthesis of NA and located in the membrane of synaptic vesicles; [34]) and of NA not only proximal, but also distal to nerve constrictions (4, 14).

Neuroanatomical tracing experiments in the central nervous sytem have shown, surprisingly, that some established and some putative neurotransmitters (glycine, gamma-aminobutyric acid [GABA], aspartate, glutamate, acetylcholine, dopamine, and serotonin) are selectively taken up by the respective nerve terminals and transported retrogradely to the cell bodies (1, 12). For these small molecules to be transported over distances of several millimeters, a stable storage complex has to be formed. Besides the amines, only glycine has been shown to be associated with vesicles during anterograde transport, in *Aplysia* neurons (42). The organelles involved in the retrograde transport of these transmitter molecules are not yet known.

Dissociated rat sympathetic neurons in a tissue culture dish divided into three chambers (7) were used to study retrograde axonal transport under well-defined conditions. We have shown that [^{3}H]NA is taken up into the varicosities, and that part of it is transported retrogradely in reserpine-resistant, mainly large, dense-core vesicles.

MATERIALS AND METHODS

Culture Procedure: Polystyrene (Lux Scientific Inc., Newbury Park, CA) or Aclar (Allied Chemical Corp., Morristown, NJ) coverslips of 25-mm diam were glued into 35-mm tissue culture dishes (Falcon Labware, Oxnard, CA) sterilized by ultraviolet irradiation, and coated with sterile collagen (calf skin, Sigma Chemical Co., St. Louis, MO). A Teflon ring separating the dish into a central chamber for the neuron cell bodies and two side chambers for axons (Fig. 1 a) was sealed onto the coverslip with silicon grease (Dow Corning high-vacuum grease; Dow Corning Corp., Midland, MI) as described by Campenot (7).

Neurons were dissociated from superior cervical ganglia of newborn rats by a 1-h incubation in 0.1% trypsin (Sigma Chemical Co.) and 0.1% collagenase (Worthington Biochemical Corp., Freehold, NJ) in Ca^{++}/Mg^{++}-free phosphate-buffered saline at 37°C, followed by 10 min in 0.5% trypsin. After two washes, the ganglia were suspended in 1 ml of complete medium and triturated with a siliconized Pasteur pipette. 5,000–10,000 Neurons were plated into the cell body chamber of each dish and cultured for 3–6 wk in a 5% CO$_2$ in air atmosphere. The growth medium was an enriched L$_{15}$, CO$_2$-based medium as described by Mains and Patterson (39), containing 50 ng/ml mouse nerve growth factor, prepared according to the modified method of Bocchini and Angeletti (54), 5% rat serum, 0.6 g/100 ml Methocel (hydroxymethyl cellulose, Dow Chemical Co., Midland, MI), penicillin (100 U/ml), streptomycin (100 µg/ml; Sigma Chemical Co.), and 10^{-5} M cytosine arabinoside (Sigma Chemical Co.; [24]).

Before the retrograde transport experiments, the density of the axonal networks in the axon chambers was checked, so that only cultures with comparable, high fiber densities were used. The axons were washed overnight in medium containing no Methocel, no cytosine arabinoside, and 0.1% bovine serum albumin (BSA; Sigma Chemical Co.) instead of rat serum (L$_{15}$-BSA), to prevent complex effects of serum constituents. For this wash, the axon chambers were filled with medium to the top, i.e., ~3 mm above the fluid level of the cell body chamber, which enabled leaking cultures to be detected and discarded.

Retrograde Transport of [^{3}H]Noradrenaline: [^{3}H]NA (l-7,8-[^{3}H]noradrenaline, specific activity 32 or 37 Ci/mmol, Amersham Corp., Radiochemical Center, Amersham, England; or 44 Ci/mmol, New England Nuclear, Boston, MA) was diluted in L$_{15}$-BSA medium to a concentration of 0.7 × 10^{-6} M and added to the axons in 100 µl per axon chamber. Cultures were returned to the incubator for 6 h. At the end of the experiment, 20 µl of medium was taken from each axon chamber and 150 µl from the slit containing the cell bodies for counting. All the remaining medium was then removed and the cell bodies were
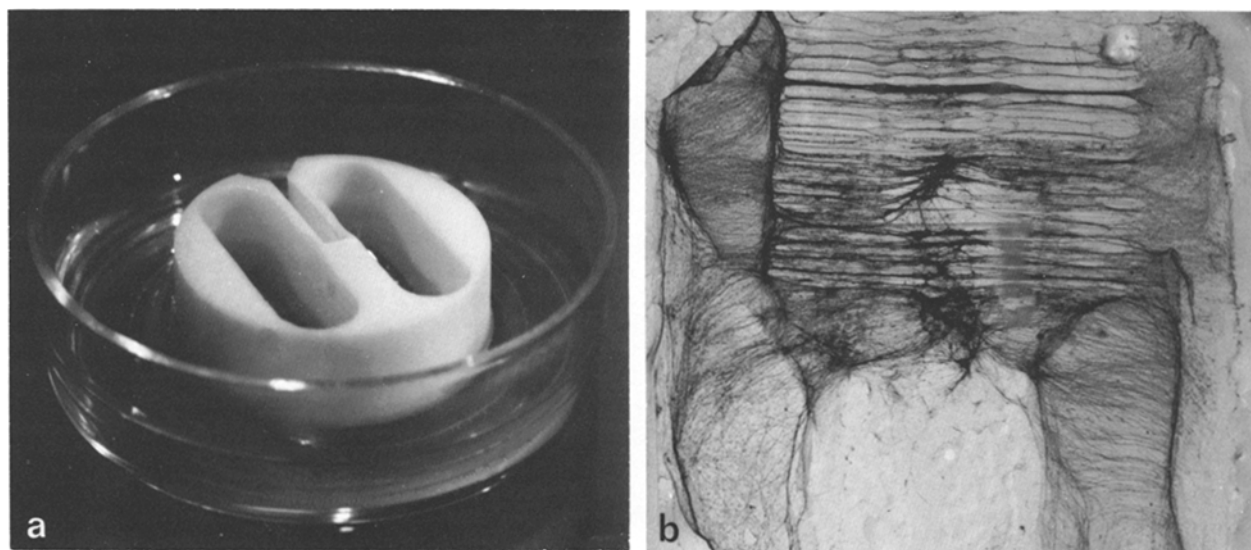

FIGURE 1   Chamber culture arrangement used in the present study (7). (a) Teflon ring with left and right axonal chambers and central slit for the cell bodies. (b) Bottom of culture dish after removing the ring. Clumps of cell bodies in the center give rise to bundles of branching axons. Methylene blue stained. × 70.

rinsed with 2 ml of L₁₅-BSA run through the slit-shaped cell body chamber. The cultures were air-dried, the Teflon ring was removed, and the coverslips were cut into left and right axon chambers, and cell body chamber, and the parts counted separately.

The amount of radioactivity in the medium of the cell body chambers at the end of the experiment indicated whether leakage occurred above the normal level (see below). Data obtained from such cultures were discarded. Due to the care that had to be taken in handling the axons and removing the previous medium from the axon chambers, the actual concentrations of [H³]NA in the axon chambers, as determined for each culture at the end of the experiments, varied from 0.3 to 0.6 × 10⁻⁶ M. Since the uptake of [³H]NA was linear between 0.3 and 0.6 × 10⁻⁶ M, the counts obtained from axons and cell bodies were normalized to 0.5 × 10⁻⁶ M for each culture.

Drug Treatments: All drugs applied to the axons were diluted in L₁₅-BSA medium; drugs applied to the neuronal cell bodies were diluted in normal growth medium. The following drugs were added with [³H]NA to the axons only: unlabeled NA (*d*-, *l*-NA; Sigma Chemical Co.), 2 × 10⁻⁴ M; desmethylimipramine (DMI; Ciba-Geigy Ltd., Basel, Switzerland) 0.7 or 7 × 10⁻⁶ M; cocaine, 10⁻⁵ M.

These drugs were used for preincubations only: reserpine (Sigma Chemical Co.), 10⁻⁶ M, 45-min incubation of axons and cell bodies, followed by a 15-min wash with L₁₅-BSA (axons) or normal growth medium (cell bodies); tranylcypromine (Röhm Pharma GmbH, Darmstadt, Federal Republic of Germany), 5 × 10⁻⁶ M for 15 min on axons only or axons and cell bodies, followed by two washes with L₁₅-BSA.

The following drugs were present during a preincubation period as well as with [³H]NA in the axon chambers: colchicine (Sigma Chemical Co.), 5 × 10⁻⁶ M on axons and cell bodies, 1-h preincubation; vinblastine (Sigma Chemical Co.), 10⁻⁵ M on axons and cell bodies, 1-h preincubation; yohimbine (Sigma Chemical Co.), 10⁻⁷ M on axons only, 15-min preincubation; *l*-propranolol (ICI-Pharma, Plankstadt, Federal Republic of Germany), 10⁻⁷ or 10⁻⁶ M on axons only, 15-min preincubation; U-0521 (catechol-o-methyl transferase [COMT] inhibitor, gift of Dr. H. Bönisch, Institute of Pharmacology and Toxicology, University of Würzburg, Federal Republic of Germany), 5 × 10⁻⁶ M on axons and cell bodies, 15-min preincubation.

Electron Microscopy: Axons were washed with L₁₅-BSA as previously described, and incubated for 6 h with 5 × 10⁻⁴ M 5-hydroxydopamine (5-OHDA; [5-OHDA · HBr]; Hoffmann-La Roche Inc., Basel, Switzerland) in L₁₅-BSA. The incubation solution was renewed after 3 h. Control cultures were treated identically but incubated with L₁₅-BSA alone. As a control for the specificity of the 5-OHDA uptake and labeling, DMI (7 × 10⁻⁶ M) was present during the tracer incubation. Reserpine pretreatment of axons and cell bodies was carried out as described above. Before fixation, the axon chambers were rinsed once with L₁₅-BSA.

Glutaraldehyde fixation (3% in 0.1 M phosphate buffer, pH 7.4) was performed for 1 h at room temperature, followed by extensive washes in 0.1 M phosphate buffer containing 5% sucrose, postfixation in 1% OsO₄, block staining in uranyl acetate, and embedding in Epon 812. The bichromate-osmium tetroxide fixation was made as described by Tranzer and Richards (62): Briefly, cultures were treated on ice with ice-cold 1% glutaraldehyde, 0.4% formalin in a sodium chromate-potassium bichromate buffer, 0.1 M, pH 7.2 for 3 min, and then washed and stored overnight in 0.2 M sodium chromate-potassium bichromate buffer, pH 6.0, at 4°C. OsO₄ (2%) impregnation was performed for 1 h at 4°C in chromate-dichromate buffer, 0.1 M, pH 7.2, followed by washes in acetate buffer, dehydration, and embedding in Epon 812.

Thin sections were cut from the axons or cell bodies parallel to the surface of the tissue culture dish and examined unstained or stained with uranyl acetate and lead citrate in a Zeiss EM 10 electron microscope.

RESULTS

Cultures

About 4 d after plating, the first axons penetrated the silicon grease barrier dividing the axon and cell body chambers. Within 2–3 wk, a dense network of fibers having many varicosities filled the axon chambers (Fig. 1*b*). No non-neuronal cells (Schwann cells or fibroblasts) were present in the axon chambers. In the cell body chambers, the neurons had a strong tendency to aggregate into variously-sized clumps. Rarely, a non-neuronal cell on the plastic surface could be seen serving as an attachment point for neurons or neuronal aggregates.

Uptake and Retrograde Transport of [³H]Noradrenaline: Pharmacological Characterization

The addition of [³H]NA (0.5 × 10⁻⁶ M) to the axon chambers was followed by uptake into the axons and varicosities and the appearance of radioactivity in the cell body chamber (proximal axons and cell bodies) within 6 h (Fig. 2). The uptake and transport of [³H]NA were blocked by the presence of a 400-fold excess of unlabeled NA or by the disruption of microtu-
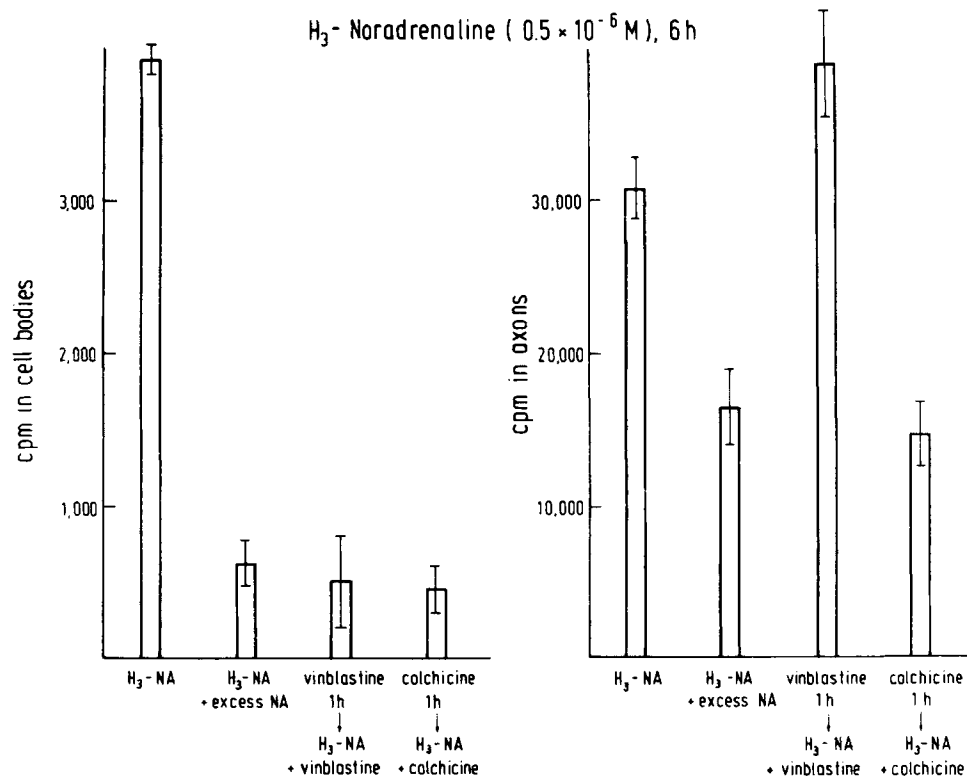

H₃- Noradrenaline (0.5 × 10⁻⁶ M), 6 h

FIGURE 2   Accumulation of radioactivity in axons and cell bodies of sympathetic neurons in chamber cultures during a 6-h exposure of their axons to [³H]NA (see Materials and Methods). Label in the cell body compartments (*left*) reflects the retrogradely transported [³H]NA. Specificity of uptake-transport is shown by adding a 400-fold excess of unlabeled NA. Vinblastine (10⁻⁵ M) or colchicine (10⁻⁶ M) blocked this retrograde transport. Values represent means ± SEM of three to four cultures.

bules by vinblastine or colchicine (Fig. 2). In contrast with colchicine, which also blocked the storage or uptake of [³H]- [NA] in the varicosities and axons, vinblastine showed a selective effect on the retrograde transport. (The higher nonspecific values [i.e., in the presence of excess unlabeled NA] for the axon chambers result largely because rigorous washing was not possible: the axons detach easily and would be lost.) Retrograde transport and accumulation of radioactivity in the cell body chamber could already be seen 1–2 h after adding [³H]NA (data not shown). Because the minimal distance between the uptake sites and the cell bodies was 2–3 mm, a minimal transport rate of 2–3 mm/h can be estimated. The mean axonal length, however, was ~8–12 mm, and the true transport velocity may therefore well be higher.

Inhibition of the NA-degrading enzymes, monoamine oxidase (MAO) and, in one experiment, also COMT by tranylcypromine and U-0521, respectively, led to a small, barely significant increase in the amounts of [³H]NA transported retrogradely (Fig. 6, MAO blockade). This result indicates that the transported radioactivity is [³H]NA rather than a product of its metabolism.

Between 0.1 and 1% of the total radioactivity added to the axons was found in the medium of the cell body chamber after 6 h of incubation. Whereas a small part of this radioactivity may represent [³H]NA or its metabolic products released from the cell bodies, the larger part originated from diffusion through the silicon grease seal. We showed that this leaked-[³H]NA did not contribute to the specific labeling of the nerve cell bodies by directly adding 0.5 × 10⁻⁸ M [³H]NA (the maximal concentration corresponding to that produced by leakage and release) to the chamber containing the nerve cell bodies. The radioactivity accumulated in the cell bodies after 6 h was <5% of the values achieved following retrograde transport.

Depolarization of the axons and varicosities by 54 mM K⁺ reproducibly led to a decrease in the amount of retrogradely transported [³H]NA, irrespective of whether the depolarization occurred 1 h before adding [³H]NA or continuously during the incubation with [³H]NA (Fig. 3). Wheat germ agglutinin (WGA), a lectin that binds to specific glycoproteins and glycolipids on the cell surface and that is taken up and transported in high amounts by these cells (M. Schwab and H. Thoenen, in preparation), seemed to slightly inhibit the retrograde accumulation of [³H]NA as well as the local accumulation in the axons (Fig. 3).

Two possibilities existed for the mechanism of the specific uptake of [³H]NA by varicosities and axons as a prerequisite for retrograde transport: (a) binding of [³H]NA to presynaptic α- or β-receptors (51) followed by internalizing the ligand-receptor complex; and (b) uptake by the DMI- and cocaine-sensitive NA pump in the plasma membrane and transfer into the terminal cytoplasm, followed by pumping into the vesicular storage compartments (30). Fig. 4 shows that neither yohimbine, a presynaptic α-blocker, nor L-propranolol, a blocker of β-receptors, abolished the retrograde transport of [³H]NA at concentrations that maximally block presynaptic adrenergic receptors (36, 51, 52). In fact, propranolol at 10⁻⁷ M enhanced the retrograde accumulation of [³H]NA. In contrast, DMI and cocaine, at the concentrations known to be effective from in vivo and in vitro experiments (29, 30, 44), effectively blocked the uptake of [³H]NA into the axons and the retrograde accumulation of [³H]NA in the cell bodies (Fig. 5). To our surprise, pretreatment of axons and cell bodies with reserpine (10⁻⁶ M, a saturating dose [8, 53]), a drug that selectively blocks the energy-dependent uptake of biogenic amines such as dopamine, noradrenaline, adrenaline, or serotonin from the cytoplasm into the vesicular storage compartments (8, 30, 31, 53), did not block the retrograde transport of [³H]NA (Fig. 6). The decrease observed in some experiments varied from insignificant to a maximal value of 40%, but was never total. In the same cultures, the terminal and axonal storage of [³H]NA was completely abolished by reserpine (Fig. 6). Identical results
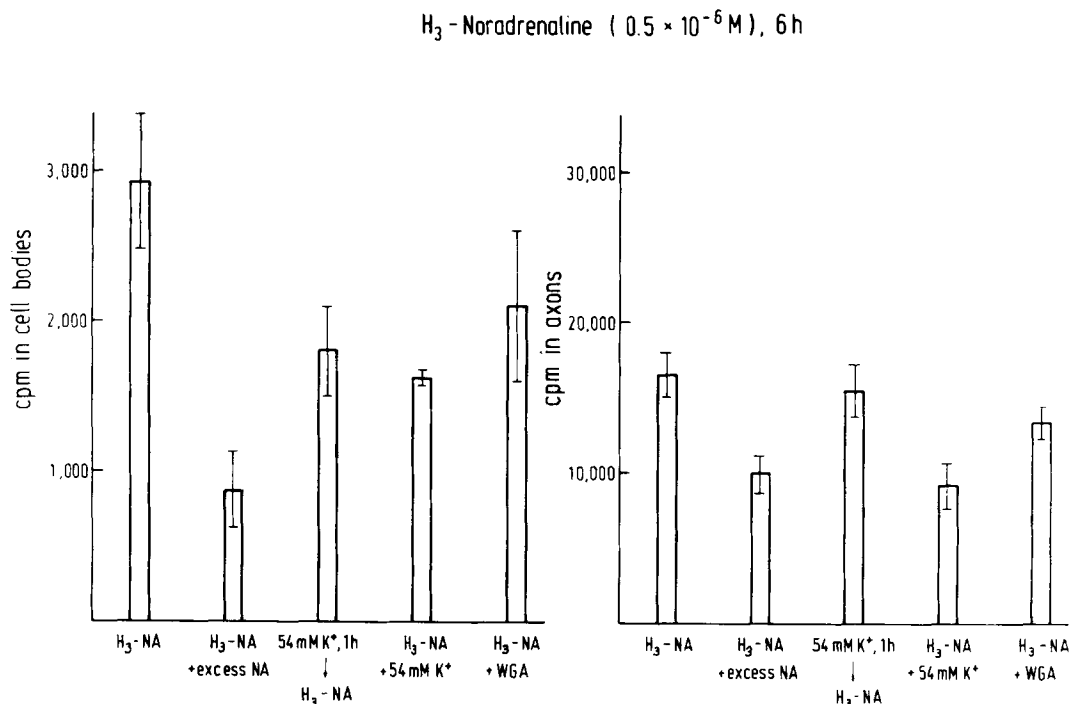
H_3 - Noradrenaline (0.5×10^{-6} M), 6h

FIGURE 3 Decrease of the retrograde transport of [³H]NA to the cell body chamber (left) by depolarizing the axons 1 h before or during the exposure to [³H]NA, or by wheat germ agglutinin (50 μg/ml). Values represent means ± SEM of three to four cultures.
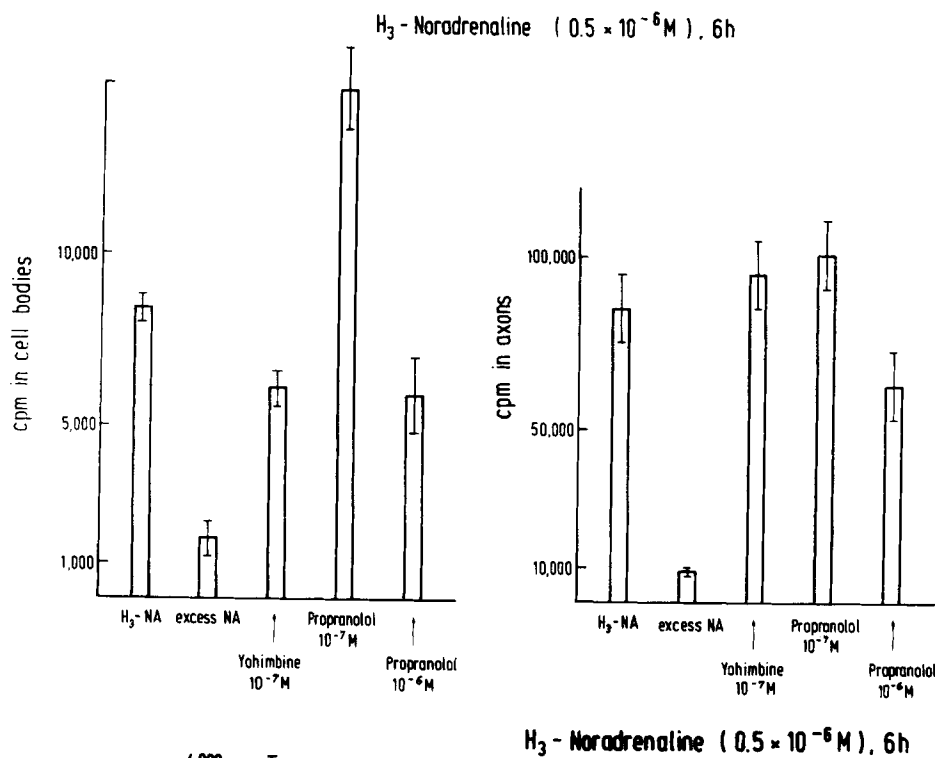
H₃ - Noradrenaline (0.5 × 10⁻⁶M), 6h

cpm in cell bodies

10,000

5,000

1,000

H₃- NA   excess NA   Propranolol 10⁻⁷M
Yohimbine 10⁻⁷M   Propranolol 10⁻⁶M

cpm in axons

100,000

50,000

10,000

H₃- NA   excess NA   Propranolol 10⁻⁷M
Yohimbine 10⁻⁷M   Propranolol 10⁻⁶M

FIGURE 4 Effects of presynaptic α- or β-adrenergic blockers on axonal uptake-storage (right) and retrograde transport (left) of [³H]NA. Drugs were applied to the axons 15 min before, as well as during, the incubation with [³H]-NA. Values represent means ± SEM of three to four cultures.
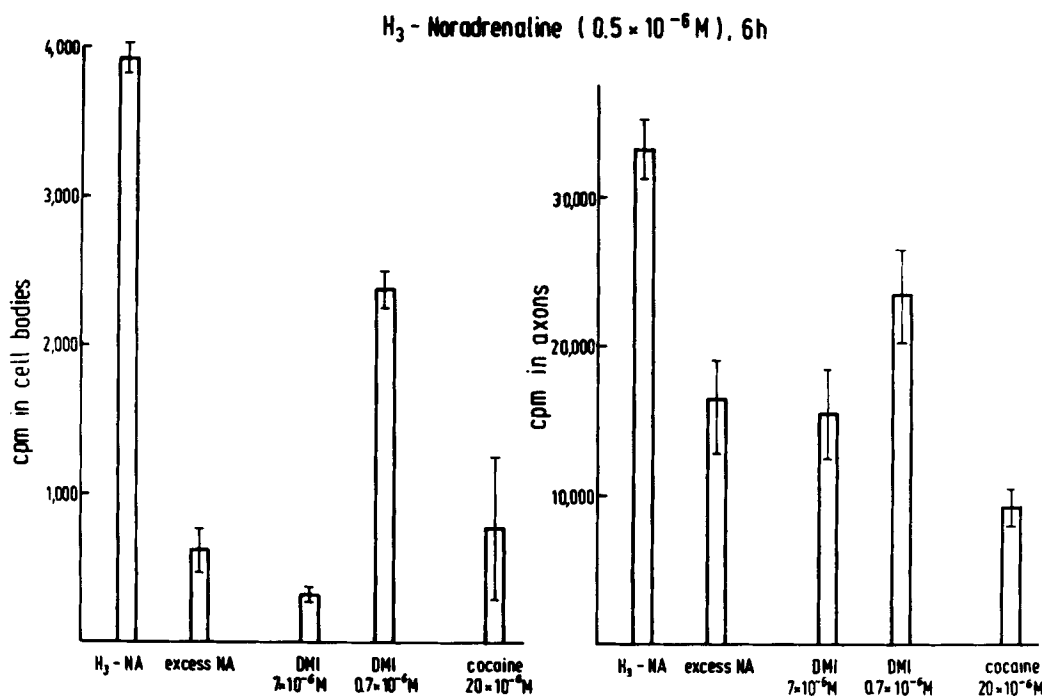
H₃ - Noradrenaline (0.5 × 10⁻⁶M), 6h

cpm in cell bodies

4,000

3,000

2,000

1,000

H₃ - NA   excess NA   DMI 7×10⁻⁶M   DMI 0.7×10⁻⁶M   cocaine 20×10⁻⁶M

cpm in axons

30,000

20,000

10,000

H₃ - NA   excess NA   DMI 7×10⁻⁶M   DMI 0.7×10⁻⁶M   cocaine 20×10⁻⁶M

FIGURE 5 Blockade of axonal uptake (right) and retrograde transport (left) of [³H]NA by DMI or cocaine. Drugs were added together with [³H]NA. Values represent means ± SEM of three to four cultures.

were also obtained with a higher concentration of reserpine (10^{-5} M). Additional reserpine treatment of the neuronal cell bodies during the incubation with [³H]NA to inactivate all newly synthesized vesicles did not influence the retrograde transport either (data not shown). These results were not influenced by pretreatment with the MAO inhibitor, tranylcypromine. Increasing the concentration of [³H]NA in the axon chambers to 1.5×10^{-6} M caused only a minor increase in the retrograde accumulation of [³H]NA in the nerve cell bodies, although the axonal labeling was greatly enhanced (Fig. 6). This result seems to indicate a saturation of the retrograde transport compartment, but, because the radioactivity present

in the nerve cell bodies probably resulted from an equilibrium between the retrograde accumulation and subsequent metabolism or release, this interpretation has to be viewed with caution.

Electron Microscopic Observations

Nontreated glutaraldehyde-fixed cultures showed the healthy appearance of the neurons under the present culture conditions. Most fibers in the axon chambers had many varicosities, showing bundles of microtubules and the total absence of ribosomes. Axosomatic and axodendritic typical asymmetric synapses were present in the cell body chambers.
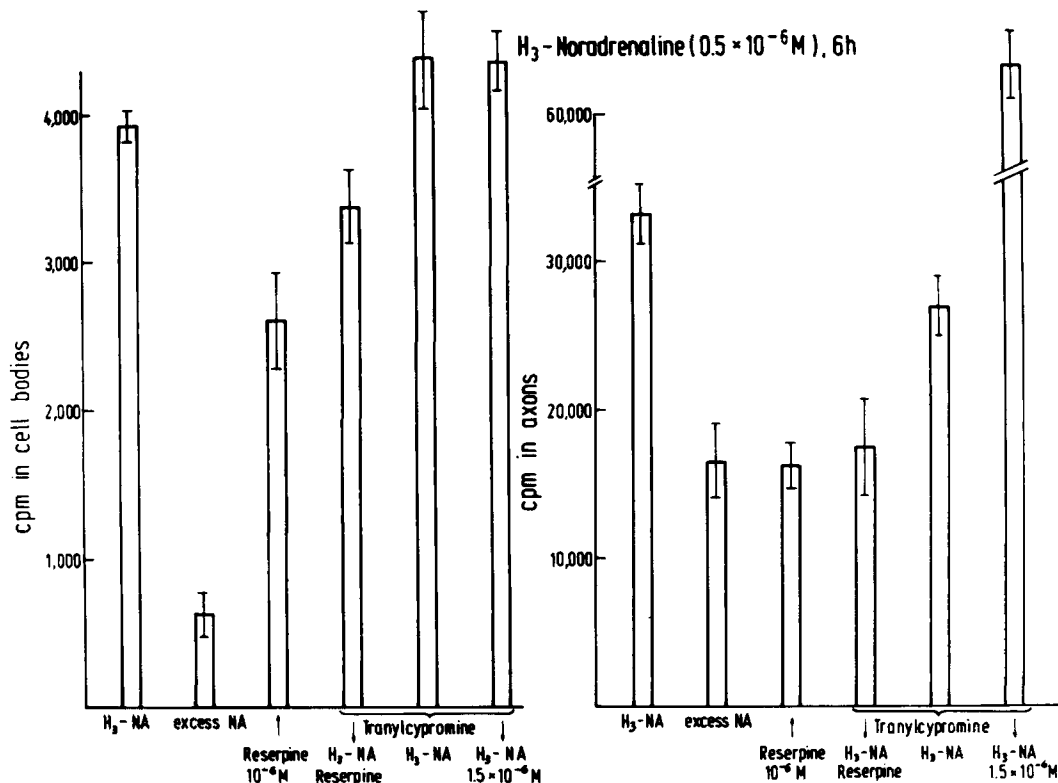
FIGURE 6 Effects of reserpine and tranylcypromine on retrograde transport and axonal accumulation of [³H]NA. Pretreatment (45 min) with reserpine (10⁻⁶ M) with or without tranylcypromine totally abolished the storage of [³H]NA in the axons (*right*) but had only a minor effect on the [³H]NA transported retrogradely (*left*). Increasing the concentration of [³H]NA to 1.5 × 10⁻⁶ M indicated saturability of the retrograde transport compartment. Values are means ± SEM of three to four cultures.

The bichromate-osmium tetroxide fixation for demonstrating catecholamines (62) revealed endogenous NA and/or dopamine as dense-cores mainly in small and large vesicles. In axons and cell bodies, small dense-core vesicles were less frequent, whereas in varicosities and synapses, most of the vesicles were small. In cultures older than 3 wk, varicosities and synapses fell into three groups according to the presence of dense-core vesicles: (a) terminals in which a majority of small vesicles had dense-cores; (b) the majority of terminals in which only some (5–20%) small vesicles had dense-cores; and (c) terminals with only clear vesicles. This observation, together with the biochemical demonstration of tyrosine hydroxylase activity as well as significant amounts of choline acetyltransferase in these cultures (R. Heumann, H. Gnahn, and M. Schwab, unpublished observations), indicate the presence of some largely adrenergic, some largely cholinergic, and a majority of dual-function neurons (35, 41) under the present culture conditions.

Incubation of the axons with 5-OHDA (5 × 10⁻⁴ M), a false transmitter that mimics the behavior of NA (but has a lower affinity for the amine uptake system) (60) for 6 h, resulted in the appearance of dense-cores in virtually all the small and large vesicles in the axonal varicosities in bichromate-osmium-fixed cultures (Fig. 7a). DMI abolished the 5-OHDA labeling, showing that it resulted exclusively from the specific uptake of 5-OHDA by the neuronal amine uptake mechanism, in spite of the high concentration of 5-OHDA used. (Glutaraldehyde fixation was not totally adequate for demonstrating all of these dense-cores. In most of the 5-OHDA experiments we therefore used bichromate-osmium tetroxide fixation, in spite of the poorer ultrastructural preservation.) Many neurons in long-term cultures in the presence of rat serum synthesized acetyl-

choline in addition to, or instead of catecholamines, but nevertheless retained the ability for uptake and vesicular storage of NA or 5-OHDA. These results confirmed the detailed descriptions of Landis (35), Wakshull et al. (66), and Reichardt and Patterson (43). Tubular or reticulum-like structures containing 5-OHDA were occasionally seen, in both axons and varicosities. In the cell body chamber, the retrogradely transported 5-OHDA was reflected by an increase in the frequency of large dense-core vesicles mainly in the axons (Fig. 7b). However, the presence of endogenous catecholamines made a clear interpretation of this experimental condition difficult.

Because the biochemical experiments showed that the retrograde transport of NA is at the most influenced only slightly by reserpine, axons and cell bodies were pretreated with reserpine. This resulted in an almost total disappearance of the dark dense-cores characteristic of endogenous catecholamines in small and large vesicles (Fig. 8). Some large vesicles containing pale dense-cores, probably of protein, glycoprotein, or peptide nature, could still be observed.

If 5-OHDA was added for 6 h to the axons of reserpine-pretreated cultures, a very clear picture emerged: in the nonvaricose parts of the axons, irrespective of their localization in the cell body or axonal chamber, the majority of the labeled organelles were large dense-core vesicles of a diameter of 700–1200 Å (Fig. 9a, Table I). In addition, some small dense-core vesicles and elongated vesicles contained 5-OHDA, whereas none of the larger cisternae, sER-like structures, or multivesicular or other prelysosomal bodies did. In the varicosities, some small vesicles contained dense-cores in addition to the labeled large vesicles (Fig. 9b). Their number was, however, lower than expected from the total proportion of small versus large vesicles (Table I). The number of labeled vesicles per varicosity
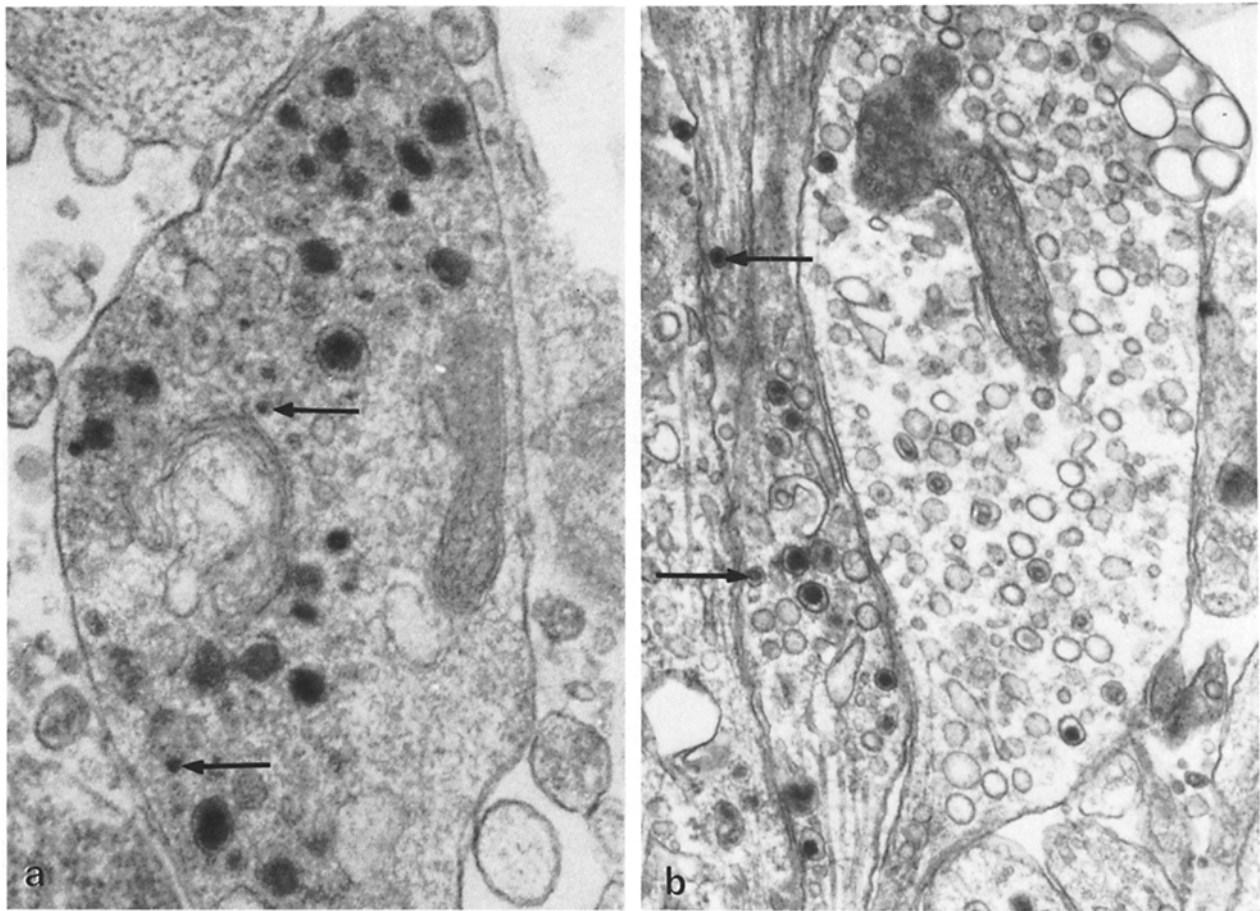
FIGURE 7 (a) Electron micrograph of a varicosity in an axon chamber after incubation with 5-OHDA. Most of the small (arrows) and large vesicles show the typical dense cores produced by 5-OHDA. Bichromate-osmium tetroxide fixation, × 60,000. (b) Axons and varicosity in the cell body chamber showing large and small (arrows) dense-core vesicles transporting 5-OHDA retrogradely from the axon chamber. Glutaraldehyde fixation. × 25,000.
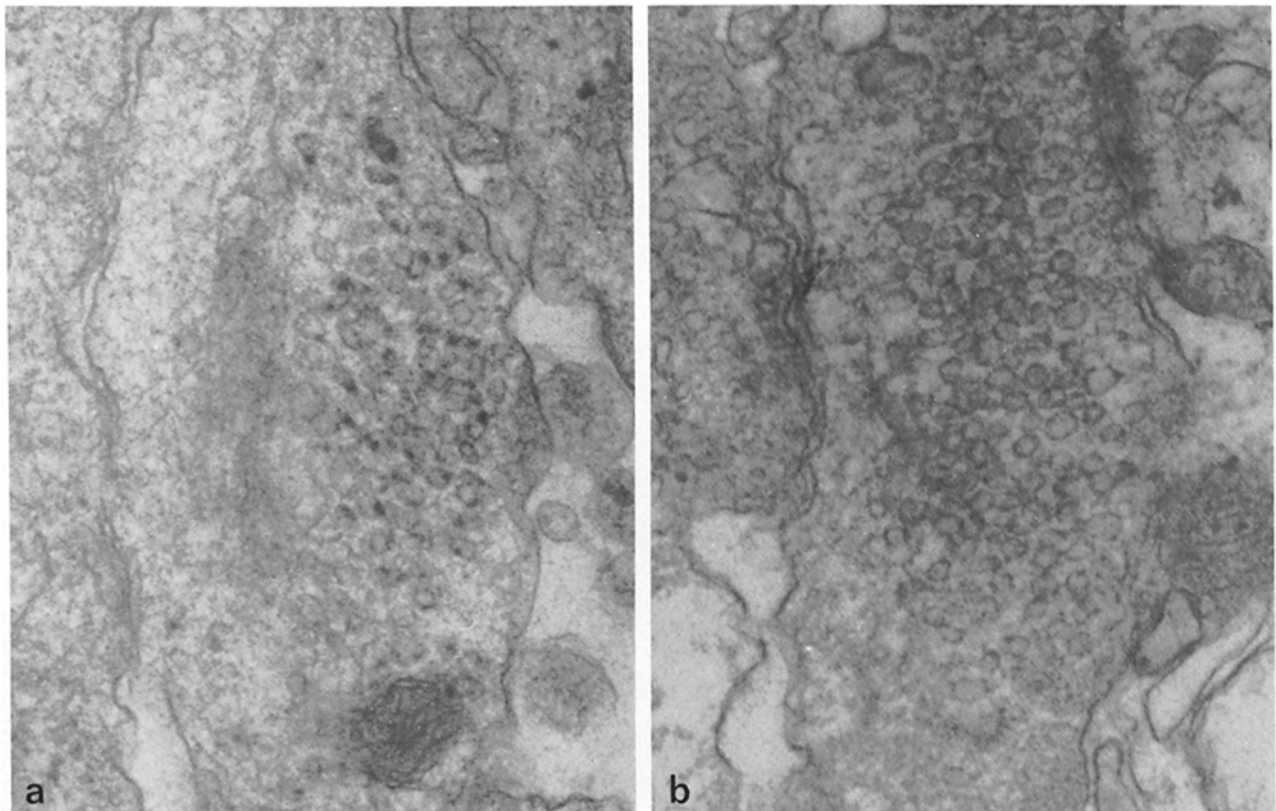

FIGURE 8 (a) Endogenous NA or dopamine in a synapse forms small dense-cores after bichromate-osmium tetroxide fixation. × 56,000. (b) Disappearance of these dense cores by treatment with reserpine. × 74,000.
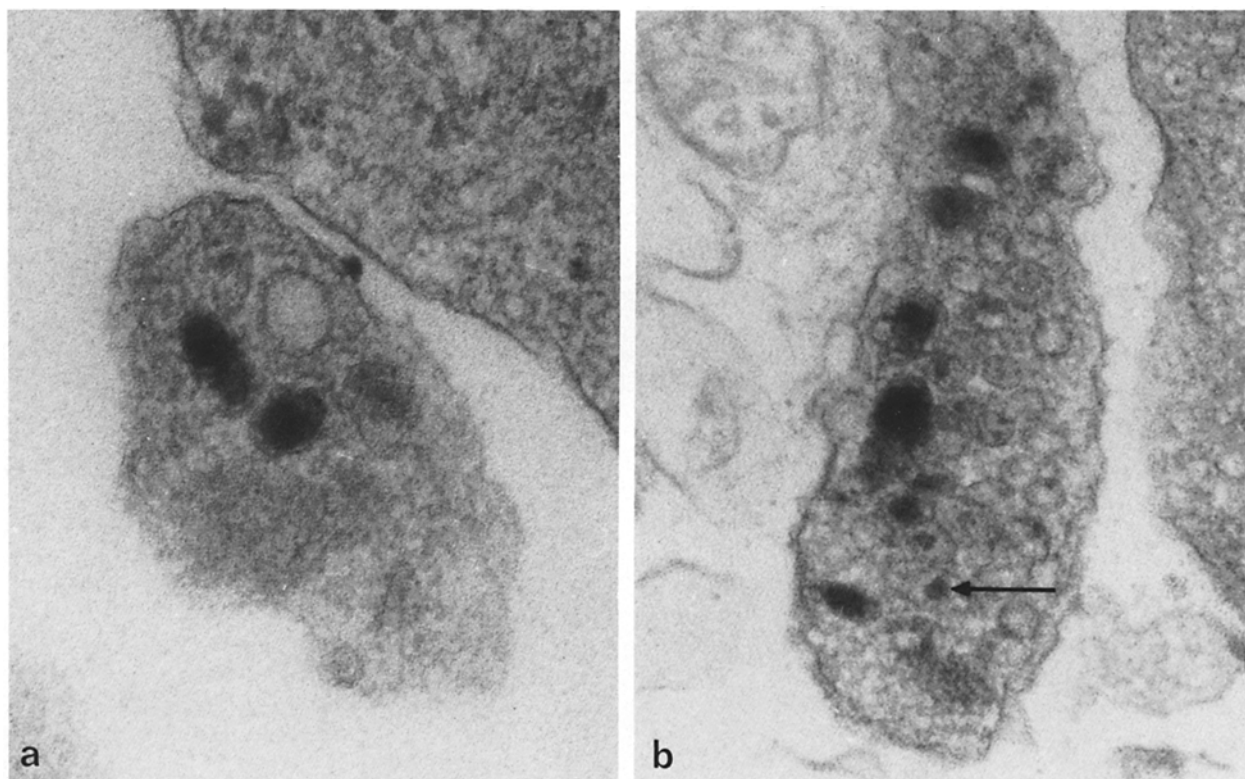
1544

FIGURE 9   Reserpine-treated cultures incubated with 5-OHDA. (a) Axon with two large dense-core vesicles transporting 5-OHDA, × 124,000. (b) Varicosity with several large and small (arrow) dense-core vesicles containing 5-OHDA. Bichromate-osmium tetroxide fixation, × 117,000.

TABLE I

Organelles Labeled by 5-OHDA in Axons and Varicosities of Reserpine-pretreated Cultures of Sympathetic Neurons

| | Percent of all labeled organelles | | | | |
	Large dense-core vesicles	Small dense-core vesicles	Elongate vesicles	Cultures analyzed	Total No. of labeled organelles
*Axon chambers					
Varicosities	36 ± 13	60 ± 14.5	4 ± 1.7	3	361
Axons	80 ± 3.6	17 ± 2	3 ± 2	3	117
Cell body chambers					
Axons	62 ± 6.5	36 ± 5	2 ± 2	3	110
‡Percent of total vesicle population					
Varicosities	11 ± 1.2	89 ± 1.2		3	1790
Axons	43 ± 1.5	57 ± 2.5		4	405

* 5-OHDA (5×10^{-4} M, 6 h) was applied to the axon chamber only.
‡ Including vesicles labeled and unlabeled, dense-cored and empty.

was generally small but quite variable.

In the cell bodies the only labeled organelles found were large dense-core vesicles. Cell body labeling was relatively low altogether, suggesting a rapid transformation of the transport organelles (e.g., by fusion with lysosomal or Golgi compartments) leading to the loss of the transported storage complex visible by electron microscopy.

DISCUSSION

Virtually all the agents so far shown to be transported by retrograde axonal transport have been macromolecular: material endogenous to the cell returning to the cell body from the axons and nerve terminals, or macromolecules taken up from the extracellular space (23, 47). One of the few exceptions is found in the brain, where some established or putative neuro-

transmitters (dopamine, serotonin, glycine, GABA, aspartate, glutamate, and acetylcholine) are accumulated by retrograde transport in the cell bodies of neurons using the corresponding, or a closely related molecule, as transmitter (1, 12). The reasons for this selectivity, and above all the compartments in which these low molecular weight substances are able to undergo retrograde axonal transport, remain unknown.

Using a chamber culture system that allowed separate access to the axons and cell bodies of dissociated sympathetic neurons in the absence of non-neuronal or target cells (7), we have been able to characterize the uptake and retrograde axonal transport of [³H]NA and 5-OHDA with regard to their pharmacological and morphological properties. [³H]NA or 5-OHDA applied to the axons and varicosities was taken up by the DMI- and cocaine-sensitive amine carrier in the plasma membrane and transferred to two different intracellular storage compartments:

a reserpine-sensitive one that comprised the large majority of the small vesicles seen under the electron microscope in the varicosities, but that also contributed some large dense-core vesicles, and a reserpine-insensitive compartment. The latter was mainly responsible for the retrograde transport to the cell bodies. Electron microscopic localization of 5-OHDA revealed mostly large dense-core vesicles but also some small dense-core vesicles as this reserpine-resistant transport organelle. The retrograde transport velocity for [³H]NA is fast (minimally 2–3 mm/h) and the retrograde transport was totally abolished by the classical blockers of fast anterograde and retrograde transport, the microtubule-disrupting agents vinblastine and colchicine. These results exclude passive intra-axonal diffusion of the amines as a possible transport mechanism. Since blocking the NA-degrading enzymes did not significantly influence our results, the transported radioactivity probably represents intact [³H]NA. Blockade of presynaptic α- and β-receptors by yohimbine and L-propanolol, respectively (51), did not decrease the retrograde transport of [³H]NA, indicating that the internalization of [³H]NA after binding to α- or β-receptors and subsequent receptor down-regulation is not how [³H]NA is taken up and transported (10, 22, 47). Interestingly, the stimulation of NA release by 54 mM K^+ decreased the accumulation of retrogradely transported [³H]NA, whereas 10^{-7} M L-propranolol increased the retrograde accumulation of [³H]NA. The stimulation of β-receptors on adrenergic nerve terminals leads to an increase in the subsequent release of NA, and L-propranolol, by blocking this positive feedback loop, has been shown to lead to a decrease of NA release (36, 51). Whether the present results reflect the specific labeling of various intracellular pools of NA and how these pools are related to retrograde transport is unknown.

Reserpine-resistant compartments for NA have been observed repeatedly in pharmacological studies (3, 18, 20, 30, 57). Because it was assumed that all storage vesicles are equal in their reserpine-sensitivity, these results were usually attributed to neuronal extravesicular binding sites. In the reserpine-treated perfused rabbit heart, this compartment was shown to be saturable and to take up NA with a higher affinity than the cocaine-sensitive pump in the plasma membrane (3). The present results clearly show that a reserpine-resistant compartment is responsible for the retrograde transport of [³H]NA and 5-OHDA, which is represented by a subpopulation of large vesicles (700–1200 Å) that, upon loading with 5-OHDA, become dense-cored. In addition, a few small vesicles (300–600 Å) in the varicosities (and rarely also in axons) are consistently resistant to reserpine pretreatment. These results confirm two earlier reports of reserpine-resistant retrograde transport of biogenic amines in the central nervous system (1, 64). Dopamine injected into the caudate nucleus of reserpine-treated rats accumulated rapidly in the dopaminergic cell bodies of the substrate nigra (64), and the fast, colchicine-sensitive retrograde transport of [³H]serotonin from the olfactory bulb to the raphe nuclei was diminished by only 40% by reserpine pretreatment (1). In contrast, no retrograde accumulation of [³H]serotonin was detectable after blockade of the neuronal uptake with fluoxetine, analogous to our results with DMI and cocaine in the adrenergic neurons (1).

The membrane of amine storage vesicles in adrenal medulla and adrenergic nerves is characterized by an energy-dependent carrier capable of transporting amines (e.g., dopamine, adrenaline, and serotonin) from the cytoplasm into the vesicles against a steep concentration gradient (30, 32–34, 68). This amine carrier is inactivated by reserpine (8, 31, 53). Because

reserpine is highly lipophilic, this inactivation is irreversible under physiological conditions (31). In addition, there is an ATP carrier also driven by the proton gradient that is produced by a Mg^{2+}-activated ATPase (68). ATP, together with Ca^{++}, have an important function in forming the intravesicular storage complex for the amines (8, 34, 68). These components are essential for an organelle able to store and transport [³H]NA or 5-OHDA. Dopamine β-hydroxylase is another important component of adrenal medullary granules and at least the large vesicles from adrenergic nerves (33, 34, 68). All these macromolecular constituents of synaptic vesicle membranes are synthesized in the cell bodies and supplied to the terminals by fast axonal transport (23). In the case of adrenergic nerves, indications for their return to the cell body by retrograde transport came from two different observations: Early in the study of axonal transport accumulation of endogenous NA was seen not only proximal but also distal to nerve ligations (13). Later it was demonstrated that dopamine-β-hydroxylase also accumulates distal to ligations (4, 40) and that antibodies to dopamine-β-hydroxylase are taken up selectively by adrenergic nerve terminals and transported by retrograde transport to the corresponding cell bodies (19, 48, 69). Most interestingly, a large part of this retrogradely transported dopamine-β-hydroxylase is enzymatically inactive, in contrast with the anterogradely transported enzyme in the same axons (40). This indicates that local modifications of components of synaptic vesicles occur in the nerve terminals and may be a direct analogy to our finding of resistance to reserpine. Instead of assuming a vesicle type with amine transport characteristics totally different from the classical reserpine-sensitive ones, loss of reserpine sensitivity could be the result of a local modification of the vesicle membrane. Strong indications for such local modifications of axonal proteins in target regions were revealed by a two-dimensional gel electrophoresis analysis of fast-transported proteins in the rabbit optic system (45). A protein (protein C, apparent mol wt 20,000) was found to shift its pI by 1 pH U in the lateral geniculate nucleus and by 2 pH U in the optic tectum as compared with its pI in the optic nerve. Such local modifications are attractive candidates for a signal for retrograde transport. What determines why a particular vesicle or organelle is transported anterogradely or retrogradely in an axon has remained enigmatic up to now.

In accordance with in vivo and in vitro observations (56, 61) we also found that large dense-core vesicles are not the only organelles in the axons containing catecholamines or capable of accumulating them. Small dense-core vesicles and elongated and tubular structures are also present, although in the varicosities small vesicles clearly represent the most common amine storage compartments (90% in our cultured cells). Formation of vesicles by budding from reticular or tubular structures, or formation of small vesicles from the membrane of large dense-core vesicles has been suggested mainly on the basis of electron microscopic observations (49, 56, 61). In the case of the large dense-core vesicles engaged in retrograde transport shown in this study, their origin in the nerve terminals could not be decided.

Extracellular macromolecules taken up by nerve terminals are transported retrogradely in large cisternae, tubular structures, or cup-shaped or multivesicular prelysosomal bodies (2, 6, 11, 23, 37, 46, 47, 63). This has been observed in many in vivo and in vitro situations, and is also true for sympathetic neurons growing in chamber cultures (9; [M. Schwab and H. Thoenen, in preparation]). These compartments are clearly different from the vesicles transporting NA or 5-OHDA, re-

flecting their different origin and function and probably also the different biochemical properties of their surrounding membranes. We would like to conclude that the vesicles transporting NA and 5-OHDA represent synaptic vesicle membrane returning to the cell body in the process of synaptic vesicle turnover.

REFERENCES

1. Araneda, S., P. Bobillier, P. Buda, and J. F. Pujol. 1980. Retrograde axonal transport following injection of ³H-serotonin in the olfactory bulb. I. Biochemical study. *Brain Res.* 196:405–415.
2. Birks, R. I., M. C. MacKey, and P. R. Weldon. 1972. Organelle formation from pinocytotic elements in neurites of cultured sympathetic ganglia. *J. Neurocytol.* 1:311–340.
3. Boenisch, H., and K. H. Graefe. 1977. Saturation kinetics of uptake and of the neuronal steady-state accumulation of ³H-(−)noradrenaline in the reserpine-pretreated rabbit heart. *Naunyn-Schmiedeberg's Arch. Pharmacol.* 297:R48a (Abstr.).
4. Brimijoin, S., and L. Helland. 1976. Rapid retrograde transport of dopamine-β-hydroxylase as examined by the stop-flow technique. *Brain Res.* 102:217–228.
5. Broadwell, R. D., C. Oliver, and M. W. Brightman. 1980. Neuronal transport of acid hydrolases and peroxidase within the lysosomal system of organelles: involvement of reticulum-like cisterns. *J. Comp. Neurol.* 190:519–532.
6. Bunge, M. B. 1977. Initial endocytosis of peroxidase or ferritin by growth cones of cultured nerve cells. *J. Neurocytol.* 6:407–439.
7. Campenot, R. B. 1979. Independent control of the local environment of somas and neurites. *Methods Enzymol.* 58:302–307.
8. Carlsson, A., N. A. Hillarp, and B. Waldeck. 1962. A Mg⁺⁺-ATP dependent storage mechanism in the amine granules of the adrenal medulla. *Med. Exp.* 6:47–53.
9. Chan, K. Y., A. H. Bunt, and R. H. Haschke. 1981. In vitro retrograde neuritic transport of horseradish peroxidase isoenzymes by sympathetic neurons. *Neuroscience.* 6:59–69.
10. Chuang, D. M., and E. Costa. 1979. Evidence for the internalization of the recognition site of β-adrenergic receptors during receptor subsensitivity induced by (−)-isoproterenol. *Proc. Natl. Acad. Sci. USA.* 76:3024–3028.
11. Chu-Wang, I. W., and R. W. Oppenheim. 1980. Uptake, intraaxonal transport and fate of horseradish peroxidase in embryonic spinal neurons of the chick. *J. Comp. Neurol.* 193:753–776.
12. Cuénod, M., P. Bagnoli, A. Beaudet, A. Rustioni, L. Wiklund, and P. Streit. 1982. Transmitter specific retrograde labeling of neurons. *In* Cytochemical Methods in Neuroanatomy. S. L. Palay and P. Chan-Palay, editors. Alan R. Liss, Inc., New York. 17–44.
13. Dahlström, A. 1965. Observations on the accumulation of NA in the proximal and distal parts of peripheral adrenergic nerves after compression. *J. Anat.* 99:677–689.
14. Dahlström, A. 1971. Axoplasmic transport. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 261:325–358.
15. de Camilli, P., S. M. Harris, W. B. Huttner, and P. Greengard. 1983. Synapsin I (protein I), a nerve terminal-specific phosphoprotein. II. Its specific association with synaptic vesicles demonstrated by immunocytochemistry in agarose-embedded synaptosomes. *J. Cell Biol.* 96:1355–1373.
16. Droz, P. 1969. Protein metabolism in nerve cells. *Int. Rev. Cytol.* 25:363–390.
17. Droz, B., H. Koenig, and L. DiGiamberardino. 1973. Axonal migration of protein and glycoproteins to nerve endings. I. Radioautographic analysis of renewal of protein in nerve endings of chicken ciliary ganglion after intracerebral injection of ³H-lysine. *Brain Res.* 60:93–127.
18. Enna, S. J., and D. A. Shore. 1974. On the nature of the adrenergic neuron extragranular amine binding site. *J. Neural Transm.* 35:125–135.
19. Fillenz, M., C. Gagnon, K. Stöckel, and H. Thoenen. 1976. Selective uptake and retrograde axonal transport of dopamine-β-hydroxylase antibodies in peripheral adrenergic neurons. *Brain Res.* 114:293–303.
20. Giachetti, A., and R. A. Hollenbeck. 1976. Extravesicular binding of noradrenaline and guanethidine in the adrenergic neurones of the rat heart: a proposed site of action of adrenergic neurone blocking agents. *Br. J. Pharmacol.* 58:497–504.
21. Goldman, J. E., K. S. Kim, and J. H. Schwartz. 1976. Axonal transport of [³H]serotonin in an identified neuron of *Aplysia Californica*. *J. Cell Biol.* 70:304–318.
22. Goldstein, J. L., R. G. W. Anderson, and M. S. Brown. 1979. Coated pits, coated vesicles, and receptor-mediated endycytosis. *Nature Lond.* 279:679–685.
23. Grafstein, B., and D. S. Forman. 1980. Intracellular transport in neurons. *Physiol. Rev.* 60:1167–1283.
24. Hawrot, E., and P. H. Patterson. 1979. Long-term culture of dissociated sympathetic neurons. *Methods Enzymol.* 58:574–584.
25. 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.
26. Heuser, J. E., and T. S. Reese. 1981. Structural changes after transmitter release at the frog neuromuscular junction. *J. Cell Biol.* 88:564–580.
27. Holtzman, E. 1976. Lysosomes, a survey. *In* Cell Biology Monographs. Springer-Verlag, Berlin. 3.
28. Holtzman, E. 1977. The origin and fate of secretory packages, especially synaptic vesicles. *Neuroscience.* 2:327–355.
29. Iversen, L. L. 1963. The uptake of noradrenaline by the isolated perfused rat heart. *Brit. J. Pharmacol.* 21:523–537.
30. Iversen, L. L. 1967. The Uptake and Storage of Noradrenaline in Sympathetic Nerves.

Cambridge University Press, Cambridge.
31. Kanner, B. I., H. Fishkes, R. Maron, I. Sharon, and S. Schuldiner. 1979. Reserpine as a competitive and reversible inhibitor of the catecholamine transporter of bovine chromaffin granules. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 100:175–178.
32. Kanner, B. I., I. Sharon, R. Maron, and S. Schuldiner. 1980. Electrogenic transport of biogenic amines in chromaffin granule membrane vesicles. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 111:83–86.
33. Klein, R. L., and H. Lagercrantz. 1981. Noradrenergic vesicles: composition and function. *In* Chemical Neurotransmission. L. Stjärne, P. Hedquist, H. Lagercrantz, and A. Wennmalm, editors. Academic Press, New York. 69–83.
34. Lagercrantz, H. 1976. On the composition and function of large dense cored vesicles in sympathetic nerves. *Neuroscience.* 1:81–92.
35. Landis, S. C. 1980. Developmental changes in the neurotransmitter properties of dissociated sympathetic neurons: a cytochemical study of the effects of medium. *Dev. Biol.* 77:349–361.
36. Langer, S. Z., M. L. Dubocovich, and S. M. Laluch. 1975. Prejunctional regulatory mechanisms for noradrenaline release elicited by nerve stimulation. *In* Chemical Tools in Catecholamine Research. O. Almgren, A. Carlsson, and J. Engel, editors. North Holland Publishing Co., Amsterdam. 2:183–191.
37. LaVail, J. H., S. Rapisardi, and I. K. Sugino. 1980. Evidence against the smooth endoplasmic reticulum as a continuous channel for retrograde axonal transport of horseradish peroxidase. *Brain Res.* 191:3–20.
38. Lentz, T. L., and J. Chester. 1982. Synaptic vesicle recycling at the neuromuscular junction in the presence of a presynaptic membrane marker. *Neuroscience.* 7:9–20.
39. Mains, R. E., and P. H. Patterson. 1973. Primary cultures of dissociated sympathetic neurons. I. Establishment of long-term growth in culture and studies of differentiated properties. *J. Cell Biol.* 59:329–345.
40. Nagatsu, I., Y. Kondo, T. Kato, and T. Nagatsu. 1976. Retrograde axoplasmic transport of inactive dopamine-β-hydroxylase in sciatic nerves. *Brain Res.* 116:277–285.
41. Patterson, P. H. 1978. Environmental determination of autonomic neurotransmitter functions. *Annu. Rev. Neurosci.* 1:1–17.
42. Price, C. H., and D. J. McAdoo. 1981. Localization of axonally transported ³H-glycine in vesicles of identified neurons. *Brain Res* 219:307–315.
43. Reichardt, L. F., and P. H. Patterson. 1977. Neurotransmitter synthesis and uptake by isolated sympathetic neurones in microcultures. *Nature Lond.* 270:147–151.
44. Rehavi, M., P. Skolnick, M. J. Brownstein, and S. M. Paul. 1982. High-affinity binding of ³H-desipramine to rat brain: a presynaptic marker for noradrenergic uptake sites. *J. Neurochem.* 38:889–895.
45. Schick Kelly, A., J. A. Wagner, and R. B. Kelly. 1980. Properties of individual nerve terminal proteins identified by two-dimensional gel electrophoresis. *Brain Res.* 185:192–197.
46. Schwab, M. E., K. Suda, and H. Thoenen. 1979. Selective retrograde transsynaptic transfer of a protein, tetanus toxin, subsequent to its retrograde axonal transport. *J. Cell Biol.* 82:798–810.
47. Schwab, M. E., and H. Thoenen. 1983. Retrograde axonal transport. *In* Handbook of Neurochemistry. Second ed. A. Lajtha, editor. Plenum Press, New York. 5.
48. Silver, M. A., and D. M. Jacobowitz. 1979. Specific uptake and retrograde flow of antibody to dopamine-β-hydroxylase by central nervous system noradrenergic neurons in vivo. *Brain Res.* 167:65–75.
49. Smith, A. D. 1973. Mechanisms involved in the release of noradrenaline from sympathetic nerves. *Br. Med. Bull.* 29:123–129.
50. Smith, R. S. 1980. The short term accumulation of axonally transported organelles in the region of localized lesions of single myelinated axons. *J. Neurocytol.* 9:39–65.
51. Starke, K. 1977. Regulation of noradrenaline release by presynaptic receptor systems. *Rev. Physiol. Biochem. Pharmacol.* 77:1–124.
52. Starke, K., and T. Endo. 1976. Presynaptic α-adrenoceptors. *Gen. Pharmacol.* 7:307–312.
53. Stjärne, L. 1964. Studies of catecholamine uptake, storage and release mechanisms. *Acta Physiol. Scand.* 62:228. (Suppl.).
54. Suda, K., Y. A. Barde, and H. Thoenen. 1978. Nerve growth factor in mouse and rat serum: correlation between bioassay and radioimmunoassay determinations. *Proc. Natl. Acad. Sci. USA* 75:4042–4046.
55. Taxi, J., and C. Sotelo. 1973. Cytological aspects of the axonal migration of catecholamines and of their storage material. *Brain Res.* 62:431–437.
56. Teichberg, S., and E. Holtzman. 1973. Axonal agranular reticulum and synaptic vesicles in cultured embryonic chick sympathetic neurons. *J. Cell Biol.* 57:88–108.
57. Thoa, N. B., G. F. Wooten, J. Axelrod, and I. J. Kopin. 1975. On the mechanism of release of norepinephrine from sympathetic nerves induced by depolarizing agents and sympathomimetic drugs. *Mol. Pharmacol.* 11:10–18.
58. Thoenen, H., and G. W. Kreutzberg. 1981. The role of fast transport in the nervous system. *Neurosci. Res. Program Bull.* 20:1–138.
59. Tomlinson, D. R. 1975. Two populations of granular vesicles in constricted postganglionic sympathetic nerves. *J. Physiol. (Lond.).* 245:727–735.
60. Tranzer, J. P., and H. Thoenen. 1967. Electron microscopic localisation of 5-hydroxydopamine (3, 4, 5-trihydroxyphenyl-ethylamine), a new false sympathetic transmitter. *Experientia (Basel).* 23:743–745.
61. Tranzer, J. P. 1972. A new amine storing compartment in adrenergic axons. *Nat. New Biol.* 237:57–58.
62. Tranzer, J. P., and J. G. Richards. 1976. Ultrastructural cytochemistry of biogenic amines in nervous tissue: methodologic improvements. *J. Histochem. Cytochem.* 24:1178–1193.
63. Tsukita, S., and H. Ishikawa. 1980. The movement of membranous organelles in axons. Electron microscopic identification of anterogradely and retrogradely transported organelles. *J. Cell Biol.* 84:513–530.
64. Ungerstedt, U., L. L. Butcher, S. G. Butcher, W.-E. Andén, and K. Fuxe. 1969. Direct chemical stimulation of dopaminergic mechanisms in the neostriatum of the rat. *Brain Res.* 14:461–471.
65. Wagner, J. A., and R. B. Kelly. 1979. Topological organization of proteins in an intracellular secretory organelle: the synaptic vesicle. *Proc. Natl. Acad. Sci. USA.* 76:4126–4130.
66. Wakshull, E., M. I. Johnson, and H. Burton. 1978. Persistence of an amine uptake system in cultured rat sympathetic neurons that use acetylcholine as their transmitter. *J. Cell Biol.* 79:121–131.
67. Wedel, R. J., S. S. Carlson, and R. B. Kelly. 1981. Transfer of synaptic vesicle antigens to the presynaptic plasma membrane during exocytosis. *Proc. Natl. Acad. Sci. USA.* 78:1014–1018.
68. Winkler, H., and E. Westhead. 1980. The molecular organization of adrenal chromaffin granules. *Neuroscience.* 5:1803–1823.
69. Ziegler, M. G., J. A. Thomas, and D. M. Jacobowitz. 1976. Retrograde axonal transport of antibody to dopamine-β-hydroxylase. *Brain Res.* 104:390–395.