GROWTH CONES OF CULTURED SYMPATHETIC NEURONS CONTAIN ADRENERGIC VESICLES

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ABSTRACT

The growth cones of dissociated rat sympathetic neurons developing in culture were fixed with potassium permanganate to visualize vesicular stores of norepinephrine through the formation of granular precipitates. It was found that growth cones contain numerous small granular vesicles (SGV) 40–60 nm in diameter. The majority of the SGV was present in the varicosity of the growth cone but SGV also occurred in filopodia. The SGV appeared in clusters or scattered throughout the varicosity. Treatment of the cultured neurons, before fixation, with reserpine, which depletes catecholamine stores by blocking uptake into vesicles, resulted in the presence of small clear vesicles. In contrast, growth cones of nonadrenergic sensory neurons dissociated from dorsal root ganglia and fixed with permanganate lacked SGV and possessed small clear vesicles. These observations indicate that the growth cones of cultured sympathetic neurons contain norepinephrine, suggest that the norepinephrine is stored in synaptic vesicles, and raise the question whether this transmitter plays a role in early axon-target cell interactions during synapse formation.

KEY WORDS   growth cone · sympathetic neuron · norepinephrine · synaptic vesicles

Axonal growth cones effect the early interactions of developing neurons and their targets; they are the first part of the neuron to seek out and contact potential target cells during synaptogenesis. Numerous morphological studies of growth cones in vivo and in vitro (for example, references 3, 4, 15, 21, 24, 26, 27) have revealed a relatively consistent ultrastructure. The growing tip consists of a bulbous enlargement or varicosity from which several to many fine fingerlike filopodia extend. The varicosity usually contains agranular reticulum, clear vesicles and vacuoles, coated vesicles, large dense-cored vesicles, mitochondria, microtubules, microfilaments, and lysomal structures, whereas the filopodia possess only a microfilamentous network and occasional membranous elements. Synaptic vesicles are not generally thought to be present in growth cones until after the acquisition of membrane specializations by the primitive synaptic contacts (3, 15, 21, 26, 27). It is difficult, however, to distinguish synaptic vesicles from other smooth membranous elements in the growth cone in the absence of membrane specializations or a cytochemical marker.

Potassium permanganate fixation provides a sensitive cytochemical marker for adrenergic vesicles. The synaptic vesicles in axonal varicosities of neurons which use norepinephrine as a neurotransmitter, when fixed with permanganate, contain granular precipitates and have been called small granular vesicles (SGV) (1, 13, 14, 22, 25). Dense granules are not present in the vesicles if before fixation, the terminal has been exposed to reserpine (1, 13, 14) which depletes norepinephrine by blocking uptake into vesicles (10) or if the

terminal has been exposed to α-methyl-tyrosine (13, 25) which depletes norepinephrine by blocking synthesis (23). In the second instance, granular precipitates reappear if the axon is then exposed to exogenous norepinephrine (13, 25). The dense granules visualized by permanganate are thus thought to represent the vesicular stores of norepinephrine which are presumably available for release by exocytosis.

Sympathetic neurons which use norepinephrine as a transmitter are known from in vivo (6, 8, 9) and in vitro (8, 19) studies to express adrenergic functions early in development. These neurons can be dissociated and grown in culture conditions which foster their adrenergic differentiation (18, 19). To determine whether vesicles present in growth cones contain neurotransmitter, young cultured sympathetic neurons were fixed with permanganate and their growth cones were examined for the presence of SGV.

MATERIALS AND METHODS

Neurons from the superior cervical ganglia of newborn rats were mechanically dissociated according to the method of Bray (2) as modified by Mains and Patterson (18). The neurons were plated onto collagen-coated Aclar cover slip inserts in Falcon tissue culture dishes and were grown in L-15 Air medium containing rat serum and nerve growth factor (18). After 8 or 48 h, the cultures were fixed for 15 min with 4% ice-cold potassium permanganate (22), stained en bloc with uranyl acetate, dehydrated with ethanol, and embedded in Epon. After removal of the cover slip, areas containing growth cones were located in the plastic wafer with the phase microscope, and were marked and mounted on blank Epon blocks. Each block was trimmed to include only one or two growth cones to facilitate their identification with the electron microscope. Serial thin sections were cut in the plane parallel to the collagen film. The first 20 sections usually contained the entire growth cone and, in most cases, all of these sections were collected on 1 × 2-mm Formvar-coated slot grids. The sections were examined without further staining. For comparison, several cultures were fixed with 3% glutaraldehyde in 0.12 M phosphate buffer, postfixed with 1% OsO_4 in phosphate buffer, stained en bloc with uranyl acetate, dehydrated, and embedded. Thin sections from these cultures were stained with lead citrate before examination.

Four cultures of dissociated sympathetic neurons were treated with 2 μM reserpine phosphate in growth medium for 2 h before fixation. In addition, sensory neurons were dissociated from the dorsal root ganglia of newborn rats, grown under conditions identical to those outlined above, and fixed with potassium permanganate after 48 h in culture.

RESULTS

Axonal growth cones were clearly recognizable in thin sections of 2-day-old cultures fixed with potassium permanganate; the axon broadened out into a varicosity from which characteristic filopodia extended (Fig. 1). There were differences, however, in the appearance of growth cones fixed with permanganate and those fixed with aldehyde-osmium tetroxide. For example, after permanganate fixation, the filopodia appeared beaded, the microfilamentous network appeared flocculent and microtubules were absent (Fig. 2). Most membranous components were present but tubular invaginations and vesicle-filled mounds (4) have not been observed.

A striking finding was the presence in the permanganate-fixed growth cones of numerous small vesicles 40–60 nm in diameter which contained granular deposits (Figs. 1 and 2). These SGV comprised 50–67% of the vesicles present in this size range, and every thin section through every growth cone examined contained them. In some growth cones, clusters of 20 or more SGV were present in several adjacent thin sections (Fig. 1). More frequently, however, the SGV were scattered through the axoplasm of the varicosity and occasionally even in filopodia (Fig. 2). Growth cones of neurons fixed 8 h after plating also contained SGV, and no obvious differences have been noted in the proportion or size of SGV in the 8- and 48-h growth cones. Behind the advancing growth cone, clusters of SGV were evident in axonal varicosities. SGV were not observed in growth cones or axonal varicosities of neurons after aldehyde-osmium fixation which is less sensitive in demonstrating norepinephrine than permanganate fixation (1, 13, 14, 22); clear vesicles with the same diameter and distribution were present.

Several cultures of dissociated sympathetic neurons were treated with reserpine phosphate before permanganate fixation. The growth cones in these cultures lacked SGV and contained instead numerous clear vesicles of the same diameter as the SGV (Fig. 3). Reserpine treatment did not obviously alter the matrix density of the large dense-cored vesicles but it did deplete their granular deposits. The growth cones of sensory neurons dissociated from dorsal root ganglia and fixed with
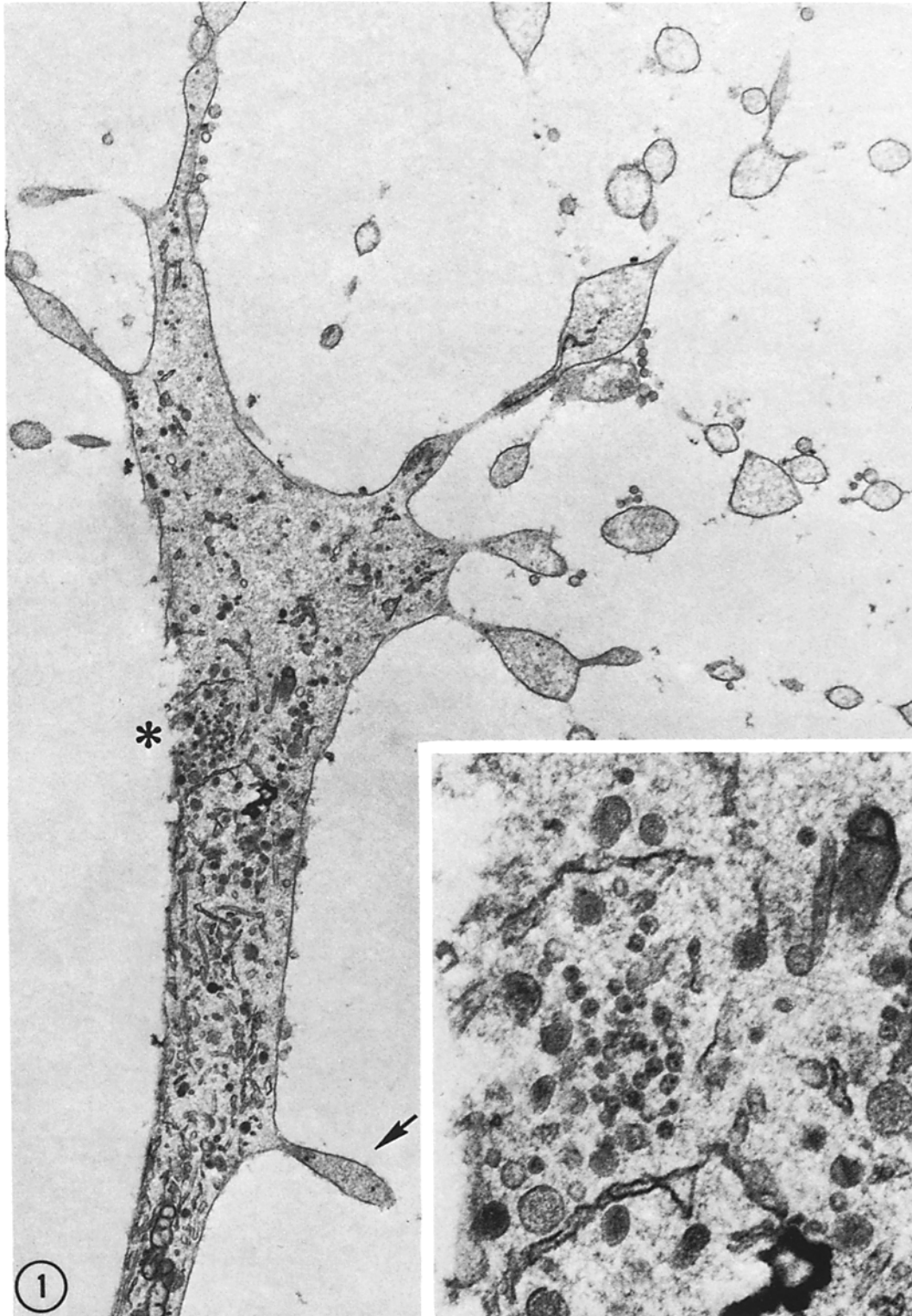
FIGURE 1 Growth cone of sympathetic neuron, 48 h in culture, permanganate fixation. The axon broadens out into the growth cone varicosity at the filopodium seen at the lower right (arrow). The number and distribution of organelles changes at this junction; whereas the axon contains cisternae of smooth endoplasmic reticulum and mitochondria aligned along the long axis, the growth cone contains a variety of large and small vesicles and smooth membranous elements all of which lack a predominant orientation. More filopodia extend from the tip of the varicosity. The asterisk marks a cluster of SGV seen at higher magnification in the inset. The granular precipitate in the SGV is denser than the adjacent cytoplasm and occurs either as a single core or as fragments. $\times$ 14,000. Inset, $\times$ 61,000.
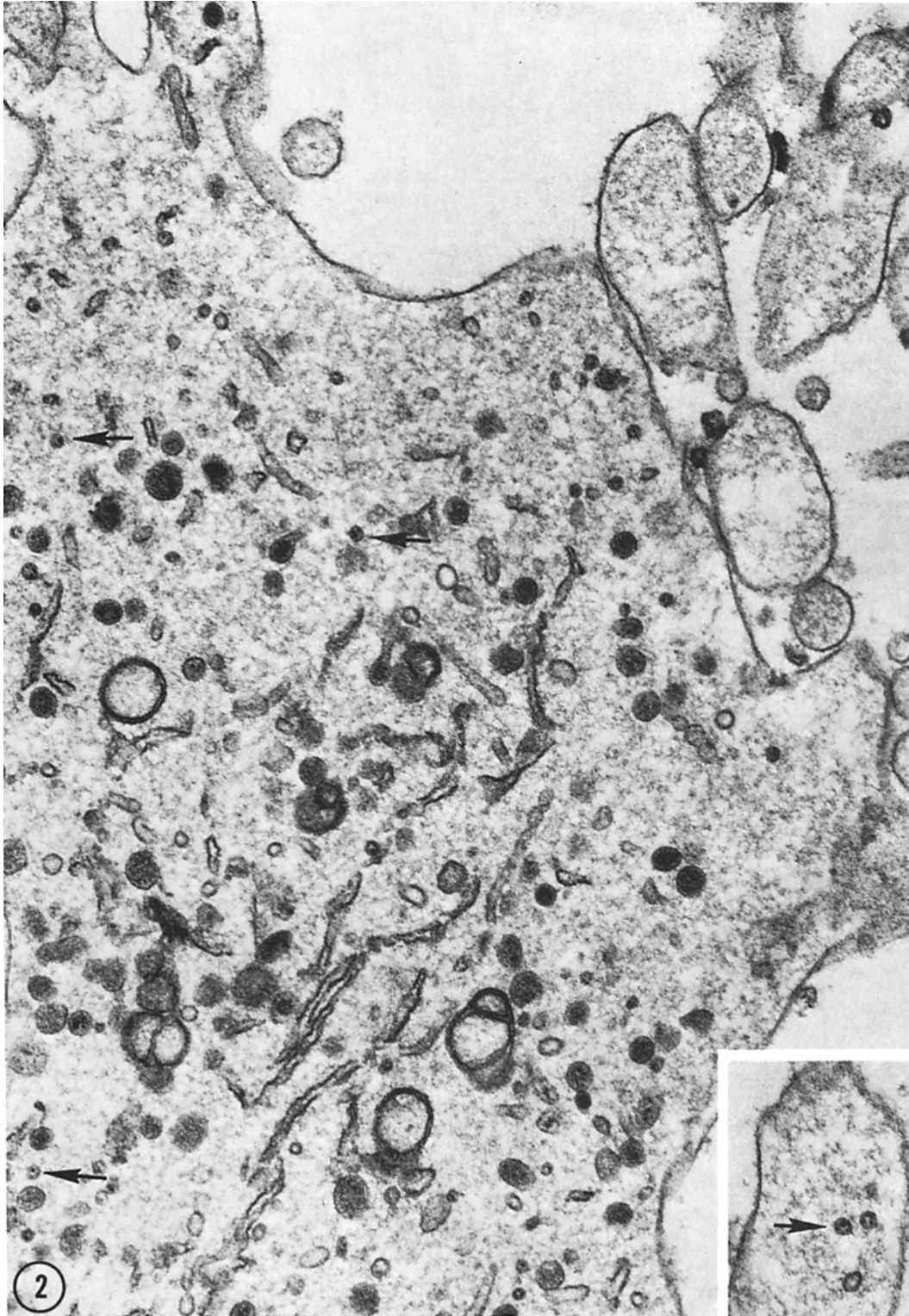
FIGURE 2 Growth cone of sympathetic neuron, 48 h in culture, permanganate fixation. SGV (arrows) are scattered through the varicosity and occasionally appear in filopodia as seen in the inset. Several of the large dense-cored vesicles contain granular deposits. × 55,000. Inset, × 61,000.
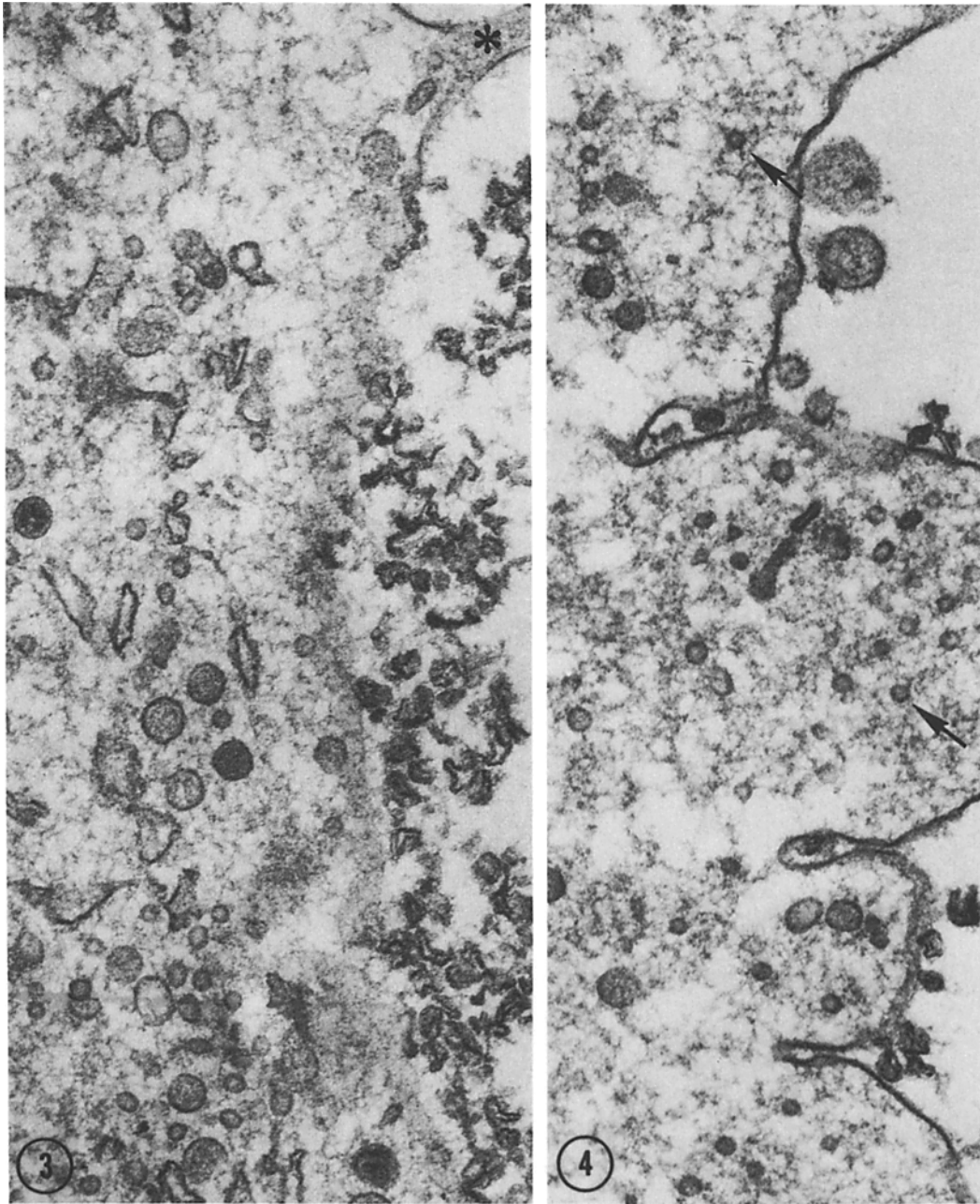
FIGURE 3   Growth cone of sympathetic neuron, 48 h in culture, reserpine pretreatment, permanganate fixation. Only small clear vesicles are present after reserpine treatment. The extracellular, electron-opaque debris present in Figs. 3 and 4 is typically observed in sections through permanganate-fixed growth cones close to the collagen substrate. The asterisk indicates a filopodium. × 61,000.

FIGURE 4   Growth cone of sensory neuron, 48 h in culture, permanganate fixation. Arrows indicate clear vesicles. × 61,000.

permanganate contained only small clear vesicles and large dense-cored vesicles lacking granular precipitate (Fig. 4).

DISCUSSION

The use of potassium permanganate as a fixative allows the demonstration of norepinephrine-containing synaptic vesicles as SGV in the peripheral and central nervous systems (1, 13, 14, 22, 25). Sympathetic neurons dissociated from the superior cervical ganglia of newborn rats and grown for several weeks in the culture' conditions used in the present experiments synthesize, store, and release norepinephrine (18, 19, 20). Single sympathetic neurons grown in microcultures have been shown with physiological techniques to secrete norepinephrine onto cardiac myocytes (12), and the same neurons are found to contain vesicles with granular deposits in synaptic varicosities after permanganate fixation (17). The present study has demonstrated that some of the vesicles in the growth cones of these dissociated adrenergic neurons contain dense granules after permanganate fixation. The SGV are observed before synaptic membrane specializations appear as the growth cone advances along the collagen substrate in the absence of a target. They appear both clustered and dispersed in the growth cone, adjacent to and distant from the axonal membrane. It is possible that the distribution of SGV in the growth cone would be affected by the presence of a suitable target. The SGV are absent from the growth cones of non-adrenergic sensory neurons, they are exactly the same size and shape as those found in mature adrenergic axonal boutons, they show the same response to reserpine as do adrenergic synaptic vesicles, and they are thus, by morphological criteria, indistinguishable from adrenergic synaptic vesicles.

Although the SGV present in growth cones are morphologically equivalent to norepinephrine-containing synaptic vesicles, it is not known whether these vesicles are functionally equivalent. Preliminary evidence is available in other systems that transmitter is released from early axonal contacts with appropriate targets, very likely even from growth cones. In vivo, for example, neuromuscular transmission has been observed in the myotomes of Xenopus laevis larvae before any specialized junctions are recognizable morphologically (16). In vitro, the initial contacts between axon and target can be identified microscopically as they occur, and Cohen (7) and Frank and Fischbach (11) have reported that transmitter

release onto myotubes can be evoked from the growing tips of spinal cord axons. Finally, Chamley et al. (5) have noted that the rate of contraction of smooth muscle cells cultured from vas deferens increased when adrenergic growth cones contacted the myocytes and have suggested that the release of norepinephrine from the growth cones could be responsible. The observations reported in this paper provide the first morphological evidence for the presence of neurotransmitter in vesicles of growth cones, suggest that these vesicles are, in fact, synaptic vesicles, and raise the possibility that the release of transmitter from growth cones at initial contacts could mediate some of the early interactions between a growing axon and its target cells during synapse formation.

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