Neurons: Secretory Activity during Limb Regeneration and Induction in the Newt

Abstract. *Material staining with aldehyde fuchsin appeared in sensory ganglion cells supplying a regenerating limb or nerve-induced blastema and in regenerating nerve fibers within the blastema. With the electron microscope, large (1000 to 2500 angstroms), dense granules were observed in the perikarya and within end bulbs of peripheral nerves. Secretory materials may be elaborated and transported by neurons during limb regeneration and induction in the newt.*

The presence of nerves at a site of limb regeneration in urodele amphibians is necessary for normal regeneration to occur (*1*). In addition, ectopic supernumerary limbs can be induced by the diversion of a peripheral nerve to an appropriate site (*2*). Little is known, however, of the mechanism of this influence, an example of trophic function, by the nerve. The most widely held theory is that the trophic effect is due to a hormonal substance elaborated by neurons. In neurons, synthesis of a substance for export, including hormones and neurohumors, is generally manifested as secretory activity (*3*). We made this study to determine whether evidence for altered or increased secretion could be detected in neurons during limb regeneration or induction in the newt *Triturus viridescens*.

At intervals of 7, 10, 14, 21, and 28 days after amputation through the lower one-third of the upper forelimb, or after deviation of the superficial and deep brachial nerves into the pectoral region as described by Bodemer (*2*), dorsal root ganglia C3, C4, and C5 and the blastema were removed. For histochemical studies, tissues were fixed in Bouin's solution or 10 percent formalin and embedded in paraffin; serial sections were made at 10 μ and stained as described below. For electron microscopy, tissues fixed in 3 percent glutaraldehyde in sodium cacodylate buffer (*p*H 7.2) were placed in 1 percent osmium tetroxide (*p*H 7.2), dehydrated, embedded in Maraglas, and sectioned on a Porter-Blum microtome. The sections were stained with lead hydroxide and examined with an RCA-EMU 3F electron microscope.

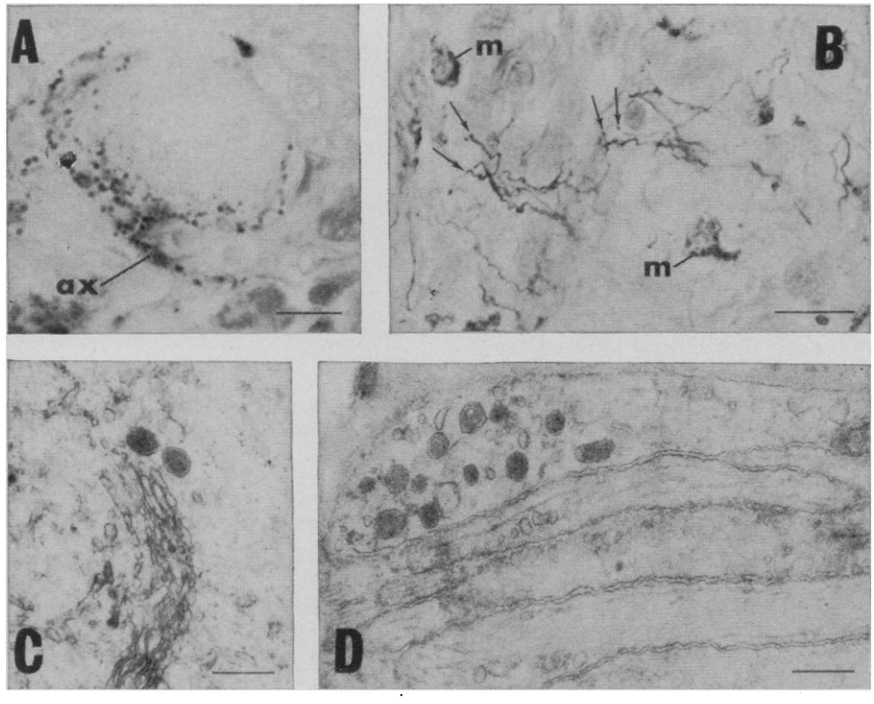

Fig. 1. (A) Neuron in dorsal root ganglion serving a regenerating limb 10 days after amputation. Stained with aldehyde fuchsin, Halmi counterstain. Note AF-positive granules in the perikaryon and proximal axon (*ax*) of this pseudounipolar cell. Bar represents 5 μ (× 1800). (B) Nerve-induced blastema 28 days after nerve deviation. Individual nerve fibers (0.2 to 0.4 μ in diameter) can be seen twining among the undifferentiated mesenchymal cells. Note occasional end bulbs at the tips of these fibers (arrows). *m*, Melanocytes. Bar represents 10 μ (× 1100). (C) Golgi apparatus of a sensory neuron 21 days after nerve deviation. At the ends of the Golgi lamellae are two large membrane-bounded dense granules. Note similarity to granules in end bulbs in (D). Bar represents 0.4 μ (× 21,500). (D) Peripheral nerves in a nerve-induced blastema 21 days after nerve deviation. The section passes near the termination of the upper fiber and through more proximal levels of the lower three fibers. These fibers are approximately 0.3 μ in diameter, corresponding to those in (B). Large dense granules are prominent in the distal end of the upper fiber. Note profiles of smooth endoplasmic reticulum, neurotubules, filaments, and small clear vesicles distinguishing these fibers from processes of other cell types. Bar represents 0.4 μ (× 21,500).

Material staining with a classical neurosecretory stain, aldehyde fuchsin (AF) [basic fuchsin, color index 677 or 42,500, crystallized with paraldehyde and used after the method of Cameron and Steele (*4*) for neurosecretory cells] was observed in sensory ganglia and blastemata during limb regeneration and induction. In the sensory neurons, small, intensely stained granules were distributed throughout the perikarya, being more concentrated toward the periphery (Fig. 1A). Both axon hillocks and proximal axons contained stained granules (Fig. 1A). Granules were most densely packed and numerous in large cells. Staining was prominent 7 to 21 days after operation, and was most intense at 10 to 14 days. In normal ganglia, only a few scattered AF-positive granules were seen in the perikarya of some neurons. A few circumferentially arranged and irregularly shaped clumps of material were lightly stained in cells of both normal and experimental ganglia.

Aldehyde fuchsin, a relatively nonspecific Schiff base which combines with –CHO, $-OSO_3H$, $-SO_3H$, $-SO_2H$, and probably –COOH groups (*5*), stains a variety of substances in addition to neurosecretory substances or their carrier molecules. Control reactions were performed to distinguish some of these other substances. For lipofuscin, Gomori's chrome-alum hematoxylin and the Schmorl reaction (*6*) were used. These methods revealed relatively large clumps of material scattered at the periphery of some neurons. Bargmann's modification of the chrome-alum hematoxylin stain, as used to demonstrate neurosecretory substances (*7*), showed material in a pattern identical to that described for lipofuscins, and presumably demonstrated the latter. These

methods also stained small clumps of material arranged in a concentric pattern around the nucleus, as did AF. The periodic acid–Schiff reaction gave only a faint generalized pink cast to the cytoplasm. Sudan Black B (saturated solution in 70 percent alcohol) demonstrated bound lipid in coarse droplets of variable size sparsely distributed throughout the cytoplasm, but most often clumped at one pole of the neuron. The pattern of staining by these methods was distinctly different from that of AF in alternate serial sections, and was observed as often in normal ganglia. In a few cells, the pattern of Sudan Black B staining resembled that seen with AF, but the number of cells stained were so few compared to the number of AF-positive cells that we could not conclude that the same structures were stained. Neurons in sections in which the oxidation with sulfuric acid and potassium permanganate was omitted were not stained with AF (although the melanin in pigment cells in these ganglia stained with or without prior oxidation).

In the blastema, regenerating nerve fibers stained homogeneously with AF (Fig. 1B). These delicate, wavy fibers, approximately 0.3 μ in diameter, were found among the mesenchymal cells beyond the original level of transection of the peripheral nerve. On occasion, they could be traced from the blastema into the epidermis or proximally to the large nerve bundles. Slight bulbous enlargements were observed at the ends of some of these fibers, probably representing end bulbs or growth cones (Fig. 1B). Staining of the regenerating fibers was observed at 14 to 28 days after amputation or nerve deviation. No staining was detected within the fibers of the larger peripheral nerves proximal to the level of transection. Connective tissue fibers stained with AF were seen in the blastema, especially surrounding arteries and muscle bundles. These could be distinguished from nerve fibers as they occurred in a branching and anastomosing network composed of unevenly stained bundles of variable size. Nerve fibers, in contrast, were uniformly small and evenly stained along their length; they occurred singly, pursued a wavy course, and could often be traced to their origin from the large peripheral nerves containing myelinated fibers.

The electron microscope revealed large membrane-bounded granules in sensory ganglia and within nerve fibers in the blastema during limb induction. In ganglion cells a few granules were found throughout the perikaryon and associated with the Golgi complex (Fig. 1C), and were most numerous in the region of the axon hillock and proximal portion of the axon. In the blastema, the end bulbs of the regenerating nerve fibers contained many granules (Fig. 1D), whereas proximal regions had few. The granules were large, averaging 1700 Å in diameter (range 1000 to 2500 Å), and were composed of moderately dense material. Granules of this size were rarely found in normal ganglion cells or normal peripheral nerves, although smaller dense vesicles occur. The granules observed during limb induction were identical to those described in regenerating nerves (*8*) which were distinguished from dense-core vesicles containing catecholamines.

Secretory activity of neurons apparently changes during limb regeneration or induction. Because this change in secretory activity occurs during a physiological process in which a hormonal substance is believed to function, it could reflect the formation and transport to the peripheral tissues of a trophic substance. This process occurs in neurons heretofore not considered capable of hormonal activity.

CONDIT B. VAN ARSDALL
THOMAS L. LENTZ
Department of Anatomy, Yale University School of Medicine, New Haven, Connecticut 06510

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Alveolar Macrophages: Reduced Number in Rats after Prolonged Inhalation of Lead Sesquioxide

Abstract. *A decreased number of alveolar macrophages was found in washings from lungs of rats inhaling small particles of lead sesquioxide for 3 to 12 months, as compared with control animals exposed to filtered air. This result contrasts with that reported by others for animals given massive exposures to various dusts for short periods of time. Because the concentrations of lead were comparable to those observed in some industrial (150 $\mu g/m^3$) or urban (10 $\mu g/m^3$) environmental conditions, the results may be significant in terms of human lung clearance processes after such exposures.*

Phagocytosis by alveolar macrophages is considered to be an important step in removal of dust particles or bacteria from the respiratory tract. For this reason the number and activity of alveolar macrophages are thought to be important aspects of pulmonary defense. The technique of harvesting alveolar macrophages from excised mammalian lungs has been used to obtain a quantitative estimate of the number of these cells (*1, 2*). With this technique we have demonstrated significant decreases in the number of alveolar macrophages that were washed from the lungs of rats after prolonged inhalation of low concentrations of very small particles of lead sesquioxide.

In contrast, LaBelle and Brieger (*2*) found that intratracheal injection of carbon particles caused a tenfold increase in the number of cells they obtained with lung washings. After termination of the exposure the rate of clearance of particles was proportional to the number of alveolar macrophages recovered in other comparable animals. Other investigators (*3, 4*) have reported obtaining increased numbers of alveolar macrophages after massive exposures to dusts for short periods of time, and accelerated clearance of dust from the lungs of animals similarly exposed.

Young adult male and female rats (Controlled-Flora, Greenacres Farm) were exposed continuously (24 hours per day) in three separate chambers (one a control chamber) to different concentrations of lead aerosol for periods up to 1 year. The particulate lead compound was generated by burning