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Inhibitors of Lysosomal Enzymes: Accumulation of Lipofuscin-Like Dense Bodies in the Brain

Abstract. *Injections of leupeptin (a thiol proteinase inhibitor) or chloroquine (a general lysosomal enzyme inhibitor) into the brains of young rats induced the formation of lysosome-associated granular aggregates (dense bodies) which closely resembled the ceroid-lipofuscin that accumulates in certain disease states and during aging. The dense material increased in a dose- and time-dependent fashion and was differentially distributed across brain regions and cell types. These observations provide clues to the origins of ceroid-lipofuscin and suggest means for studying the consequences of its accumulation.*

During aging there is an accumulation in neurons and other cell types of a heterogeneous group of pigment granules referred to as lipofuscin (*1*). A similar material, usually termed "ceroid," develops in cells during a number of pathological conditions ranging from anoxia to vitamin deficiency (*2*), and is characteristic of a group of genetically inherited diseases called the "neuronal ceroid lipofuscinoses" (*3*). The origins and causes of ceroid or lipofuscin accumulation, as well as the consequences for the functioning of the cell, are poorly understood. The granules are rich in peroxidated lipids and polymerized phospholipids (*4*), and a combination of histochemical and ultrastructural evidence indicates that they are related to lysosomes (*5*). This evidence has led to the hypothesis that peroxidation of membrane lipids induced by free radicals blocks the normal degradation of organelles and vesicles by lysosomes, resulting in the accumulation of residual bodies that are classified as ceroid-lipofuscin (*6*). Alternatively, it has been proposed that ceroid-lipofuscin accumulation results from a defect in intracellular catabolism itself, followed by intralysosomal peroxidation and polymerization of the undigested material (*7*). We now report that inhibitors of lysosomal enzymes cause a rapid and massive increase in ceroid-lipofuscin in the brain cells of young rats.

Leupeptin and chloroquine, two well-established inhibitors of lysosomal proteinases (*8, 9*), were injected into the brains of Sprague-Dawley rats (40 to 60 days of age) with a constant infusion technique. The rats were implanted with osmotic minipumps (Alzet) that were attached to cannulae leading into the lateral ventricle of each rat (*10*). The pumps contained leupeptin (acetyl-leupeptin, Boehringer Mannheim, 8, 20, or 40 mg/ml; 2, 3, and 12 rats, respectively), chloroquine (Sigma, 40 mg/ml; 4 rats) or saline (9 mg/ml; 4 rats) and operated at a steady rate of 0.5 μl/hour. After 2 weeks most of the animals were killed; three rats implanted with leupeptin-filled pumps (40 mg/ml) were allowed to survive for 8 to 10 weeks. In addition, two rats each received daily intraventricular injections of leupeptin (0.5 mg/5 μl), chloroquine (0.5 mg/5 μl), or saline (0.045 mg/5 μl) for four consecutive days, and two rats received single intraventricular injections of leupeptin (0.5 mg/5 μl) and were killed 8 hours later. Most animals were killed by perfusing with a mixed aldehyde fixative (*11*), and samples from various brain regions were dissected and processed for electron microscopy. Remaining portions of these brains were either sectioned on a freezing microtome (at 20 μm thick) or were embedded and sectioned in paraffin (at 6 μm thick) or glycol methacrylate (at 1 to 2 μm thick). The following stains were then employed (*12*): periodic acid Schiff (PAS), Nile

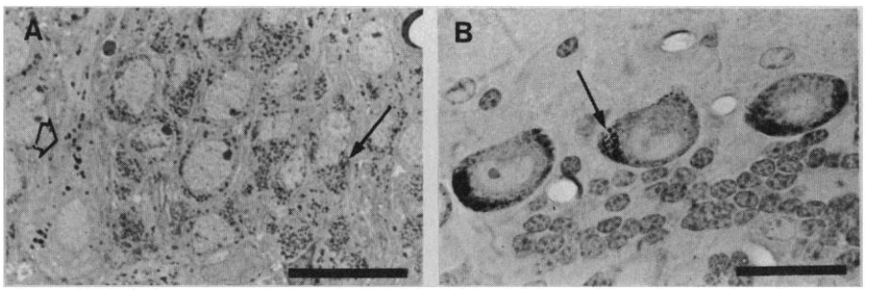

Fig. 1. Bright-field photomicrographs from the brains of two rats that received a continuous infusion of leupeptin (0.5 mg/day) into the lateral ventricle via a cannula attached to a mini-osmotic pump. (A) A 1-μm-thick section (in epon) through the dentate gyrus showing dense granular inclusions in granule cells (solid arrow) and in a pyramidal-like interneuron (open arrow); toluidine blue stain. (B) A 1-μm section (in glycol methacrylate) through cerebellum showing densely staining PAS-positive inclusions in Purkinje cells (arrow). Scale bars, 25 μm.

blue sulfate, toluidine blue, oil red O, Sudan black B, Schmorl's ferric ferricyanide method, AFIP method for lipofuscin, and carbol fuchsin; the tissue was also examined for autofluorescence. The remaining rats were perfused with a solution of buffered Formalin (10 percent) and processed for light microscopy. Light- and electron-microscopic analyses were also carried out on four 2-month-old and two 24-month-old untreated rats.

Figure 1A is a photomicrograph of a toluidine blue–stained 1-μm-thick plastic section of the dentate gyrus of an animal killed after 14 days of infusion with leupeptin. The cytoplasm of many of the cells contains granules of densely stained material. This was observed throughout the brains of nine similarly treated rats, as well as in the chloroquine-treated rats. However, regional variations were apparent; in each subdivision of the brain, certain cell types or subregions were clearly more affected than others. Large cells such as cerebellar Purkinje cells (Fig. 1B) and neocortical pyramidal cells of motor cortex were among those exhibiting the most dramatic effects. The material ("dense bodies") was stained by the PAS technique, Nile blue sulfate, oil red O, Sudan black B, and Schmorl's method, indicating that, like ceroid-lipofuscin, it contained high concentrations of lipids and glycogen (*12*). Further, the dense bodies emitted a yellowish autofluorescence in the hippocampus and cerebellum within the range of emission spectra reported for autofluorescence of various lipopigments (*13*). Further study will be needed to determine if subtle differences exist in the staining properties of the material induced by leupeptin as compared to that induced by chloroquine and among the dense bodies found in various brain regions. Treatment with the highest concentration of leupeptin (40 mg/ml) also resulted in the selective degeneration and disappearance of populations of cerebellar Purkinje cells. This effect was manifested as patches of cells in which some Purkinje neurons were absent while others appeared shrunken and in advanced stages of degeneration.

Electron microscopy of portions of hippocampus confirmed the lipofuscin-like nature of the dense bodies and the extent of the increase in number produced by leupeptin and chloroquine (Fig. 2). These electron-opaque structures are osmiophilic, enclosed by a single membrane, and are within the range of the dimensions (approximately 0.5 to 3 μm) of lipofuscin residual bodies seen in aged animals (*14*). The ultrastructure of the organelles varied but usually could be assigned to one of the several categories of lipofuscin bodies that have been described (*15*).

Granular aggregates of varying sizes were common and generally seen in conjunction with electron-opaque lysosomal bodies. These aggregates comprised approximately 80 percent of the material found in dentate gyrus granule cells of leupeptin-treated rats and approximately 20 percent of that in chloroquine-treated rats. Curvilinear profiles, patterns resembling fingerprints, and vacuoles were less common with either treatment, accounting for approximately 20 percent of the material in leupeptin- and 10 to 20 percent of the material in chloroquine-treated rats. Membranous whorls similar to those found in Tay-Sachs disease (*16*) were numerous in rats infused with chloroquine (about 60 to 70 percent of the total ceroid-lipofuscin material), but were rarely observed in leupeptin-treated animals (*17*).

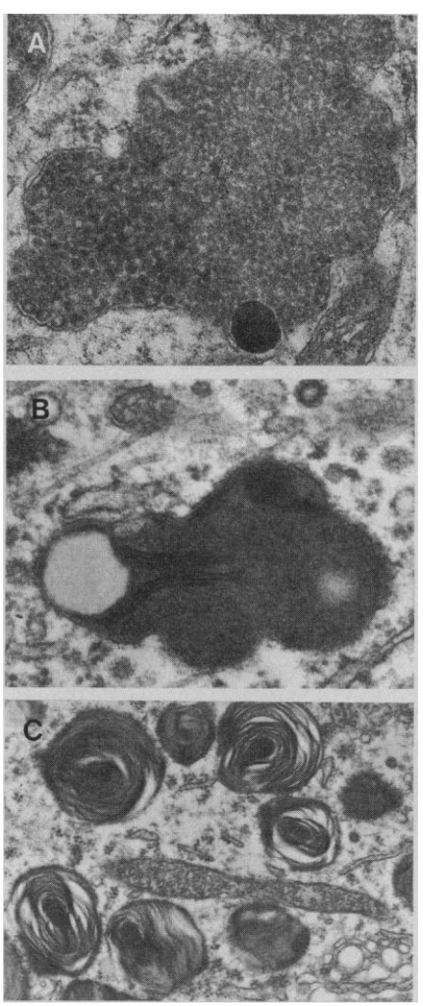

Fig. 2. Electron micrographs showing fine morphology of the chemically induced lipofuscin-like substances. (A) Leupeptin-induced granular substance associated with intact lysosome from a dentate gyrus granule cell of the same rat as in Fig. 1A. (B) Chloroquine-induced heterogeneous compound containing fine granular substance, membrane, and vacuole from dentate gyrus granule cell of a rat treated like those in Fig. 1 but with chloroquine. (C) Chloroquine-induced membranous whorls from the same section as in (B).

The induction of ceroid-lipofuscin by leupeptin is both dose and time dependent. In an electron microscopic analysis of dentate gyrus granule cells, granular aggregates were absent or barely detectable in the cells of animals treated with 8 mg of leupeptin per milliliter but were prominent in the cells of animals treated with 20 mg/ml and extensive at higher doses. The highest dose of leupeptin was more effective than chloroquine at any concentration tested. The effects of leupeptin had a rapid onset. In a series of animals killed at various times after the intraventricular administration of leupeptin, a small increase in dense bodies was observed at 8 hours of treatment with a substantial accumulation by 72 hours. Animals allowed to survive for 8 to 10 weeks after emptying of the pumps had appreciably less ceroid-lipofuscin material as compared to rats examined at 10 to 14 days after implantation of pumps. Thus, the effect of leupeptin was partially reversible.

The appearance and behavior of most drug-treated rats was not dramatically different from that of normal rats. However, rats receiving continuous infusion of chloroquine or leupeptin at the highest doses (40 mg/ml) for 2 weeks appeared more lethargic and less well-groomed than normal rats.

Leupeptin is a small peptide that reversibly binds to thiol proteinases and thereby inhibits their activity (*8*); chloroquine causes a pronounced elevation of intralysosomal *p*H, and thus indirectly blocks the acidic proteinases (*9*). Leupeptin and chloroquine thus block lysosomal enzymes in different ways, which suggests that the increased ceroid-lipofuscin material observed after treatment with either agent results from an interruption of lysosomal metabolism. However, leupeptin also inhibits nonlysosomal proteases and little is known of the nonspecific effects of chloroquine. It remains possible that the drugs exert their common effect through a different and unknown mechanism.

The speed and magnitude of the accumulation of ceroid-lipofuscin after leupeptin administration was surprising. If the dense bodies represent incompletely degraded organelles (such as endoplasmic reticulum, mitochondria, or coated vesicles) then the turnover of these structural elements must proceed at a

very rapid rate. Alternatively, it is possible that a feedback system exists such that incomplete degradation accelerates autophagocytosis. However, with the exception of the Purkinje cells, we detected no obvious disturbances in the ultrastructure of neurons containing ceroid-lipofuscin. This would not be expected if an increased rate of organelle breakdown occurred unless it were balanced by an equivalent change in the rate of synthesis.

These results may have implications for the etiology of the group of inherited neuronal ceroid lipofuscinoses (NCL) (*3*). Not only do the granular and curvilinear profiles of the leupeptin-induced ceroid-lipofuscin closely resemble those found in patients with NCL diseases, but also, in both leupeptin-treated rats and patients there is a focal degeneration of Purkinje cells in the cerebellum. Perhaps the accumulation of pigment in nerve cells of individuals with NCL is caused by a defective (or absent) lysosomal proteinase, in much the same way that defective lipases, galactosidases, or other enzymes cause the accumulation of various substances in the hyperlipoproteinemias, Tay-Sachs disease, or other inherited lysosomal storage disorders (*18*).

Finally, our findings suggest a means for studying the functional consequences of dense body accumulation. Since leupeptin is a reversible inhibitor, the drug is probably removed from the brain once the osmotic pump has been emptied. By comparing differentially affected cell populations in the same animal (during and after drug treatment) as well as in animals exposed to different concentrations of drugs, it should be possible to arrive at correlations between the accumulation of lipofuscin and various structural and functional measures. These approaches, coupled with recent evidence of biochemical changes associated with ceroid-lipofuscin accumulation (*19*), could lead to the development of experimentally accessible models for studying the role of dense body accumulation in NCL diseases and aging.

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10. Stereotaxic coordinates for cannula implantation were: incisor bar at +5 mm above horizontal zero, 1 mm anterior to the bregma, 1.3 mm lateral to the bregma, and 3.3 mm ventral to the brain surface.
11. For electron microscopy, animals were perfused with a fixative containing 2.5 percent each of paraformaldehyde and glutaraldehyde in 0.1*M* phosphate buffer, *p*H 7.2. Portions of these brains were used for light microscopy; additional animals were perfused with 10 percent buffered Formalin for light microscopy.
12. Toluidine blue reveals ribonucleic acid; PAS reveals glycogen and other carbohydrates (prior digestion of tissue with α-amylase did not remove the dense PAS-positive staining). Nile blue sulfate stains neutral fats and fatty acids [G. G. Brown, Ed., *Primer of Histopathologic Technique* (Appleton-Century-Crofts, New York, 1969)]. Oil red O and Sudan black B reveal lipids; Schmorl's method, the AFIP (Air Force Institute of Pathology) method, and carbol fuchsin (the Long Ziehl-Neelsen method) reveal lipofuscin [L. G. Luna, Ed., *Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology* (McGraw-Hill, New York, 1968)].
13. Several authors have defined categories of emission spectra of ceroid-lipofuscin: J. H. Dowson, *J. Microsc.* **128**, 261 (1982); ——— and S. J. Harris, *ibid.* **123**, 249 (1981); H. S. Garg, Y. C. Awasthi, S. K. Srivastava, *J. Neurosci. Res.* **6**, 771 (1981). In our study, autofluorescence was observed in frozen and paraffin- and glycol methacrylate–processed tissue sections with a Zeiss Universal Microscope having a 100-watt mercury lamp and fluorescence filter package 487702. This filter combination produces ultraviolet excitation with a peak at 365 nm and allows visualization of fluorescence at 420 nm and above. Several categories of lipopigment with different fluorescence spectra are visible with this technique.
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Novel Pharmacology of Substance K–Binding Sites: A Third Type of Tachykinin Receptor

Abstract. *The tachykinins are a family of peptides with the carboxyl terminal amino acid sequence Phe-X-Gly-Leu-Met-NH_2. Three major mammalian tachykinins have been identified—substance K, neuromedin K, and substance P—but only two tachykinin receptors have been postulated. Three tachykinins were labeled with radioiodinated Bolton-Hunter reagent and their binding characteristics were determined in crude membrane suspensions from several tissues. In cerebral cortex labeled eledoisin exhibited high-affinity binding that was inhibited by tachykinins in a manner indicating a definitive SP-E receptor site. In gastrointestinal smooth muscle and bladder, high-affinity binding of labeled substance P was inhibited in a pattern indicating a definitive SP-P site. In intestinal smooth muscle and bladder, however, labeled substance K and labeled eledoisin were both bound in a pattern indicating a preference for substance K itself. The results suggest the existence of three distinct types of tachykinin receptors: SP-P, SP-E, and SP-K.*

The tachykinin family of peptides is characterized by the carboxyl terminal amino acid sequence Phe-X-Gly-Leu-Met-NH_2. The molluscan salivary gland peptide eledoisin and the amphibian skin peptides physalaemin and kassinin are members of this family, as is the mammalian putative neurotransmitter substance P (*1*).

In addition to substance P, two novel tachykinins have recently been identified in mammals. Neuromedin K (also called neurokinin β) has been isolated from spinal cord (*2, 3*), as has a kassinin-like neuropeptide named substance K (also called neurokinin α) (*2, 4*). Nawa *et al.* (*5*) used bovine striatal messenger RNA and bacterial cloning to identify a substance P precursor molecule, α-preprotachykinin, which contains only one copy of substance P, and a second precursor, β-preprotachykinin, which con-