References and Notes

1. I. Creese, D. R. Burt, S. H. Snyder, *Science* **192**, 481 (1976).
2. J. Stevens, *Arch. Gen. Psychiatry* **29**, 177 (1973).
3. Task Force on Late Neurological Effects of Antipsychotic Drugs, *Am. J. Psychiatry* **137**, 1163 (1980).
4. G. Chouinard, B. D. Jones, L. Annagle, *ibid.* **135**, 1409 (1978).
5. J. Gerlach, *Psychopharmacologia* **45**, 51 (1975); K. Jellinger, in *Neurotoxicology*, L. Rozin, H. Shiraki, H. Gorevic, Eds. (Raven, New York, 1977), pp. 25–42.
6. E. R. Weibel, *Stereological Methods* (Academic Press, New York, 1978), vol. 1, p. 101.
7. A. Campbell and R. J. Baldessarini, *Life Sci.* **29**, 1341 (1981).
8. V. B. Domesick, L. Stinus, P. Paskevich, *Neuroscience* **8**, 743 (1983).
9. F. M. Benes and R. J. Barrnett, *Brain Res.* **150**, 277 (1978).
10. C. E. Ribak, J. Vaughn, K. Saito, R. Barber, E. Roberts, *ibid.* **116**, 287 (1976).
11. V. M. Pickel, *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **38**, 2374 (1979); ———, T. H. Joh, D. Reis, S. E. Leeman, R. J. Miller, *Brain Res.* **160**, 387 (1979).
12. M. DiFiglia, N. Aronin, S. E. Leeman, *Brain Res.* **233**, 381 (1981).

13. We thank J. Lipinski for his early hypotheses, which proved to be instrumental to the initiation of these experiments. We also thank S. Matthyse for advice regarding appropriate statistical analyses, W. Nauta and R. Baldessarini for their helpful comments on the manuscript, and J. Davidson for her technical assistance. Supported by NIMH grants 1K01 MH00423-01 and MH31154-06.

23 February 1983; revised 29 April 1983

Neurofilament Accumulation Induced in Synapses by Leupeptin

Abstract. *The hypothesis that the usual absence of neurofilaments in synaptic terminals is due to their degradation by the calcium-activated protease present in axoplasm was tested by injecting leupeptin, which inhibits the protease, into the optic tectum of goldfish kept at 15° and at 25°C. The resulting accumulation of neurofilaments in synaptic terminals provides in vivo evidence in support of the hypothesis. The significance of these results and the potential uses of this pharmacological tool are discussed.*

Two major unsolved questions in neurobiology are: What happens to the cytoskeleton when it reaches the synaptic terminals? and How is the growth of axons regulated and what makes them stop growing when they reach their target cells? The cytoskeleton consisting of neurofilaments and microtubules is generated in the neuronal soma and transported along the axons at a rate of 1 to 4 mm per day (*1*). This process is considered to play a major role in axonal growth, which occurs at a rate that is approximately the same as the rate of delivery of cytoskeletal elements (*2*). Axoplasmic transport, however, occurs throughout the life of the neuron, even when the neuron is not growing, but neurofilaments and microtubules are usually not present in synaptic terminals. Therefore, some mechanism must exist to prevent the accumulation of neurofilaments and microtubules in synaptic terminals.

When neurofilaments are found in synaptic terminals, they are often in the form of annuli which, in sections of silver-impregnated material, are seen as dark neurofibrillary rings (*3*, *4*). Such rings occur only sporadically in the central nervous system of most vertebrates (*3*, *5*). However, in the brains of poikilotherms, such as lizards, bullfrogs, and goldfish, that have been subjected to low temperatures, the rings are numerous and quantitatively related to the environmental temperature (*6*, *7*). In seeking to explain this phenomenon in goldfish, Roots and Bondar (*8*) postulated that when the neurofilaments reached the synaptic terminals they were subjected to enzyme-mediated breakdown; during prolonged exposure to low temperatures, enzyme activity was inhibited either by a change in *p*H during acclimation or by the low temperatures, so that the breakdown of neurofilaments was slower than the delivery of neurofilaments to the terminals by axoplasmic transport. After the discovery of a calcium-activated endogenous protease in the axoplasm of invertebrate giant axons (*9*), Lasek and Hoffman (*2*) proposed a model that would explain not only the usual absence of neurofilaments in synaptic boutons but also how the growth of axons is regulated and terminated when the target cells are reached. According to the model, when the growth cone meets a target cell, differentiation of a synaptic terminal is initiated and mechanisms that degrade the cytoskeleton are activated. Lasek and Hoffman (*2*) proposed that within the axon the endogenous protease is inactive, but upon entering the axon terminal it is activated by calcium ions and disassembles the neurofilaments by cleaving the polypeptide backbone. An integral assumption in the model is that the calcium ion concentration in the axon differs from that in the terminal. The calcium ions in the axon terminals could originate from the agranular reticulum or the mitochondria and, once transmission begins, from the extracellular space, since there is evidence that calcium enters axon terminals in association with transmitter release (*10*) and membrane depolarization (*11*). Because microtubule disassembly can be brought about in vitro by low concentrations of calcium, the increased concentration of calcium in the terminals could also alter the equilibrium between polymerized microtubules and free tubulin (*12*). Axons from a wide variety of animals are now known to contain a calcium-activated protease (*13*, *14*). This enzyme is capable of disrupting neurofilaments and of cleaving neurofilament proteins in vitro. The finding that calcium-activated protease is present in various types of axons and is not restricted to invertebrate giant axons lends credence to the general applicability of the hypothesis.

The experiment described here was designed to test the hypothesis that neurofilaments are degraded in synaptic terminals in vivo by a calcium-activated protease. If the hypothesis is correct, inhibition of the protease by an inhibitor such as leupeptin would result in the accumulation of neurofilaments in the synaptic terminals.

Leupeptin was injected into the right optic tectum of goldfish maintained at 15° or at 25°C at a dose of 1.2 mg per 100 g of body weight. Depending on weight, 5 to 12 μl of a solution of leupeptin in teleost saline (50 mg/ml) (*15*) was injected. Control fish received saline

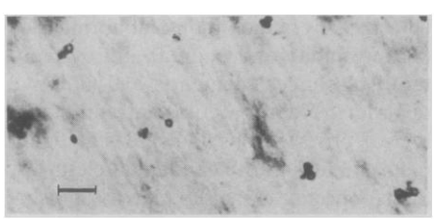

Fig. 1. Optic tectum 72 hours after injection of leupeptin in a goldfish kept at 15°C. Neurofibrillary rings are darkly stained. Scale bar, 5 μm.

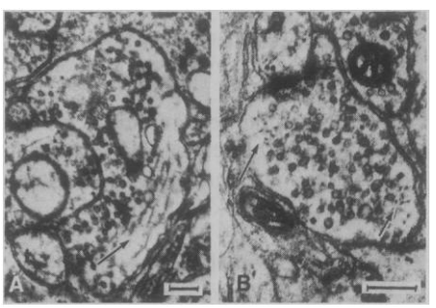

Fig. 2. Synaptic terminals in the optic tectum 48 hours after injection of leupeptin in a goldfish kept at 25°C. (A) Neurofilaments seen in a longitudinal plane (arrow). (B) Two groups of transversely sectioned neurofilaments approximately 1 μm apart (arrows). These represent a cross section of a neurofibrillary ring seen by light microscopy (Fig. 1). Scale bars, 300 nm.

alone (*16*). The fish were killed 48 or 72 hours later, and the brains were removed and fixed in 2.5 percent glutaraldehyde in Karlsson and Schultz's phosphate buffer (*17*) (36 hours at 4°C). The injected tectum was bisected sagittally, with one-half processed for light microscopy and one-half for electron microscopy. For light microscopy, serial sagittal sections (10 μm) were stained by a modification (*7*) of the silver impregnation method of Holmes (*18*). Neurofibrillary rings, approximately 1 μm in diameter, that appeared to be identical to those occurring in fish kept at 5°C for prolonged periods were observed in the fish receiving leupeptin but not in the controls (Fig. 1). Moreover, electron microscopy revealed neurofilaments in the synaptic terminals of the leupeptin-treated fish but not of the control fish (Fig. 2). Thus, the injection of leupeptin into the optic tectum does result in the accumulation of neurofilaments. These observations not only support the hypothesis that the rings appearing in fish after long periods at 5°C are the result of the inhibition of a protease, but provide evidence in vivo in support of the Lasek-Hoffman hypothesis. Neurotubules were also observed in the terminals of leupeptin-treated fish. They occur also in some terminals of fish exposed to 5°C for long periods. There is evidence from studies in vitro that under some conditions the calcium-activated protease found in the peripheral nerve of rats may degrade microtubule as well as neurofilament protein (*14*). Hence the disaggregation of microtubules in axon terminals may be brought about by both a higher calcium ion concentration, as postulated by Lasek and Hoffman (*2*), and the action of a calcium-activated protease as indicated by the present experiments.

The regulation of neurofilament degradation has many implications for neuropathology (*19*). Studies in which leupeptin is used in vivo may help to answer questions about how accumulations of neurofilaments affect axoplasmic transport and synaptic transmission. The loss of plasticity in the nervous system during development and aging has been related to changes in cytoskeletal elements (*20*); the use of leupeptin in vivo and in tissue culture should facilitate the elucidation of the regulatory mechanisms involved. Regeneration, another expression of neural plasticity, could be studied by observing the effects of leupeptin on the reestablishment of optic axon connections in the optic tectum during regeneration of the optic nerve of goldfish. Thus, the demonstration that leupeptin inhibits endogeneous calcium-activated proteases in vivo provides a pharmacological tool that may be used to answer questions on the control of the growth of axons in normal and pathological conditions and on the plasticity of the nervous system (*21*).

BETTY I. ROOTS
Department of Zoology and *Erindale College, University of Toronto, Mississauga, Ontario, Canada L5L 1C6*

References and Notes

1. P. A. Weiss and H. B. Hiscoe, *J. Exp. Zool.* **107**, 315 (1948); B. Droz and C. P. Leblond, *J. Comp. Neurol.* **121**, 325 (1963); R. J. Lasek, *Int. Rev. Neurobiol.* **13**, 289 (1970); P. N. Hoffman and R. J. Lasek, *J. Cell Biol.* **66**, 351 (1975).
2. R. J. Lasek and P. N. Hoffman, in *Cell Motility*, R. Goldman, T. Pollard, J. Rosenbaum, Eds. (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1976), p. 1021.
3. E. J. Gray and R. W. Guillery, *J. Physiol. (London)* **157**, 581 (1961).
4. Evidence for the equivalence of neurofibrillary (argyrophilic) rings and annuli of neurofilaments was presented by E. G. Gray and R. W. Guillery (*3*) and in the papers listed in (*5*). The observation that Bodian's silver method is specific for neurofilament proteins [P. Gambetti, L. Autilio-Gambetti, S. Ch. Papasozomenos, *Science* **213**, 1521 (1981)] and the electron microscopic examination of identified silver stained rings (R. L. Bondar and B. I. Roots, in preparation) establishes the equivalence beyond doubt.
5. M. Colonnier and R. W. Guillery, *Z. Zellforsch. Mikrosk. Anat.* **62**, 333 (1964).
6. B. B. Boycott, E. G. Gray, R. W. Guillery, *Proc. R. Soc. London Ser. B* **154**, 151 (1961); H. D. Potter, *J. Neurocytol.* **2**, 29 (1973); ——— and G. S. Hafner, *J. Comp. Neurol.* **155**, 409 (1975).
7. R. L. Bondar and B. I. Roots, *Exp. Brain Res.* **30**, 577 (1977).
8. B. I. Roots and R. L. Bondar, *J. Neuropathol. Exp. Neurol.* **36**, 453 (1977).
9. D. S. Gilbert, B. J. Newby, B. H. Anderton, *Nature (London)* **256**, 586 (1975).
10. B. Katz and R. Miledi, *J. Physiol. (London)* **207**, 789 (1970); R. Llinás, J. R. Blinks, C. Nicholson, *Science* **176**, 1127 (1972).
11. P. R. Baker, A. L. Hodgkin, E. B. Ridgway, *J. Physiol. (London)* **218**, 709 (1971); A. L. Hodgkin and R. D. Keynes, *ibid.* **138**, 253 (1957).
12. R. J. Lasek and M. M. Black [in *Mechanisms, Regulation, and Special Functions of Protein Synthesis in the Brain*, S. Roberts, A. Lajtha, W. H. Gispen, Eds. (Elsevier, Amsterdam, 1977), p. 161] showed that cytoskeletal proteins are not degraded in transit as suggested by Weiss and Hiscoe (*1*) and must therefore be disassembled when they reach the terminals.
13. W. W. Schlaepfer and M. B. Hasler, *Brain Res.* **168**, 299 (1979); M. N. Malik *et al.*, *Life Sci.* **29**, 795 (1981).
14. K. Kamakura, S. Ishiura, H. Sugita, Y. Toyokura, *J. Neurochem.* **40**, 908 (1983).
15. J. Z. Young, *Pubbl. Stn. Zool. Napoli* **12**, 425 (1933).
16. Forty-two experimental and six control fish were used. Two of the experimental fish died.
17. U. Karlsson and R. L. Schultz, *J. Ultrastruct. Res.* **12**, 160 (1965).
18. W. Holmes, *Anat. Rec.* **86**, 157 (1943).
19. W. S. Schlaepfer, *Progress in Neuropathology*, H. M. Zimmerman Ed. (Raven, New York, 1979), vol. 4, p. 101.
20. R. J. Lasek and J. R. Morris, in *Biological Functions of Microtubules and Related Structures*, H. Sakai and H. Mohri, Eds. (Academic Press, New York, 1982), p. 329.
21. This work was reported at the meeting of the American Society for Neurochemistry in March 1983 [B. I. Roots, *Trans. Am. Soc. Neurochem.* **14**, 216 (1983)].
22. I thank K. Gaebel and R. C. Cameron for technical assistance, and the United States–Japan Cooperative Cancer Research Program for providing the leupeptin used in the preliminary experiments. Supported by grant A-6052 from the National Science and Engineering Research Council of Canada.

3 May 1983

Regional Brain Concentrations of Neuropeptides in Huntington's Chorea and Schizophrenia

Abstract. *To ascertain whether Huntington's chorea and schizophrenia are associated with specific regional alterations in neurotensin, somatostatin, and thyrotropin-releasing hormone, the concentrations of these putative neurotransmitters were measured by radioimmunoassay in postmortem brain samples from patients with Huntington's chorea or schizophrenia. Compared to 50 patients without psychiatric or neurological disease, the patients with Huntington's chorea showed significantly elevated concentrations of all three neuropeptides in the nucleus caudatus. In the nucleus accumbens somatostatin levels were increased threefold, while in the amygdala thyrotropin-releasing hormone levels were elevated. In contrast, the schizophrenics exhibited reduced levels of thyrotropin-releasing hormone in two frontal cortical regions, reduced somatostatin levels in one frontal cortical area, and increased neurotensin levels in one frontal cortical area. None of the differences between the diseased brains and the controls could be accounted for by differences in age, sex, or time between death and autopsy.*

Anticipating that the etiology of Huntington's chorea and schizophrenia might be elucidated, as was Parkinson's disease (*1*), by detailed biochemical analysis of postmortem brain samples, researchers have sought for biochemical abnormalities in the brains of patients with these disorders. Abnormal concentrations of some neurotransmitters have been observed, but the pathophysiological significance of this is not known. Huntington's chorea is inherited as an autosomal dominant trait (*2*) and is characterized by major motor abnormalities (hypertonicity and chorea) and by mental disturbances (dementia and affective disorder). Neuropathological and radiological observations indicate significant neuronal loss in the caudate nucleus (*2*). Schizophrenia, a major behavioral disorder that affects approximately 0.6 percent of the population (*3*), may be associated with neuropathological alterations of the limbic forebrain. Its active phase