Lesions to the mesencephalic reticular formation attenuate long-term habituation of the acoustic startle response (*6, 13*), but to a much lesser extent than did our lesions to the cerebellar vermis. In addition, the decerebrate rat with an intact cerebellum shows no long-term but significant short-term habituation in this response system (*14*). If we assume that these various lesions interfere with the same pathway, a useful working assumption, we can conclude that structures rostral to the cerebellum are involved and that the vermis is an area of convergence or focus, but we do not know whether the vermis is on the ascending or descending limb of the pathway. There are numerous pathways into and out of the cerebellum that could mediate these effects. There are rich auditory projections to the vermis (*15, 16*), which were removed by our vermal aspirations. The vermis projects widely into the reticular formation and throughout the brainstem and receives rich projections from more rostral brain areas (*17*). Whatever the rostral limb of this pathway may be, it is not the classical auditory pathway (*18*). It is interesting to note that the cerebellar vermis, in contrast to the cerebellar hemispheres and related structures, is not directly involved in conditioning of the rabbit eyelid response to an acoustic stimulus (*10*).

Our data contribute to the evidence that the cerebellum is involved in subtle and complex ways in many behavioral processes (*19*). Snider and Stowell (*16*) noted no sensory defects following cerebellar damage in spite of its rich auditory, visual, and tactile projection areas. They wondered "... whether loss of the cerebellar representations of these three exteroceptive systems does not produce objective and subjective effects which are so subtle that they have escaped present methods of study." We believe long-term habituation may be one of those subtle behavioral effects.

REFERENCES AND NOTES

1. R. F. Thompson and W. A. Spencer, *Psychol. Rev.* **73**, 16 (1966); P. M. Groves and R. F. Thompson, *ibid.* 77, 419 (1970).
2. W. H. Thorpe, *Learning and Instinct in Animals* (Harvard Univ. Press, Cambridge, MA, 1956).
3. H. D. Kimmel, in *Habituation*, vol. 1, *Behavioral Studies*, H. V. S. Peeke and M. J. Herz, Eds. (Academic Press, New York, 1973), p. 219; L. Petrinovich, *ibid.*, p. 141.
4. M. Davis, *J. Comp. Physiol. Psychol.* **78**, 260 (1972); S. E. File, *Q. J. Exp. Psychol.* **25**, 96 (1973); R. N. Leaton and W. P. Jordan, *J. Comp. Physiol. Psychol.* **92**, 803 (1978).
5. R. N. Leaton, *J. Comp. Physiol. Psychol.* **87**, 1157 (1974); *J. Exp. Psychol. Anim. Behav. Processes* **2**, 248 (1976).
6. W. P. Jordan and R. N. Leaton, *J. Comp. Physiol. Psychol.* **96**, 170 (1982); *Behav. Neurosci.* **97**, 710 (1983).
7. M. Davis *et al.*, *J. Neurosci.* **2**, 791 (1982).
8. R. F. Thompson *et al.*, *Annu. Rev. Neurosci.* **6**, 447 (1983).
9. E. R. Kandel and J. H. Schwartz, *Science* **218**, 433 (1982).
10. D. A. McCormick and R. F. Thompson, *ibid.* **223**, 296 (1984).
11. D. A. Haley *et al.*, *Soc. Neurosci. Abstr.* **9**, 643 (1983); D. G. Lavond *et al.*, *ibid.*, p. 636.
12. A similar startle apparatus has been described previously (*5*). The 20 by 12 by 14 cm steel-rod and Plexiglas chamber was sandwiched between compression springs attached to a rigid superstructure. Vertical displacement of the chamber induced a voltage in the attached transducer which was digitized, rectified, and integrated by a microcomputer system into 200-msec epochs. Startle amplitude was taken as the difference between the computer output for the 200-msec epochs before and after stimulus onset.
13. D. S. Leitner *et al.*, *Physiol. Behav.* **26**, 259 (1981).
14. R. N. Leaton *et al.*, *Behav. Neurosci.* **99**, 901 (1985).
15. R. S. Dow and R. Anderson, *J. Neurophysiol.* **5**, 363 (1942); C.-M. Huang *et al.*, *Brain Res.* **244**, 1 (1982).
16. R. S. Snider and A. Stowell, *J. Neurophysiol.* 7, 331 (1944), p. 351.
17. S. L. Palay and V. Chan-Palay, *Cerebellar Cortex* (Springer-Verlag, Berlin, 1974).
18. W. P. Jordan and R. N. Leaton, *Physiol. Behav.* **28**, 253 (1982).
19. P. J. Watson, *Psychol. Bull.* **85**, 944 (1978).
20. L. J. Pellegrino *et al.*, *A Stereotaxic Atlas of the Rat Brain* (Plenum, New York, ed. 2, 1979).

21. This research was supported in part by a grant from the Faculty Research Committee of Dartmouth College. R.N.L. was an Honorary Research Fellow in the Department of Anatomy and Embryology, University College London, London, England, during preparation of the manuscript. We thank that department for support and assistance and J. O'Keefe, S. File, M. Fanselow, and T. Tighe for reading a version of this manuscript.

22 August 1985; accepted 15 January 1986

Calcium Antagonist Receptors in Cardiomyopathic Hamster: Selective Increases in Heart, Muscle, Brain

JOHN A. WAGNER, IAN J. REYNOLDS, HARLAN F. WEISMAN, PAMELA DUDECK, MYRON L. WEISFELDT, SOLOMON H. SNYDER*

The Syrian cardiomyopathic hamster has a hereditary disease in which a progressive myocardial necrosis mimics human forms of cardiac hypertrophy. Lesions are associated with calcium overload and can be prevented with the calcium antagonist verapamil. Numbers of receptor binding sites for calcium antagonists in heart, brain, skeletal muscle, and smooth muscle were markedly increased in cardiomyopathic hamsters. The uptake of calcium-45 into brain synaptosomes was also increased in cardiomyopathic hamsters. The increase in calcium antagonist receptors and related voltage-sensitive calcium channels may be involved in the pathogenesis of this cardiomyopathy.

THE SYRIAN CARDIOMYOPATHIC (CM) hamster (BIO 14.6, Bio Research, Cambridge, Massachusetts) is an inbred strain with a hereditary abnormality in skeletal and cardiac muscle involving ventricular and atrial hypertrophy with subsequent development of congestive heart failure (*1*). This strain has been used as a model for certain disturbances, such as hypertrophic obstructive cardiomyopathy and Freidrich's ataxia, the latter involving brain pathology as well as cardiac hypertrophy (*2*). Calcium overload of myocytes has been implicated in the etiology of the cardiac abnormalities in these hamsters. The calcium concentration in cardiac myocytes of CM hamsters is elevated (*3*), and calcium antagonist drugs such as verapamil are the most effective agents in relieving cardiac dysfunction (*4*). Calcium entry into myocytes and neurons (*5*) occurs through voltage-sensitive calcium channels (VSCC), which are blocked by calcium antagonist drugs of several classes including nitrendipine, verapamil, and diltiazem. Specific receptors for these drugs can be labeled with radioligand binding techniques (*5, 6*) and voltage-dependent calcium entry measured by $^{45}Ca^{2+}$ flux determinations. We now report a selective increase in numbers of calcium antagonist receptors in heart, brain, skeletal muscle, and smooth muscle of CM hamsters. In addition, synaptosomal preparations from CM hamster brain show increased calcium uptake, suggesting a link to VSCC.

[^{3}H]Nitrendipine labels the dihydropyridine class of calcium antagonist receptors (*5*). Both heart and brain exhibit 50 to 100 percent increased [^{3}H]nitrendipine binding in 30-day-old CM hamsters compared with age- and sex-matched random-bred controls (Fig. 1). Scatchard analysis indicates that the augmentation is in the number of binding sites (B_{max}) with virtually no change in affinity (K_D). [^{3}H]Desmethoxyverapamil labels high- and low-affinity forms of a second type of calcium antagonist receptor, which is allosterically linked to the dihydropyridine site (*6*). Saturation analysis reveals more than a twofold increase in numbers of both the high- and low-affinity [^{3}H]desmethoxyverapamil sites in brain. Binding affinity is decreased, but to a lesser extent. Because of

J. A. Wagner, I. J. Reynolds, S. H. Snyder, Departments of Neuroscience, Pharmacology and Experimental Therapeutics, and Psychiatry and Behavioral Sciences, Johns Hopkins Medical Institutions, Baltimore, MD 21205. H. F. Weisman, P. Dudeck, M. L. Weisfeldt, Division of Cardiology, Department of Medicine, Johns Hopkins Medical Institutions, Baltimore, MD 21205.

*To whom correspondence should be addressed.

high levels of nonspecific binding, we were unable to detect a low-affinity site in heart. However, B_{max} of the high-affinity site was doubled, as in brain.

CM hamsters display abnormalities in other types of muscle as well as cardiac muscle (*1*). Calcium antagonist receptor binding has been reported in skeletal and smooth muscles (*7*). Both skeletal and smooth muscles exhibited increased levels of [^{3}H]nitrendipine binding in CM hamsters compared with age- and sex-matched controls (Table 1). Scatchard analysis indicated that the augmentation was in B_{max} with no statistically significant change in K_D.

Dihydropyridine receptor binding is inhibited allosterically by verapamil and related drugs, whereas the calcium antagonist diltiazem and similar agents increase binding (*5*). To ascertain whether the allosteric regulation of dihydropyridine binding is altered in CM hamsters, we explored the effects of methoxyverapamil (gallopamil, D-600), the dihydropyridine nifedipine, and fostedil (BK-966), a diltiazem-like drug (*8*). Inhibition of [^{3}H]nitrendipine binding by methoxyverapamil, stimulation by fostedil, and inhibition by nifedipine display essentially identical concentration-response curves in CM and control hamsters (Fig. 2), suggesting that presumed subunit interactions of the receptor complex are not altered.

To determine whether these abnormalities are selective for calcium antagonist receptors, we measured ligand binding to several neurotransmitter receptors (Table 1). In the brain, [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB) binding to muscarinic cholinergic receptors, [^{3}H]WB-4101 binding to α_1 adrenoceptors, [^{3}H]spiperone binding to dopamine D_2 receptors, [^{3}H]phenylisopropyladenosine ([^{3}H]PIA) binding to adenosine A_1 receptors, and ^{125}I-cyanopindolol binding to β-adrenoceptors was essentially the same in CM hamsters as in control animals. In the heart we also found no statistically significant difference between CM hamsters and controls in [^{3}H]QNB or ^{125}I-cyanopindolol binding.

To ascertain whether other membrane proteins associated with the control of ion flux are altered, we monitored [^{3}H]phorboldibutyrate ([^{3}H]PDBU) binding to protein kinase C, a calcium-regulated site, and [^{3}H]ouabain binding to Na^+,K^+-dependent adenosine triphosphatase (ATPase) in both brain and heart (Table 1). Binding associated with protein kinase C and the sodium-potassium ATPase is essentially the same in CM hamsters and controls for both brain and heart.

If the augmented numbers of calcium antagonist receptors reflect an increase in the number or function of voltage-dependent calcium channels, one might anticipate an increase in voltage-dependent calcium fluxes. Accordingly, we measured the uptake of $^{45}Ca^{2+}$ in synaptosomal preparations from the brain (Fig. 3); $^{45}Ca^{2+}$ uptake in synaptosomes in the presence of 5 m*M* potassium was essentially the same in CM hamsters and controls. To assess voltage-dependent calcium flux, we measured the uptake of $^{45}Ca^{2+}$ after depolarizing the synaptosomal preparations with 57.5 m*M* potassium. In depolarized synaptosomes, $^{45}Ca^{2+}$ uptake was increased significantly in CM hamsters compared with controls. However, the technique used to assess $^{45}Ca^{2+}$ flux in synaptosomes does not differentiate between uptake through VSCC and transport mediated by the sodium-calcium antiport system.

A critical question is whether the observed abnormalities are secondary to cardiac damage. This is improbable for two reasons. The cardiac pathology becomes most apparent after 35 to 40 days of age (*1*). We compared calcium antagonist receptor binding and $^{45}Ca^{2+}$ synaptosomal uptake in 30-day, 60-day, and 90-day-old hamsters. [^{3}H]Nitrendipine binding and synaptosomal $^{45}Ca^{2+}$ uptake were augmented to essentially the same extent in animals at all three ages. Examination of hearts from these hamsters, by light microscopy, confirms that cardiac abnormalities are minimal in 30-day

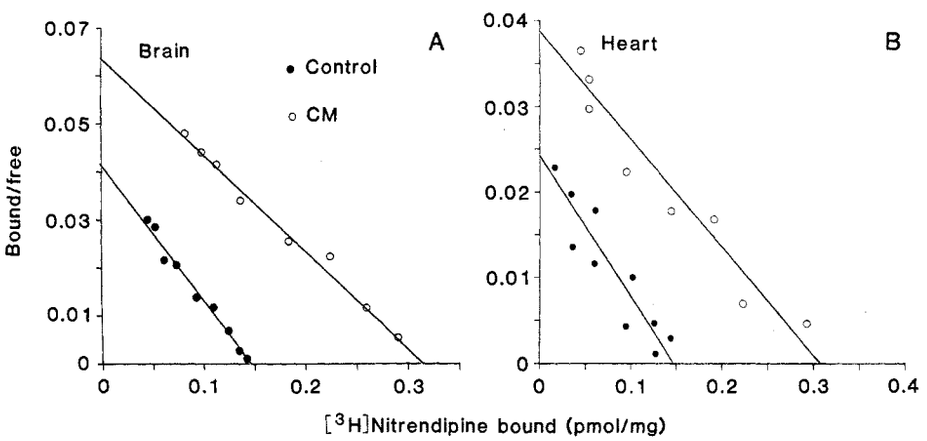

Fig. 1. Saturation analysis of [^{3}H]nitrendipine binding in (A) brain and (B) heart of control (●) and CM (○) hamsters. [^{3}H]Nitrendipine binding was assayed (*15*) with 0.2 to 0.5 mg of protein per tube. The concentration of nitrendipine varied between 0.05 and 100 n*M*. The data shown represent typical experiments, performed in duplicate. Experiments were repeated five to seven times. The total number of binding sites (B_{max}) increased in CM hamsters with no change in affinity (Student's *t* test, two-tailed, $P < 0.05$).

Table 1. Calcium antagonist drug-binding constants in cardiomyopathic hamsters. Nitrendipine binding was assayed as described in the legend to Fig. 1. Skeletal and smooth muscle membranes were prepared as described (*16*). (−)Desmethoxyverapamil binding was assayed in similar membrane preparations by existing methods (*17*). The results are expressed as mean ± SEM for groups of eight to ten animals of each strain for each tissue. No statistically significant differences in labeling with the following ligands was seen between control and CM hamsters in brain ($P > 0.2$): [^{3}H]quinuclidinyl benzylate, [^{3}H]WB-4101, [^{3}H]phorbol dibutyrate, [^{3}H]spiperone, [^{3}H]1-phenylisopropyladenosine, [^{3}H]ouabain, and ^{125}I-iodocyanopindolol, and in heart: [^{3}H]quinuclidinyl benzylate, [^{3}H]phorbol dibutyrate, [^{3}H]ouabain, and ^{125}I-iodocyanopindolol (*18*). These ligands were measured in four to six animals of each strain. Comparisons between CM and control hamsters were made with two-tailed Student's *t* tests.

Tissue	Ligand	Affinity binding sites	K_D (n*M*)		B_{max} (fmol/mg protein)	
			Control	CM	Control	CM
Brain	[^{3}H]nitrendipine		0.32 ± 0.04	0.30 ± 0.03	115 ± 7	184 ± 12*
	[^{3}H]desmethoxy-verapamil	High	0.66 ± 0.05	1.25 ± 0.15†	270 ± 19	710 ± 48*
		Low	32 ± 7	96 ± 13†	3400 ± 561	7610 ± 928‡
Heart	[^{3}H]nitrendipine		0.60 ± 0.13	0.46 ± 0.14	187 ± 36	355 ± 44‡
	[^{3}H]desmethoxy-verapamil	High	1.64 ± 0.41	1.69 ± 0.79	79 ± 35	158 ± 54
Skeletal muscle	[^{3}H]nitrendipine		4.4 ± 0.6	4.4 ± 0.9	1500 ± 205	2300 ± 320†
Smooth muscle	[^{3}H]nitrendipine		1.2 ± 0.2	1.0 ± 0.1	250 ± 57	483 ± 11‡

*$P < 0.001$. †$P < 0.05$. ‡$P < 0.01$.

hamsters and become progressively severe in 60- and 90-day animals. In both 60- and 90-day animals, extensive tissue necrosis and infiltration with inflammatory cells was apparent, whereas such abnormalities were much reduced in 30-day hamsters.

Second, the fact that abnormalities in calcium antagonist receptors and $^{45}Ca^{2+}$ uptake occur in the brain indicates that the described abnormalities are not secondary to cardiac pathology. It is not yet possible to link conclusively distinct behavioral patterns to occupation of calcium antagonist receptors in brain. However, various studies suggest that calcium channel blockers and activators can influence behavior (*9*). Because of the increased levels of calcium antagonist receptors in brain, the CM strain may be useful for assessing the behavioral significance of VSCC in brain. Preliminary analysis of open-field behavior reveals a higher level of general activity in CM hamsters than in controls. Friedrich's ataxia, a genetic disorder with cardiac hypertrophy resembling the abnormalities in CM hamsters, also involves central nervous system disorders.

The augmented number of calcium antagonist receptor binding sites and the increased voltage-dependent calcium accumulation in CM hamster tissues suggest that these abnormalities may be responsible for the pathologic alterations. The increased influx of calcium through VSCC may cause calcium overload, which appears linked to the cardiac pathology in CM hamsters. Histologically, Syrian hamster cardiomyopathy resembles reperfusion necrosis, which is associated with calcium overload (*10*). In addition, isoproterenol stimulates calcium influx and produces a cardiomyopathy that closely resembles the abnormalities in CM hamsters (*11*). Endogenous calcium levels are greatly increased in hearts of CM hamsters (*3*). CM hamsters display a prolonged cardiac action potential that seems to be related to augmented activity of voltage-dependent calcium channels (*12*). Finally, of the many drugs that have been administered to CM hamsters, calcium antagonists have been the most effective in relieving abnormalities in cardiac function, whereas the β-adrenoceptor antagonist propranolol is ineffective (*4*). Similar abnormalities in VSCC's may be involved in human conditions, such as hypertrophic obstructive cardiomyopathy, whose pathophysiology resembles that of CM hamsters.

Could the increased number of calcium channels be secondary to some other primary abnormality? A reduced activity of sodium-potassium ATPase has been reported in CM hamsters (*13*). This reduced catalytic activity of the enzyme is not necessarily incompatible with the normal levels of [^{3}H]ouabain binding observed in this study, as these reflect the number of enzyme molecules. Conceivably, the sodium-potassium ATPase changes are secondary to calcium overload of cells, causing mitochondrial damage and thus depleting energy resources. Microvascular spasm followed by reperfusion might cause calcium overload. Although no specific histological abnormalities in microvascular structure have been identified, functional spasm of the microcirculation has been demonstrated (*14*). Increased microvascular spasm observed in CM hamsters may be secondary to the augmented voltage-dependent calcium uptake by vascular smooth muscle. It has not yet been possible to distinguish between a secondary effect on heart by vascular smooth muscle or a direct effect on heart. This issue can be addressed by calcium flux studies.

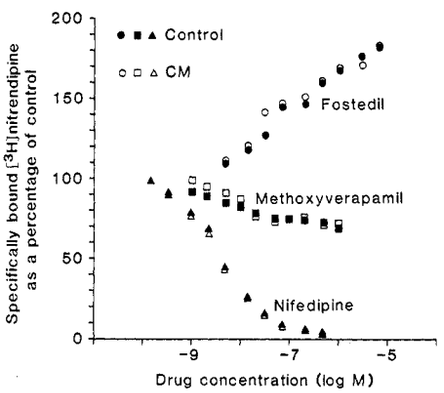

Fig. 2. Interaction of drugs that allosterically modulate [^{3}H]nitrendipine binding in brain membranes. Interactions with the dihydropyridine-labeled receptor complex were similar in control and CM hamsters.

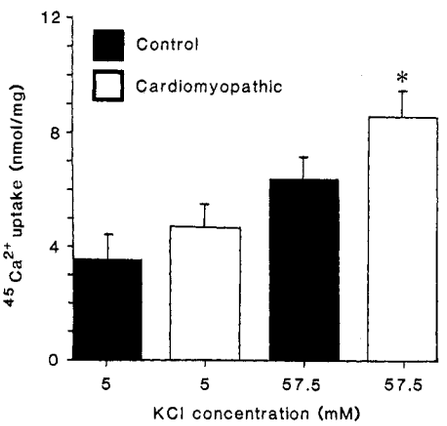

Fig. 3. Synaptosomal $^{45}Ca^{2+}$ uptake, measured as described (*19*). Values are the means of eight to ten experiments performed in triplicate, presented as mean ± SEM. The asterisk indicates a significant difference from control, Student's *t* test, two-tailed, $P < 0.05$.

REFERENCES AND NOTES

1. F. Homberger, J. R. Baker, C. W. Nixon, R. Whitney, *Med. Exp.* **6**, 339 (1962); F. Homberger and E. Bajusz, *J. Am. Med. Assoc.* **212**, 604 (1970); E. W. Gertz, *Progr. Exp. Tumor Res.* **16**, 242 (1972); G. Jasmin and L. Proschek, *Muscle Nerve* **5**, 20 (1982).
2. J. Azari, T. Reisine, A. Barbeau, H. I. Yamamura, R. Huxtable, *J. Can. Sci. Neurol.* **6**, 223 (1979); H. J. Smith and A. Nuttall, *Cardiovasc. Res.* **19**, 181 (1985).
3. E. Bajusz and K. Lossnitzer, *Trans. N.Y. Acad. Sci. Ser. II* **30**, 939 (1968); K. Lossnitzer, J. Janke, B. Hein, M. Stauch, A. Fleckenstein, *Recent Adv. Stud. Card. Struct. Metab.* **6**, 207 (1975).
4. G. Jasmin and E. Bajusz, *Recent Adv. Stud. Card. Struct. Metab.* **6**, 219 (1975); J.-L. Rouleau *et al.*, *Circ. Res.* **50**, 405 (1982).
5. F. J. Ehlert, W. R. Roeske, E. Itoga, H. I. Yamamura, *Life Sci.* **30**, 2191 (1982); J. G. Sarmiento, R. A. Janis, R. A. Colvin, D. J. Triggle, A. M. Katz, *J. Mol. Cell Cardiol.* **15**, 135 (1983); K. M. M. Murphy, R. J. Gould, B. L. Largent, S. H. Snyder, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 860 (1983); H. Glossmann, D. R. Ferry, A. Goll, M. Rombusch, *J. Cardiovasc. Res.* **6**, S608 (1984).
6. A. Goll, D. R. Ferry, J. Striessnig, M. Schober, H. Glossmann, *FEBS Lett.* **176**, 371 (1984); D. R. Ferry, A. Goll, C. Gadow, H. Glossmann, *Naunyn-Schmiedeberg's Arch. Pharmacol.* **327**, 183 (1984); J. Ptasienski, K. K. McMahon, M. M. Hosey, *Biochem. Biophys. Res. Commun.* **129**, 910 (1985); I. J. Reynolds, A. M. Snowman, S. H. Snyder, in preparation.
7. M. Fosset, E. Jaimovitch, E. Delpont, M. Lazdunski, *J. Biol. Chem.* **258**, 6086 (1983); J.-P. Galizzi, M. Fosset, M. Lazdunski, *Eur. J. Biochem.* **144**, 211 (1984); G. T. Bolger *et al.*, *J. Pharmacol. Exp. Ther.* **225**, 291 (1983); A. K. Grover, G. Y. Kwan, E. Luchowski, E. E. Daniel, D. J. Triggle, *J. Biol. Chem.* **259**, 2223 (1984).
8. T. Morita, T. Kanazawa, K. Ito, T. Nose, *Arzneim. Forsch.* **32**, 1043 (1982); R. F. Kauffman, B. G. Conery, D. W. Robertson, *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **43**, 449 (1984).
9. S. L. Dubovsky, R. D. Franks, M. Lifschitz, P. Cohen, *Am. J. Psychiatry* **139**, 502 (1982); R. J. Gould, K. M. M. Murphy, I. J. Reynolds, S. H. Snyder, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 5122 (1983); *Am. J. Psychiatry* **141**, 352 (1984); A. J. Giannini, W. L. Houser, R. H. Loiselle, M. C. Giannini, W. A. Price, *ibid.*, p. 1602; W. B. Mendelson *et al.*, *Sleep* **7**, 64 (1984); G. T. Bolger, B. A. Weissman, P. Skolnick, *Naunyn-Schmiedeberg's Arch. Pharmacol.* **328**, 373 (1985).
10. A. L. Shen and R. B. Jennings, *Am. J. Pathol.* **67**, 417 (1977).
11. S. Bloom and D. L. Davis, *ibid.* **69**, 459 (1972); A. Fleckenstein, J. Janke, H. J. Doring, O. Pachinger, *Recent Adv. Stud. Card. Struct. Metab.* **2**, 455 (1973); M. C. Welty, J. D. Welty, M. J. McBroom, *J. Mol. Cell Cardiol.* **14**, 353 (1982).
12. K. L. Rossner and H. G. Sachs, *Cardiovasc. Res.* **12**, 436 (1978).
13. V. Panagia *et al.*, *ibid.* **18**, 567 (1984).
14. S. M. Factor, T. Minase, S. Cho, R. Dominitz, E. H. Sonnenblick, *Circulation* **66**, 342 (1982).
15. [^{3}H]Nitrendipine assays were performed as described [R. J. Gould, K. M. M. Murphy, S. H. Snyder, *Mol. Pharmacol.* **25**, 235 (1984)]. Tissues were removed and rinsed in ice-cold 0.9% NaCl. Organs were then homogenized in 50 m*M* hydroxyethylpiperazine ethanesulfonic acid (Hepes), *p*H 7.4, with a Brinkmann Polytron at half-maximal setting for 15 seconds. Homogenates were then filtered through four layers of cheesecloth and centrifuged at 48,000*g* for 15 minutes. Pellets were washed twice with Hepes before being used. Diluted membrane preparations (0.2 to 0.5 mg of protein per tube) were incubated with 0.05 to 100 n*M* nitrendipine (New England Nuclear; original specific activity 81.3 Ci/mmol) in the presence or absence of 500 n*M* unlabeled nitrendipine to define nonspecific binding. Assays were terminated after 1 hour at room temperature (22° to 24°C) by filtration over glass fiber filter strips (No. 32, Schleicher & Schuell, Keene, NH). Filters were washed with three 4-ml aliquots of ice-cold 50 m*M* NaCl and counted with a Beckman LS2800 liquid scintillation counter at an efficiency of 55%. An iterative curve-fitting program was used to perform the saturation analyses [G. A. McPherson, *Comput. Programs Biomed.* **67**, 107 (1983)].

16. Hamster hind-leg skeletal muscle and the smooth-muscle portion of esophagus were removed and rinsed in ice-cold 0.9% NaCl. Muscles were homogenized in 260 m*M* sucrose, 50 m*M* Hepes, pH 7.4, through the use of a Brinkmann Polytron at half-maximal setting for 30 seconds. Homogenates were centrifuged at 1000*g* for 10 minutes. The supernatant was filtered through four layers of cheesecloth and centrifuged at 10,000*g* for 20 minutes. The supernatant was centrifuged at 100,000*g* for 60 minutes. The resulting pellet was used for [^{3}H]nitrendipine binding assays as above. Other smooth muscles including bladder were prepared as above and assayed for [^{3}H]nitrendipine binding. These tissues also displayed the same trend of increased binding in the CM hamsters compared with controls.
17. Tissue for (−)desmethoxyverapamil binding was prepared as described (*14*). Protein (0.5 to 1.0 mg) was added to tubes containing 0.1 to 200 n*M* (−)[^{3}H]desmethoxyverapamil (a gift from Knoll AB, Ludwigshafen, FRG, original specific activity 83 Ci/mmol) in the presence or absence of 1 μ*M* methoxyverapamil. After 1 hour at room temperature (22° to 24°C) reactions were terminated by filtration over glass fiber filters pretreated with 0.5% polyethyleneimine to reduce nonspecific filter binding. Membranes were washed and trapped radioactivity determined as above.
18. D. Landowne and J. M. Ritchie, *J. Physiol. (London)* **207**, 529 (1970); H. I. Yamamura and S. H. Snyder, *Proc. Natl. Acad. Sci. U.S.A.* **71**, 1725 (1974); D. C. U'Prichard, D. A. Greenberg, S. H. Snyder, *Mol. Pharmacol.* **13**, 454 (1977); P. E. Dreidger and P. M. Blumberg, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 567 (1980); K. M. M. Murphy and S. H. Snyder, *Mol. Pharmacol.* **22**, 250 (1982); S. L. Petrovic, J. K. McDonald, G. D. Snyder, S. M. McCann, *Brain Res.* **261**, 243 (1983).
19. Synaptosomes were prepared by the method of Hajos [*Brain Res.* **93**, 485 (1975)]. Membranes, removed from the 0.32 to 0.8*M* sucrose interface, were diluted by 2.5- to 3-fold with nondepolarizing (basal) buffer (composition, m*M*: NaCl, 145; KCl, 5; $CaCl_2$, 1.2; $MgCl_2$, 1.2; Hepes, 10: *p*H adjusted to 7.4 with Trizma) and centrifuged at 9,000g for 20 minutes. The pellet was resuspended in basal buffer and preincubated at 30°C for 20 minutes. Uptake of $^{45}Ca^{2+}$ was initiated by addition of 100-μl aliquots of tissue (1 to 2 mg of protein) to 0.9 ml basal or stimulating (in which 57.5 m*M* KCl was iso-osmotically substituted for NaCl) buffer containing $^{45}Ca^{2+}$. Reactions were terminated after 10 seconds by the addition of 4 ml of ice-cold basal buffer followed by filtration over glass fiber filters. Trapped radioactivity was assessed as above. Voltage-stimulated uptake was defined as the difference between accumulated $^{45}Ca^{2+}$ in the presence of stimulating and basal buffer.
20. Supported by Ischemic Heart Disease Specialized Center of Research grant HL-17655 to M.L W. and USPHS grants MH-18501 and NS-16375 and research scientist award DA-00074 to S.H.S. J.A.W. was supported by an NSF graduate fellowship. We thank A. M. Snowman for technical assistance and D. C. Dodson for secretarial assistance.

16 September 1985; accepted 27 December 1985

Gene Transfer and Molecular Cloning of the Human NGF Receptor

MOSES V. CHAO, MARK A. BOTHWELL, ALONZO H. ROSS, HILARY KOPROWSKI, ANTHONY A. LANAHAN, C. RANDALL BUCK, AMITA SEHGAL

Nerve growth factor (NGF) and its receptor are important in the development of cells derived from the neural crest. Mouse L cell transformants have been generated that stably express the human NGF receptor gene transfer with total human DNA. Affinity cross-linking, metabolic labeling and immunoprecipitation, and equilibrium binding with ^{125}I-labeled NGF revealed that this NGF receptor had the same size and binding characteristics as the receptor from human melanoma cells and rat PC12 cells. The sequences encoding the NGF receptor were molecularly cloned using the human Alu repetitive sequence as a probe. A cosmid clone that contained the human NGF receptor gene allowed efficient transfection and expression of the receptor.

NERVE GROWTH FACTOR (NGF) and its receptor play an essential role in the survival and maintenance of sympathetic and sensory neurons. NGF action is initiated by the interaction of NGF with a specific receptor on the neuronal cell surface. Receptors for NGF have also been found on nonneuronal derivatives of the neural crest, including melanoma cells (*1, 2*), pheochromocytoma cells (*3*), Schwann cells, and neurofibroma cells (*2*). The NGF receptor has been characterized in sympathetic nerve cells (*4*), pheochromocytoma (PC12) cells (*3, 5, 6*), and melanoma cells (*2, 7*). Immunoprecipitation and photoaffinity cross-linking studies indicate that it has a molecular size of 70,000 to 80,000 daltons (*2, 7*) and can also exist in a dimeric form of about 200,000 daltons (*2, 5, 7*). The receptor can occur in both high and low affinity forms (*3, 8*). Further characterization of the human receptor indicates that it is a glycoprotein and is phosphorylated on serine resides (*9*).

DNA-mediated gene transfer has been used to express and clone the human NGF receptor. Unlike other techniques, this method does not depend on messenger RNA (mRNA) enrichment, but requires the availability of an appropriate recipient cell line and a means of identifying cells which have taken up and expressed the gene. To screen the recipient cells, we used an immunological rosette assay in which cells are incubated with monoclonal antibody to human NGF receptor (*2*) and then with second antibody [rabbit antibody to mouse immunoglobulin G (IgG)] coupled to erythrocytes. This assay is quite sensitive since rosettes can be observed with the human neuroblastoma cell line SY5Y, which expresses fewer than 1000 receptors per cell (*10*). When mouse fibroblast L cells are assayed in the same manner no rosettes are observed.

High molecular weight DNA was isolated from human neuroblastoma SY5Y, human melanoma A875 cells, or human T cells and mixed with the purified herpesvirus thymidine kinase (pTK) gene to produce a calcium phosphate precipitate for transformation of mouse fibroblast L cells (Ltk$^-$) (*11*). After 2 weeks in HAT [hypoxanthine (15 μg/ml), aminopterin (1 μg/ml), and thymidine (5 μg/ml) selection medium, the tk$^+$ colonies were screened with the rosette assay (*12*). Positive colonies were observed at a frequency of 1 per 12,000 tk$^+$ colonies with DNA from all three cell types. The positive L cell colonies were isolated with cloning cylinders and purified by two rounds of rescreening with the rosette assay. The primary transformants were then grown in bulk, and four clones were analyzed further for the presence of NGF receptor (see below). HAT selection was maintained throughout the purification and growth of the cells. The transformant lines were stable over many generations and did not lose the foreign NGF receptor gene or display decreased receptor expression.

To reduce the amount of extraneous human DNA, one of the primary transformants was transferred through a second round of transformation. The positive cell line N21, which was generated from human neuroblastoma DNA, was used as the source of DNA for a second round of transformation. High molecular weight DNA was isolated from N21 and used with pTK for transformation into mouse Ltk$^-$ cells. A secondary transformant, N21-11, was isolated with HAT selection and the rosette assay.

We used affinity cross-linking with ^{125}I-labeled NGF (*5–7*) to test for NGF receptor in the L cell transformants that had been identified by the rosette assay. Cells in suspension were incubated with ^{125}I-labeled NGF at 0°C and then treated with ethyldimethylisopropylcarbodiimide to cross-link

M. V. Chao, C. R. Buck, A. Sehgal, Department of Cell Biology and Anatomy and Division of Hematology and Oncology, Cornell University Medical School, New York, NY 10021.
M. A. Bothwell and A. A. Lanahan, Department of Physiology and Biophysics, University of Washington, Seattle, WA 98195.
A. H. Ross and H. Koprowski, Wistar Institute, Philadelphia, PA 19104.