

# **India Research Foundation Symposium Series**

**Volume 3**

## **Ecological Factors and the Nervous System**

**Edited by**

**Lloyd A. Hornecke  
Victor H. Negri  
Albert J. Farca  
Maria Hadjiconstantinou**



**LIBRARY**

NOV 21 1990

National Institutes of Health



**Trophic Factors  
and the Nervous System**

---

**FIDIA Research Foundation Symposium Series  
Volume 3**

## FIDIA Research Foundation Symposium Series

---

Allosteric Modulation of Amino Acid Receptors: Therapeutic Implications, FIDIA Research Foundation Symposium Series, Volume 1, E. A. Barnard and E. Costa, editors, 1989.

Neurochemical Pharmacology—A Tribute to B. B. Brodie, FIDIA Research Foundation Symposium Series, Volume 2, E. Costa, editor, 1989.

Trophic Factors and the Nervous System, FIDIA Research Foundation Symposium Series, Volume 3, L. A. Horrocks et al., editors, 1990.

# Trophic Factors and the Nervous System

---

## FIDIA Research Foundation Symposium Series Volume 3

### Editors

Lloyd A. Horrocks

Norton H. Neff\*

Allan J. Yates\*\*

Maria Hadjiconstantinou\*

The Ohio State University

College of Medicine

Departments of Physiological Chemistry,

Pharmacology\* and Pathology\*\*

Columbus, Ohio

Raven Press  New York

52  
36  
89

Raven Press Ltd., 1185 Avenue of the Americas, New York, New York 10036

© 1990 by Raven Press, Ltd. All rights reserved. This book is protected by copyright. No part of it may be reproduced, stored in a retrieval system, or transmitted, in any form or by any means, electronical, mechanical, photocopying, or recording, or otherwise, without the prior written permission of the publisher.

Made in the United States of America

Library of Congress Cataloging-in-Publication Data

Trophic factors and the nervous system : satellite to the 20th Annual Meeting of the American Society for Neurochemistry / editors, Lloyd A. Horrocks . . . [et al.]

p. cm. — (Fidia Research Foundation symposium series : v. 3)

Based on an international symposium held on Mar. 12–14, 1989 at the Ohio State University, sponsored by the Ohio State University Neuroscience Program.

Includes bibliographical references.

Includes index.

ISBN 0-88167-671-3

1. Nerve growth factor—Congresses. 2. Neurotrophic function—Congresses. I. Horrocks, Lloyd A. II. American Society for Neurochemistry. Meeting (20th : 1989 : Ohio State University) III. Ohio State University. Neuroscience Program. IV. Series.

[DNLM: 1. Nerve Growth Factors—congresses. 2. Nervous Systems—growth & development—congresses. 3. Neurons—physiology—congresses. W1 FI321F v. 3 / WL 102 T8554 1989]

OP552.N36T76 1990

591.1'88—dc20

DNLM/DLC

for Library of Congress

90-8799

CIP

The material contained in this volume was submitted as previously unpublished material, except in the instances in which credit has been given to the source from which some of the illustrative material was derived.

Great care has been taken to maintain the accuracy of the information contained in the volume. However, neither Raven Press nor the editors can be held responsible for errors or for any consequences arising from the use of the information contained herein.

Materials appearing in this book prepared by individuals as part of their official duties as U.S. Government employees are not covered by the above-mentioned copyright.

9 8 7 6 5 4 3 2 1

# Preface

Modern research on neurotrophic factors had its inception in 1951 when Levi-Montalcini and Hamberger reported on the selective growth stimulating effects of mouse sarcoma on the sensory and sympathetic nervous system of the chick embryo. Neurotrophic factors are now recognized as essential for the growth and development of the nervous system and for the repair of neural tissue after injury. Some neurodegenerative diseases may be the consequences of a deficiency of neurotrophic factors or their receptor systems. Indeed, these factors are just beginning to be used for the treatment of some human neurodegenerative disorders, particularly nerve growth factor for Alzheimer Disease. Because of the importance of neurotrophic factors and the research interests of various groups on campus, the focus of the symposium was "Trophic Factors and the Nervous System."

The symposium was divided into six sections: Growth and Trophic Factors of Neural Origin; Trophic Factor Receptors; Post-receptor Phenomena; Neurite Outgrowth and Growth Cones; and Neuronal Regeneration and Recovery. Experts from six countries summarized their research and provided the audience with expectations of future advances in the field. More than 160 attended the symposium which was held in Rhodes Hall Auditorium, The College of Medicine. This volume is a collection of the presentations prepared by the speakers. We are grateful to the FIDIA Research Laboratories, Abano Terme, Italy, for including this volume as part of the FIDIA Research Series.

---

L. A. Horrocks  
N. A. Neff  
A. J. Yates  
M. Hadjiconstantinou

# Acknowledgments

On March 12–14, 1989, The Ohio State University Neuroscience Program sponsored an international symposium on "Trophic Factors and the Nervous System." This was a satellite of the 20th annual meeting of the American Society for Neurochemistry. The event marked the commencement of the graduate program, Doctor of Philosophy in Neuroscience from The Ohio State University. An organized interest in the neurosciences dates back to 1965 when Dr. Leopold Liss organized the Central Ohio Chapter of the Society for the Neurosciences. Over the years interest has grown so that today specialized training leading to a Ph.D. is available in the neurosciences. This is a campus-wide faculty and student effort. The current program would not have been possible without the scientific, administrative and leadership skills of William E. Hunt and James S. King. The faculty and students express their sincere gratitude to all those who worked so hard to develop this important new graduate program.

The Local Scientific Committee and Editors express sincere appreciation to the following for their support: The Ohio State University College of Medicine; The Ohio State University Office for Research and Graduate Studies; The Ohio State University Spinal Cord Injury Research Center; The Ohio State University Alzheimer's Disease Center; and the FIDIA Research Foundation.

# Contents

## I. Growth and Trophic Factors of Neural Origin

Neurotransmitters, Neuronal Third Messengers and Multigene Transcriptional Activation E. Costa	3
Comparison of Exogenous and Endogenous Gangliosides as Modulators of Neuronal Differentiation Robert W. Ledeen, Gusheng Wu, Kuldeep K. Vaswani and Michael S. Cannella	17
Platelet-derived Growth Factor: Role in Gliogenesis and in the Development of Glioblastoma Christer Betsholtz, Monica Nistér, Carl-Henrik Heldin and Bengt Westermark	35
Growth-associated Triggering Factors and Central Nervous System Regeneration M. Schwartz, V. Lavie, A. Cohen, A. Harel, A. Solomon and M. Belkin	47
Brain-derived Heparin-binding Growth Factors and their Oncogenic Homologs Ing-Ming Chiu, Per Sandberg and Wen-Pin Wang	57
Biological and Immunochemical Properties of Recombinant Human NGF L. Callegaro, E. Bigon, G. Vantini, A. Di Martino, S. D. Skaper, A. Leon and G. Toffano	75

## II. Trophic Factor Receptors

Regulation of Nerve Growth Factor Gene Expression: <i>In Vitro</i> and <i>In Vivo</i> Studies Italo Moccia, Roberto Dal Toso, Alexander G. Yakovlev, Maria A. De Bernardi and Michele Fabrazzo	85
Cell-specific Expression of NGF Receptors in the Basal Forebrain C. F. Dreyfus, P. Bernd, H. J. Martínez, S. J. Rubin and I. B. Black	95

<b>The Nerve Growth Factor Receptor</b> J. Regino Perez-Polo	107
<b>Receptors for Gangliosides on Rat Brain Membranes: Specificity, Regional and Subcellular Distribution</b> Michael Tiemeyer and Ronald L. Schnaar	119
<b>Bioactive Gangliosides Modulating Transmembrane Signaling</b> Sen-itiroh Hakomori, Yasuyuki Igarashi, Hisao Nojiri, Eric Bremer, Nobuo Hanai and Gustavo A. Nores	135

### **III. Post-receptor Phenomena**

<b>Neuronal Plasticity and Melanocortins</b> R. Gerritsen van der Hoop and W. H. Gispen	161
<b>Effects of Gangliosides on the Growth and Differentiation of HL-60 Human Leukemia Cells</b> Robert K. Yu	175
<b>Nerve Growth Factor-sensitive Phosphorylations and the Action of K- 252a on PC12 Cells</b> Shinichi Koizumi, Tatsuro Mutoh, Alexey Ryazanov, Brian B. Rudkin and Gordon Guroff	195
<b>How Do Neurites Grow? Clues from NGF-regulated Cytoskeletal Phosphoproteins</b> John M. Aletta, Hensin Tsao and Lloyd A. Greene	203
<b>Consequences to CNS Cholinergic Neurons of <i>In Vivo</i> Nerve Growth Factor Administration</b> Silvio Varon, Theo Hagg, James Conner, Barry Fass, H. Lee Vahlsing and Marston Manthorpe	219

### **IV. Neurite Outgrowth and Growth Cones**

<b>Intracellular Calcium and the Control of Neuronal Growth and Form</b> S. B. Kater, L. R. Mills and P. B. Guthrie	231
<b>Trophic Regulation of Myelinogenesis</b> A. L. Gard, R. Bansal and S. E. Pfeiffer	247
<b>Synaptic Plasticity in the Adult Sacral Spinal Cord: Effects of Lesions and Hormones</b> Michael S. Beattie, Jacqueline C. Bresnahan and M. Gail Leedy	263

## V. Neuronal Regeneration and Recovery of Function

Energy Depletion, Calcium and the Cytoskeleton: A Model for Trophic Intervention	279
B. T. Stokes, Q. Li, R. A. Altschuld, B. E. Batten and D. K. Anderson	
Recovery of Dopaminergic Function Following MPTP-induced Neurodegeneration by Exogenous GM <sub>1</sub> Ganglioside	293
M. Hadjiconstantinou, F. B. Weihmuller, J. P. Bruno, A. P. Mariani and N. H. Neff	
Degeneration and Regeneration of Basal Forebrain Cholinergic Neurons	307
A. C. Cuello, L. Garofalo, R. L. Kenigsberg, D. Maysinger, E. P. Pioro and A. Ribeiro-da-Silva	
Development of Cholinergic Neurons in Septum, Nucleus Basalis, Striatum and Pons in Culture and their Response to Nerve Growth Factor	327
F. Hefti, J. Hartikka, E. O. Junard and B. Knusel	
Monosialoganglioside Effects Following Cerebral Ischemia: Relationship with Anti-neuronotoxic and Pro-neuronotrophic Properties	339
M. S. Seren, M. Lipartiti, A. Lazzaro, R. Rubini, S. Mazzari, L. Facci, G. Vantini, R. Zanoni, A. Zanotti, G. Bonvento and A. Leon	
Subject Index	349



# Contributors

## **John M. Aletta**

Laboratory of Cellular and Molecular Neurobiology  
Columbia University College of Physicians and Surgeons  
New York, NY 10032

## **R. A. Altschuld**

The Ohio State University College of Medicine  
Columbus, OH 43210

## **D. K. Anderson**

University of Cincinnati College of Medicine  
Cincinnati, OH 45267

## **R. Bansal**

Department of Microbiology  
University of Connecticut Health Center  
Farmington, CT 06032

## **B. E. Batten**

The Ohio State University College of Medicine  
Columbus, OH 43210

## **Michael S. Beattie**

Division of Neurosurgery, Department of Anatomy and Neuroscience Program  
The Ohio State University College of Medicine  
Columbus, OH 43210

## **M. Belkin**

Goldschleger Eye Research Institute  
Tel Aviv University Sackler School of Medicine  
Sheba Medical Center  
Tel Hashomer  
ISRAEL

## **P. Bernd**

Department of Anatomy and Cell Biology  
SUNY Health Science Center  
Brooklyn, NY 11203

## **Christer Betsholtz**

Department of Pathology  
University Hospital  
S-751 85 Uppsala  
SWEDEN

## **E. Bigon**

FIDIA Research Laboratories  
Via Ponte della Fabbrica 3/A  
35031 Abano Terme (PD)  
ITALY

## **I. B. Black**

Division of Developmental Neurology  
Cornell University Medical College  
New York, NY 10021

## **G. Bonvento**

FIDIA Research Laboratories  
Via Ponte della Fabbrica 3/A  
35031 Abano Terme (PD)  
ITALY

**Eric Bremer**

*Department of Immunology/  
Microbiology  
Rush University  
Chicago, IL 60612*

**A. Cohen**

*Department of Neurobiology  
Weizmann Institute of Science  
Rehovot  
ISRAEL*

**Jacqueline C. Bresnahan**

*Department of Anatomy and  
Neuroscience Program  
The Ohio State University College of  
Medicine  
Columbus, OH 43210*

**James Conner**

*Department of Biology  
School of Medicine  
University of California, San Diego  
La Jolla, CA 92093*

**J. P. Bruno**

*Department of Psychology  
College of Social and Behavioral  
Sciences  
Department of Psychiatry  
The Ohio State University College of  
Medicine  
Columbus, OH 43210*

**E. Costa**

*FIDIA-Georgetown Institute for the  
Neurosciences  
Georgetown University  
Washington, D.C. 20007*

**L. Callegaro**

*FIDIA Research Laboratories  
Via Ponte della Fabbrica 3/A  
35031 Abano Terme (PD)  
ITALY*

**A. C. Cuello**

*Department of Pharmacology and  
Therapeutics  
McGill University  
Montreal, Quebec H3G 1Y6  
CANADA*

**Michael S. Cannella**

*Departments of Neurology and  
Biochemistry  
Albert Einstein College of Medicine  
Bronx, NY 10461*

**Roberto Dal Taso**

*FIDIA-Georgetown Institute for the  
Neurosciences  
Georgetown University School of  
Medicine  
Washington, D.C. 20007*

**Ing-Ming Chiu**

*Department of Internal Medicine  
The Ohio State University  
Davis Medical Research Center  
Columbus, OH 43210*

**Maria A. De Bernardi**

*FIDIA-Georgetown Institute for the  
Neurosciences  
Department of Biochemistry and  
Molecular Biology  
Georgetown University School of  
Medicine  
Washington, D.C. 20007*

**A. Di Martino**

FIDIA Research Laboratories  
Via Ponte della Fabbrica 3/A  
35031 Abano Terme (PD)  
ITALY

**C. F. Dreyfus**

Division of Developmental Neurology  
Cornell University Medical College  
New York, NY 10021

**Michele Fabrazzo**

FIDIA-Georgetown Institute for the  
Neurosciences  
Georgetown University School of  
Medicine  
Washington, D.C. 20007

**L. Facci**

FIDIA Research Laboratories  
Via Ponte della Fabbrica 3/A  
35031 Abano Terme (PD)  
ITALY

**Barry Fass**

Department of Biology  
School of Medicine  
University of California, San Diego  
La Jolla, CA 92093

**A. L. Gard**

Department of Microbiology  
University of Connecticut Health  
Center  
Farmington, CT 06032

**L. Garofalo**

Department of Pharmacology and  
Therapeutics  
McGill University  
Montreal, Quebec H3G 1Y6  
CANADA

**R. Gerritsen van der Hoop**

Division of Molecular Neurobiology  
Rudolf Magnus Institute and Institute  
of Molecular Biology and Biotechnology  
University of Utrecht  
Vondellaan 6  
Utrecht  
THE NETHERLANDS

**W. H. Gispen**

Division of Molecular Neurobiology  
Rudolf Magnus Institute and Institute  
of Molecular Biology and Biotechnology  
University of Utrecht  
Vondellaan 6  
Utrecht  
THE NETHERLANDS

**Lloyd A. Greene**

Laboratory of Cellular and Molecular  
Neurobiology  
Columbia University College of  
Physicians and Surgeons  
New York, NY 10032

**Gordon Guroff**

Section on Growth Factors  
National Institute of Child Health and  
Human Development  
National Institutes of Health  
Bethesda, MD 20892

**P. B. Guthrie**

Department of Anatomy and  
Neurobiology  
Program in Neuronal Growth and  
Development  
Colorado State University  
Fort Collins, CO 80523

**M. Hadjiconstantinou**

Departments of Pharmacology and  
Psychiatry  
The Ohio State University College of  
Medicine  
Columbus, OH 43210

**Carl-Henrik Heldin**

Ludwig Institute for Cancer Research  
Biomedical Center  
S-751 23 Uppsala  
SWEDEN

**Theo Hagg**

Department of Biology  
School of Medicine  
University of California, San Diego  
La Jolla, CA 92093

**Lloyd A. Horrocks**

Department of Physiological Chemistry  
Ohio State University College of  
Medicine  
Columbus, OH 43210

**Sen-itiroh Hakomori**

The Biomembrane Institute and  
University of Washington  
Seattle, WA 98119

**Yasuyuki Igarashi**

The Biomembrane Institute and  
University of Washington  
Seattle, WA 98119

**Nobuo Hanai**

Kyowa Hakko Kogyo Co.  
Tokyo 194  
JAPAN

**E. O. Junard**

Department of Neurology  
University of Miami  
Miami, FL 33101

**A. Harel**

Department of Neurobiology  
Weizmann Institute of Science  
Rehovot  
ISRAEL

**S. B. Kater**

Department of Anatomy and  
Neurobiology  
Program in Neuronal Growth and  
Development  
Colorado State University  
Fort Collins, CO 80523

**J. Hartikka**

Department of Neurology  
University of Miami  
Miami, FL 33101

**R. L. Kenigsberg**

Department of Pharmacology and  
Therapeutics  
McGill University  
Montreal, Quebec H3G 1Y6  
CANADA

**F. Hefti**

Department of Neurology  
University of Miami  
Miami, FL 33101

**B. Knusel**

Department of Neurology  
University of Miami  
Miami, FL 33101

**Shinichi Koizumi**

Section on Growth Factors  
National Institute of Child Health and  
Human Development  
National Institutes of Health  
Bethesda, MD 20892

**V. Lavie**

Department of Neurobiology  
Weizmann Institute of Science  
Rehovot  
ISRAEL

**A. Lazzaro**

FIDIA Research Laboratories  
Via Ponte della Fabbrica 3/A  
35031 Abano Terme (PD)  
ITALY

**Robert W. Ledeen**

Departments of Neurology and  
Biochemistry  
Albert Einstein College of Medicine  
Bronx, NY 10461

**M. Gail Leedy**

Department of Anatomy  
The Ohio State University College of  
Medicine  
Columbus, OH 43210

**A. Leon**

FIDIA Research Laboratories  
Via Ponte della Fabbrica 3/A  
35031 Abano Terme (PD)  
ITALY

**Q. Li**

The Ohio State University College of  
Medicine  
Columbus, OH 43210

**M. Lipartiti**

FIDIA Research Laboratories  
Via Ponte della Fabbrica 3/A  
35031 Abano Terme (PD)  
ITALY

**Marston Manthorpe**

Department of Biology  
School of Medicine  
University of California, San Diego  
La Jolla, CA 92093

**A. P. Mariani**

Laboratory of Neurophysiology  
NINCDS, NIH  
Bethesda, MD 20892

**H. J. Martínez**

Division of Developmental Neurology  
Cornell University, Medical College  
New York, NY 10021; and  
Instituto de Investigaciones Clínicas  
Universidad del Zulia Maracaibo  
VENEZUELA

**D. Maysinger**

Department of Pharmacology and  
Therapeutics  
McGill University  
Montreal, Quebec H3G 1Y6  
CANADA

**S. Mazzari**

FIDIA Research Laboratories  
Via Ponte della Fabbrica 3/A  
35031 Abano Terme (PD)  
ITALY

**L. R. Mills**

Department of Anatomy and  
Neurobiology  
Program in Neuronal Growth and  
Development  
Colorado State University  
Fort Collins, CO 80523

**Italo Mocchetti**

Department of Anatomy and Cell  
Biology  
Georgetown University School of  
Medicine  
Washington, D.C. 20007

**Tatsuro Mutoh**

Section on Growth Factors  
National Institute of Child Health and  
Human Development  
National Institutes of Health  
Bethesda, MD 20892

**N. H. Neff**

Department of Pharmacology  
The Ohio State University College of  
Medicine  
Columbus, OH 43210

**Monica Nistér**

Department of Pathology  
University Hospital  
S-751 85 Uppsala  
SWEDEN

**Hisao Nojiri**

The Biomembrane Institute and  
University of Washington  
Seattle, WA 98119

**Gustavo A. Nores**

Institut fur Physiologische Chemie I  
Philipps-Universitat-Marburg  
D-3550 Marburg/Lahn  
WEST GERMANY

**J. Regino Perez-Polo**

University of Texas Medical Branch  
Galveston, TX 77550

**S. E. Pfeiffer**

Department of Microbiology  
University of Connecticut Health  
Center  
Farmington, CT 06032

**E. P. Pioro**

Department of Pharmacology and  
Therapeutics  
McGill University  
Montreal, Quebec H3G 1Y6  
CANADA

**A. Ribeiro-da-Silva**

Department of Pharmacology and  
Therapeutics  
McGill University  
Montreal, Quebec H3G 1Y6  
CANADA

**S. J. Rubin**

Division of Developmental Neurology  
Cornell University Medical College  
New York, NY 10021

**R. Rubini**

FIDIA Research Laboratories  
Via Ponte della Fabbrica 3/A  
35031 Abano Terme (PD)  
ITALY

**Brian B. Rudkin**

Section on Growth Factors  
National Institute of Child Health and  
Human Development  
National Institutes of Health  
Bethesda, MD 20892

**Alexey Ryazanov**

Institute of Protein Research  
Academy of Sciences of the USSR  
142292 Pushcino, Moscow Region  
USSR

**Per Sandberg**

Department of Internal Medicine  
The Ohio State University  
Davis Medical Research Center  
Columbus, OH 43210

**Ronald L. Schnaar**

Departments of Pharmacology and  
Neuroscience  
The Johns Hopkins University School of  
Medicine  
Baltimore, MD 21205

**M. Schwartz**

Department of Neurobiology  
Weizmann Institute of Science  
Rehovot  
ISRAEL

**M. S. Seren**

FIDIA Research Laboratories  
Via Ponte della Fabbrica 3/A  
35031 Abano Terme (PD)  
ITALY

**S. D. Skaper**

FIDIA Research Laboratories  
Via Ponte della Fabbrica 3/A  
35031 Abano Terme (PD)  
ITALY

**A. Solomon**

Goldschleger Eye Research Institute  
Tel Aviv University Sackler School of  
Medicine  
Sheba Medical Center  
Tel Hashomer  
ISRAEL

**B. T. Stokes**

The Ohio State University College of  
Medicine  
Columbus, OH 43210

**Michael Tiemeyer**

Departments of Pharmacology and  
Neuroscience  
The Johns Hopkins University School of  
Medicine  
Baltimore, MD 21205

**G. Toffano**

FIDIA Research Laboratories  
Via Ponte della Fabbrica 3/A  
35031 Abano Terme (PD)  
ITALY

**Hensin Tsao**

Laboratory of Cellular and Molecular  
Neurobiology  
Columbia University College of  
Physicians and Surgeons  
New York, NY 10032

**H. Lee Vahlsing**

Department of Biology  
School of Medicine  
University of California, San Diego  
La Jolla, CA 92093

**Gusheng Wu**

Departments of Neurology and  
Biochemistry  
Albert Einstein College of Medicine  
Bronx, NY 10461

**G. Vantini**

FIDIA Research Laboratories  
Via Ponte della Fabbrica 3/A  
35031 Abano Terme (PD)  
ITALY

**Allan J. Yates**

Department of Pathology  
Ohio State University College of  
Medicine  
Columbus, OH 43210

**Silvio Varon**

Department of Biology  
School of Medicine  
University of California, San Diego  
La Jolla, CA 92093

**Alexander G. Yakovlev**

FIDIA-Georgetown Institute for the  
Neurosciences  
Georgetown University School of  
Medicine  
Washington, D.C. 20007

**Kuldeep K. Vaswani**

Departments of Neurology and  
Biochemistry  
Bronx, NY 10461

**Robert K. Yu**

Department of Biochemistry and  
Molecular Physics  
Medical College of Virginia  
Virginia Commonwealth University  
Richmond, VA 23298

**Wen-Pin Wang**

Department of Internal Medicine  
The Ohio State University  
Davis Medical Research Center  
Columbus, OH 43210

**R. Zanoni**

FIDIA Research Laboratories  
Via Ponte della Fabbrica 3/A  
35031 Abano Terme (PD)  
ITALY

**Bengt Westermark**

Ludwig Institute for Cancer Research  
Biomedical Center  
S-751 23 Uppsala  
SWEDEN

**A. Zanotti**

FIDIA Research Laboratories  
Via Ponte della Fabbrica 3/A  
35031 Abano Terme (PD)  
ITALY

**F. B. Weihmuller**

Department of Psychology  
The Ohio State University College of  
Social and Behavioral Sciences  
Columbus, OH 43210

# I. Growth and Trophic Factors of Neural Origin



# Neurotransmitters, Neuronal Third Messengers and Multigene Transcriptional Activation

E. Costa

FIDIA-Georgetown Institute for the Neurosciences, Georgetown University,  
Washington, D.C. 20007

**R**esearch on retroviral oncogenes and their cellular progenitors, proto-oncogenes, has provided specific nucleic acid and protein probes that have become important tools in studying the transmitter-mediated regulation of a simultaneous multigene expression in neurons. Such multigene transcriptional activation in multiplying cells provides the coordination in the synthesis of specific proteins operative in cell differentiation and growth, and may contribute to the regulation of morphological changes related to learning in mature neurons.

Almost one hundred years ago, Ramon y Cajal (20) suggested a causal association between learning and structural modification of neurons. He postulated that the duration of structural changes induced by learning was related to the memory consolidation of that learning experience. Hence, one might surmise that during learning a specific cue impinges upon synaptic receptors that coordinates the simultaneous transcription of functionally related genes to allow for the formation of morphological modification in postsynaptic neurons. These synaptically generated changes in DNA transcription may bring about increases in the expression of enzymes or other regulatory proteins modifying cytosol or neuronal membrane characteristics during learning. Such structural changes may last longer than the increase in the translation of the specific mRNA(s) transcribed in excess as a result of learning. This time discrepancy relates to the half-life of the translated proteins. Hence, one might infer that not only the DNA transcriptional activation, but also the turnover of specific proteins, may contribute to the duration of the memory consolidation.

As a model of DNA transcription associated with medium term memory consolidation (days) we will use the transsynaptic induction of tyrosine hydroxylase (TH) in adrenal medulla which is mediated by the second messenger cAMP (10). In this model, acetylcholine (Ach), which is released from splanchnic nerve terminals to activate nicotinic receptors and their associate cationic channels, operates as the first messenger (1). During sustained activation of cholinergic axons, mod-

ulatory peptides may be co-released from splanchnic nerve terminals. These peptides, by increasing chromaffin cell cAMP content, activate protein kinase A (PKA) which, in addition to the increased influx of  $\text{Ca}^{2+}$  brought about by cationic channel activation, may function as the specific second messenger generated by the combined receptor signals responsible for DNA transcription activation (12,13).

When learning is associated with morphological changes (i.e. presynaptic ending duplication or synaptic spine formation) it may be required that a set of genes encoding for the proteins participating in these morphological changes be activated. Although these changes last for a finite time, once the morphological modification is determined it becomes permanent because the turnover of the morphological modification constituents becomes routine as if they were original components of the membrane.

In searching for the synaptically generated cues that orchestrate the simultaneous expression of sets of genes encoding for functionally related proteins that are required to bring about the above mentioned morphological neuronal modifications occurring during learning, we investigated whether the gene program causing structural neuronal modifications during learning is similar to that expressing structural changes during ontogenetic neuronal differentiation. Hence, we studied whether a transcriptional activation of immediate early genes (protooncogenes) (21), which is operative in ontogenetic differentiation, could be triggered by the stimulation of those N-methyl D-aspartic acid (NMDA)-sensitive glutamate receptors that have been implicated in long-term memory. In fact, this subtype of glutamatergic transmission has received more positive support than any other synaptic mechanism as a putative mediator of plasticity responses occurring during long lasting memory consolidation. We found that these NMDA receptors increase the transcription of *c-fos* (23,24) and *c-jun* mRNA (Szekely, A.M. and Grayson, D., this laboratory, unpublished). The translation products thus generated quickly revert to the nucleus where they act as third messengers and enhance the transcription of a set of genes regulating the expression of functionally related protein (see Fig. 4). Thus, easily inducible genes may orchestrate the simultaneous synthesis increase of those functionally related structural proteins that are required to bring about the morphological modification associated with long term memory consolidations.

#### **ACTIVATION OF cAMP FORMATION: ROLE IN THE REGULATION OF TYROSINE HYDROXYLASE (th) FUNCTION AND BIOSYNTHESIS**

Functional neuronal changes due to an increase in the number of enzymes or of other cytosolic protein molecules regulating various aspects of neuronal membrane function may result from specific synaptic cues that increase the strength of synaptic activity without changing the primary transmitter quanta participating in the synaptic transduction. We now know that in some synapses, at critical frequency of

stimulation, a polytypic signalling can be generated (12). Thus, specific synaptic cues (neuropeptide modulators) may be co-released with the primary transmitter when the afferent impulse frequency increases. This cotransmission may be involved in triggering synaptically induced DNA transcription (12). When synaptic activity induces protein synthesis in postsynaptic neurons, this induction is termed trans-synaptic. But transmitter-regulated protein synthesis may also occur as a result of autoreceptor activation. Here the formation of second messengers may in turn generate tertiary chemical signals which, by reaching the nucleus antidromically, may activate gene expression. In neuronal networks, the change in the number of enzyme molecules occurring in a single neuron can bring about an alteration in the neuronal circuit characteristics. This is particularly evident if the enzyme that is transcriptionally activated is rate limiting in the biosynthesis of a primary transmitter. Such circuitry changes can modify the projection target of a given stimulus and thereby shift the response between neuronal populations of a given neuronal map functioning as the final target. This change in stimulus projection characteristics may parallel the duration of the transsynaptically induced increase in the neurotransmitter turnover rate. Neuronal TH is a target enzyme for transsynaptic regulatory mechanisms that can be transcriptionally activated (1,10) or post-translationally modified (25). Post-transcriptional modification of TH usually involves an enzyme phosphorylation that brings about changes in the TH affinity constant for the pteridines or other cofactors (25). Usually these changes are related temporally to the duration of the shifts in afferent neuronal activity. In contrast, when TH transcription is transsynaptically induced, the time course of this change lasts longer than the increase in the TH synthesis rate, for its duration also reflects the half-life of the newly synthesized peptide molecules (10). Thus, these changes may outlast a short increase (1 to 2 hours) in the afferent synaptic strength by a week to 10 days (Fig. 1). Hence, extrapolating from the adrenal model to models of memory consolidation in brain, if the transcriptional activation of TH generates putative memory engrams, they may last for 5 to 10 days.

In 1973 (9), while studying the transsynaptically induced increase of the number of medullary TH molecules, we found that a transsynaptically induced increase of cAMP content lasting 60 minutes or longer could begin to increase the number of TH molecules after a time interval of about 18 hours (Fig. 1). Hence, we became interested in investigating the molecular nature of the intervening events (10). On the basis of *in vivo* studies using intact and denervated adrenal glands combined with injections of nicotinic receptor agonists and antagonists (10), we concluded that the evidence obtained (Fig. 1) supported the inference that a prompt increase of cAMP content mediated the induction of TH via an activation and translocation to the nucleus of cytosolic PKA. We studied in some detail the time-related changes in the cytosolic content of PKA catalytic subunits after the inducing stimulus (10). We found that PKA activation was related to the duration of the cAMP increase. Moreover, the duration of the PKA activation determined the type of TH response. A short-lasting PKA activation could phosphorylate TH and increase its affinity for co-enzymes, thereby activating TH catalytic power (25). In contrast, a longer

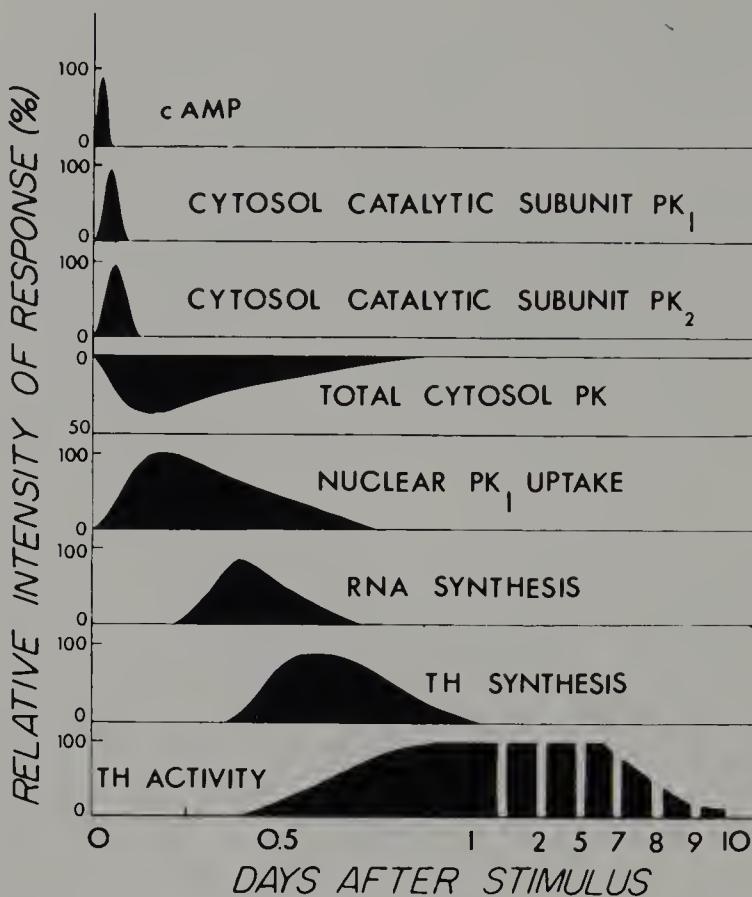


FIG. 1. Temporal sequence of molecular events taking place in chromaffin cells of adrenal medulla deriving from *in vivo* transsynaptic induction of TH. After the application of the stimulus (exposure of rats to 4 degrees, injection of either reserpine or carbachol) cAMP increases for about 90 minutes. The cytosolic PKA (type 1 and 2) are activated in 2-3 hrs. The total cytosol PKA activity is decreased for about 14 hrs while the content of nuclear PKA (type 1) is increased beginning 2 hrs after the stimulus application and lasts several hours. This increase precedes the increase in RNA synthesis and that of TH synthesis. The TH activity remains higher than normal for about 1 week. (From ref. 10.)

lasting increase of cAMP content could trigger an activation and nuclear translocation of PKA leading to a transcriptional activation of TH genes (10). The synaptic cue involved in such transcriptional regulation was not revealed by these experiments. In fact, the short pulse carbachol given to chromaffin cells in culture could neither induce TH (16) nor increase cAMP content. In contrast, a TH induction could be obtained by adding 8-Br-cAMP to this cell culture (16). On the basis of evidence on the coexistence of the neuropeptide Vasoactive Intestinal

Peptide (VIP) with Ach autonomic peripheral nerves (12,13), we suggest that VIP may be a cotransmission signal to be considered as a putative cue that changes synaptic strength in the cholinergic synapse of adrenal medulla and that, together with Ach, may be operative as a first messenger in the transcriptional activation of TH genes. This working hypothesis is consistent with findings that in cultures of chromaffin cells a short pulse of carbachol could not increase cAMP or induce TH while the addition of VIP could increase cAMP and TH (M. Olasmaa, this laboratory, *unpublished*). We are now studying whether the induction of TH occurs when carbachol is added with VIP to the chromaffin cell cultures.

As previously mentioned, 8-Br-cAMP could induce TH biosynthesis in chromaffin cells cultures with a cascade of events identical to that described (Fig. 1) in our *in vivo* experiments with adrenal medulla. The translocation of PKA catalytic subunits to the nucleus was in fact documented by our earlier studies (16). This translocation of PKA has been shown both *in vitro* (16) and *in vivo* (10) to be essential for the induction of TH. Moreover, when the translocation is inhibited by colchicine, the induction of TH is also inhibited (Fig. 2). We can infer that the translocated PKA may phosphorylate a specific nuclear protein; perhaps, the cAMP-responsive element binding protein (CREB) (6), which, in the phosphorylated state, may form a homodimer that acts as a third messenger to enhance transcriptional rates of the TH gene (Fig. 3). CREB protein presumably binds to cAMP-responsive element (CRE) that functions as a regulatory domain and is located upstream from the 5' end of the transcribed regions of the TH gene. It is currently believed that within this gene an 8 base palyndrome -TGACGTCA-5' may represent the binding site for CREB protein. By fusing the 5' flanking region of the TH gene to the chloramphenicol acetyltransferase (CAT) gene and transfected this hybrid gene into pheochromocytoma cultures, the region of the TH gene -272 + 27 conferred induction of cat by forskolin (17). This suggests that phosphorylation of a nuclear protein, perhaps CREB (Fig. 3), mediates TH induction by cAMP (17).

## CASCADE OF EVENTS IN THE TRANSSYNAPTIC INDUCTION OF TYROSINE HYDROXYLASE: ROLE OF THIRD NUCLEAR MESSENGER PHOSPHORYLATION AND HOMODIMERIZATION

Adrenal medullary cells, similarly to most eukaryotic cells, form and degrade cAMP and contain cAMP-dependent protein kinase, the natural target for the cyclic nucleotide second messenger. Paul et al. (19) demonstrated a rise of cAMP in the adrenals of rats immobilized for several hours; this change precedes the induction of medullary TH. But these investigators suggested that the cAMP increase was occurring in adrenal cortex and excluded a role of this second messenger in the induction of TH (19). However, we could show that several stimuli that induce medullary TH as a delayed response also cause an immediate increase of medullary cAMP lasting about 1 hour or longer (10). The duration of the increase

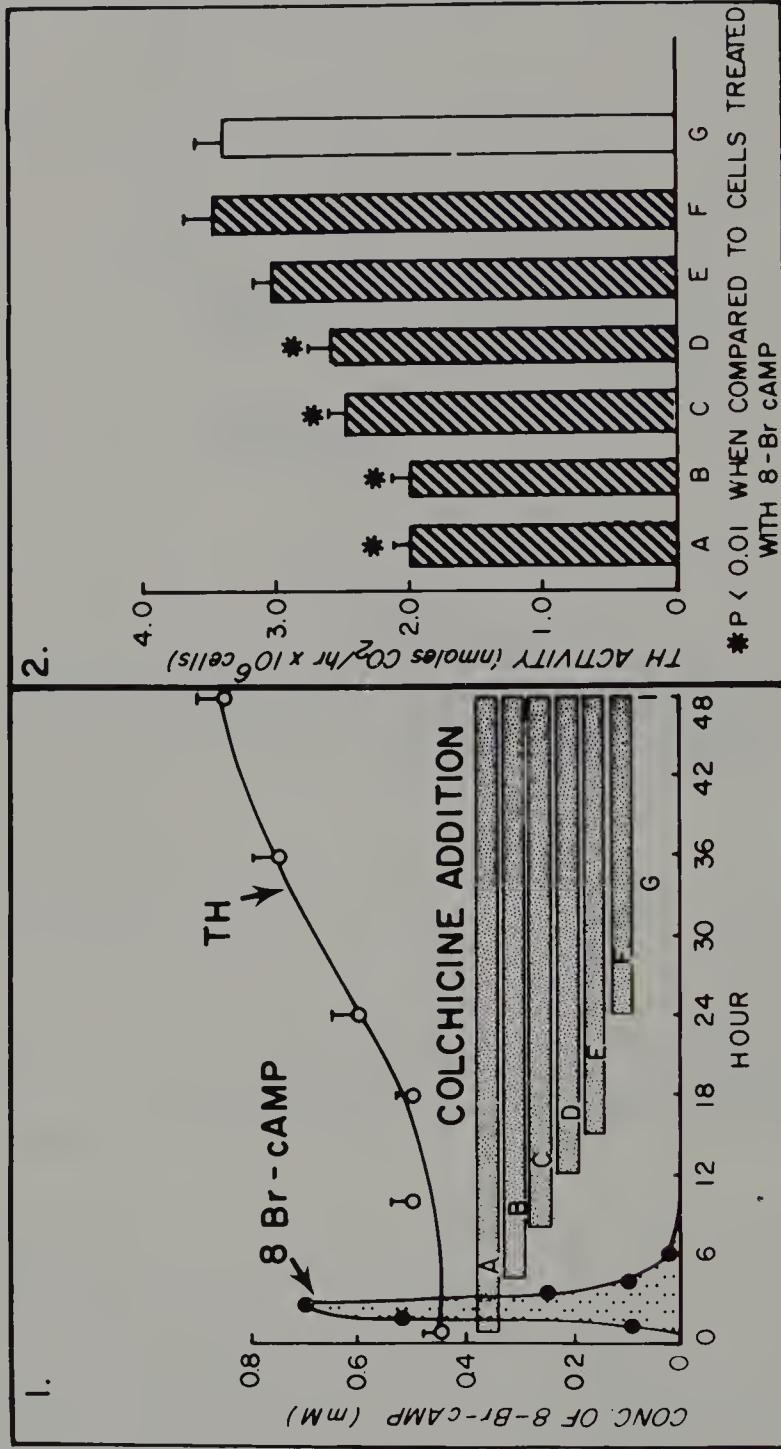
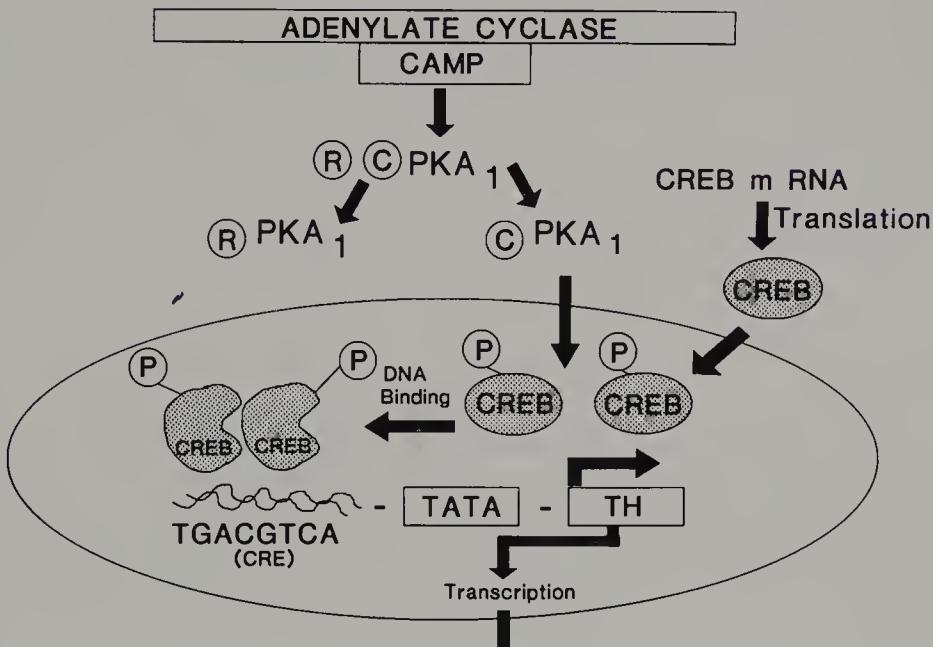


FIG. 2. Colchicine inhibition of TH induction by 8-Br-cAMP: time factor. **Panel 1:** Experimental procedure time schedule of colchicine addition. Colchicine 10 mM was added at different times after the addition of 8-Br-cAMP. TH was determined at different times after 8-Br-cAMP in the presence and absence of colchicine. **Panel 2:** TH activity measured at 48 hrs after 8-Br-cAMP. Open column TH in culture not treated with colchicine. (From ref. 16.)



**FIG. 3.** Diagrammatic representation of the mechanisms involved in the induction of TH in adrenal medulla. Probably a cotransmitter (VIP), together with Ach the primary transmitter, increases cAMP synthesis rate. CREB, =cAMP responsive element binding protein; CRE, =cAMP responsive element (an 8 base palindrome TGACGTCA).

is independent of the stimulus duration. In view of current evidence on possible multiple signalling in adrenal medulla cholinergic transmission (12,13), one might surmise that the induction of TH could be related to an increase in the synaptic strength of cholinergic transmission due to the participation of specific synaptic cues (VIP?) in the form of multiple synaptic signalling.

There are two types of PKA in the cytosol of chromaffin cells, type 1S and 2 (3). Only the catalytic subunits of type 1 can be translocated to the nucleus of chromaffin cells; those of PKA type 2, though activated by cAMP, remain in the cytosol (3). Evidence that this nuclear translocation of PKA type 1 is important was shown by *in vitro* experiments with blockers of neurotubular protein function given at various times after a dose of 8-Br-cAMP, which was used as a TH-inducing stimulus (16). Presumably, nuclear translocation of PKA type 1 requires a normal function of this neurotubular protein. As shown by Kumakura et al. (16), the inhibition of PKA translocation and TH induction by colchicine is curtailed if this drug is given 2 to 12 hours after the inducing stimulus (8-Br-cAMP application). In fact, when colchicine is applied 14 and 24 hours after 8-Br-cAMP, that is, after

PKA (type 1) translocation has already occurred (16), the TH induction remains operative despite colchicine treatment (Fig. 2). This experiment (Fig. 2) excludes a nuclear action of colchicine on transcriptional activation of TH.

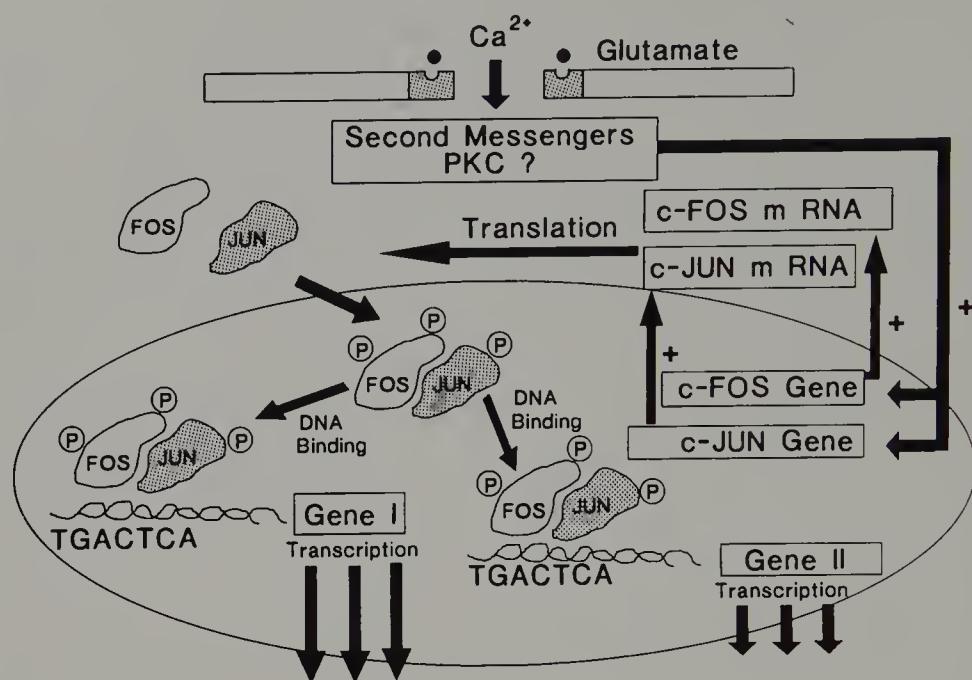
Lewis and colleagues (17) have studied by nuclear run-on assay the TH gene transcription after PKA activation with forskolin or 8-Br-cAMP in a pheochromocytoma cell line transfected with a construct that includes the 5'-flanking region of the TH gene. Both compounds increase the TH gene transcription provided the construction includes the first 272 bases of the 5' flanking region, supporting previous work from our laboratory on the role of cAMP in the activation of the TH gene (10). The 5' end flanking region of the TH gene was fused to the bacterial gene for CAT, and the hybrid gene incorporated in a plasmid containing a promoter was transfected also into GH<sub>4</sub> pituitary cells (17). Also in this cell line, a region of the regulatory sequence of TH gene -272 + 27 conferred induction of CAT expression by forskolin, a specific activator of adenylate cyclase that induces cAMP increase. It is possible (see Fig. 3) that cAMP regulates the expression of TH genes by phosphorylating CREB that enhances DNA transcription by binding to the 8 base palindrome CRE mentioned earlier. CREB is a nuclear protein of 43 kDa molecular mass whose transcriptional efficacy is regulated by PKA phosphorylation and the formation of a homodimer (6). CREB includes at least three consensus sites for phosphorylation by a PK (6). These sites are located in proximity of the CREB N terminus and form a cluster of PKA, PKC and PK II "consensus" for phosphorylation. The proximity of these phosphorylation sites to one another might suggest that they interact positively (or negatively) to regulate CREB bioactivity (6). It is not understood at the present time whether phosphorylation of all three sites is required to cause CREB dimerization and transcriptional activation capacity.

In summary, recent work has confirmed our early discovery (3,9,10,16) that cAMP-dependent phosphorylation of specific nuclear regulatory proteins can modulate transcription of TH genes. Now our suggestion has been corroborated by precise information not only concerning the regulatory 5' flanking structure of the TH gene, but also concerning the molecular mechanisms and the 8 nucleotide palindrome operative sites. One might generalize that phosphorylation of CREB by PKA 1 translocated to the nucleus after a stimulation of a transmitter receptor located in the outer part of neuronal membranes may be a third messenger mechanism used by a transmitter to modulate expression of genes. However, the mechanisms that regulate CREB biosynthesis and availability remain to be elucidated.

## THE IMMEDIATE EARLY INDUCIBLE GENES: THE BIOSYNTHESIS OF THIRD NUCLEAR MESSENGERS OPERATIVE IN TRANSMITTER-MEDIATED MULTIGENE EXPRESSION

The mechanism described above on CREB action in transmitter-mediated gene expression delineates a new concept on genomic regulation by synaptic cues, which

is based on the third messenger function carried out by the translation product of specific regulatory genes. While it is not known whether the CREB gene is activated or whether CREB is always turning over in the nucleus, it is known that multigene expression is carried out by activation of protooncogene mRNA (2,4,15). These immediate early inducible genes usually maintain a low level of basal expression in neurons. It is known that in the central nervous system, an immediate increase in their transcriptional levels is brought about by metrazol convulsions (18,22), sensory stimulation (14) and kindling (5). In primary cerebellar neuronal culture, *c-fos* can be induced by stimulation of glutamate NMDA-type receptors (23,24). In cells undergoing multiplication, *c-fos* expression can be induced by a diverse range of extracellular stimuli and provide a critical function in growth processes (11). Thus, a nerve growth factor (NGF) promotes neuronal differentiation of PC12 pheochromocytoma cells by immediately and transiently stimulating *c-fos* protooncogene transcription (7). In these cells also, the stimulation of neuronal Ach receptors induces rapid and transient transcription of the *c-fos* protooncogene (8). However, *c-fos* gene transcriptional activation by transmitter receptor activation proceeds via a different pathway from the gene activation by NGF. Nicotine receptor activation requires  $\text{Ca}^{2+}$  (8) influx while this influx is not required by NGF (7). Since PC12 cells undergo multiplication, one cannot assess from these experiments whether in neurons that do not multiply, the induction of *c-fos* has a specific functional role. Perhaps in mature neurons where growth and differentiation have a low level of expression, the immediate and transient early gene induction has an important role in regulating the gene expression with specific functional significance in mechanisms of structural modification related to learning and memory. They probably represent a nuclear language coordinated by specific synaptic cues. The translation products of immediate early inducible genes revert to the nucleus where they enhance the simultaneous expression of mRNA(s) encoding for proteins that might attend the morphological changes occurring in neurons during learning (Fig. 4). In mature neurons, where differentiation and growth are complete, the simultaneous coordination of synthesis of specific proteins may regulate the small changes in neuronal morphology that occur during learning and are responsible for long-term memory. Thus, glutamate-induced synaptic plasticity can be mediated by the general mechanism postulated in Figure 4. Perhaps gangliosides and other phospholipids facilitate neuronal plasticity by facilitating mechanisms included in Figure 4. In considering the coordination of protein synthesis to elicit neuronal modifications associated with learning, our attention was first attracted to the study of *c-fos*. This is a member of the immediate early inducible gene set that functions in coupling short-term synaptic signals to long-term adaptive responses that are probably also encoded in a genomic program used by the neurons during differentiation. In fact, formation of synaptic spines or presynaptic ending duplication and other morphological changes used in ontogenetic differentiation reappear in association with learning under the regulation of NMDA receptor. For these morphological changes to occur, it is required that certain genes are simultaneously and transcriptionally regulated. Transcription activation of *c-fos* and *c-*



**FIG. 4.** Schematic representation of the role of *c-fos-jun* gene induction in multigene expression elicited by stimulation of the NMDA-sensitive glutamate receptors in granule cerebellar cells. As demonstrated by Kouzarides and Ziff (15), the leucine residues that stabilize the interaction (dimerization) between *fos* and *jun* proteins through a leucine zipper can bind to the TGACTCA consensus better than either protein alone. This consensus site is located in the flanking region of various genes upstream from the 5' end of the transcribed part of the gene.

*jun* genes leads to an increase of their nucleotropic translation products, which act as a nuclear third messenger. This function requires the post-translational phosphorylation of these proteins. This process terminates a chain of events initiated by the transduction of an extraneuronal first messenger (glutamate) by a specific NMDA-type synaptic membrane receptor.

The *c-fos* mRNA translation product after a post-translational phosphorylation occurring in the nucleus participates in the formation of a nuclear hetero protein dimer by coupling to the translation product of *c-jun* mRNA, another product of the transcriptional activation of an immediate early inducible gene (Fig. 4). The translation products of *c-fos* and *c-jun* mRNA bind to each other through the leucine zipper to form the heterodimer (15). The leucine zipper is formed by ionic bonding between specific amino acid sequences including at least seven leucine present in each of the two protein forming the heterodimer (15). This heterodimer binds to a specific DNA consensus site (-TGACTCA-5') located upstream to the

5' end of the gene region that is transcribed. It should be noted that this 7 base palyndrome is similar to the 8 base palyndrome CRE discussed earlier. By binding to the 7 base DNA palyndrome, also termed AP-I binding site (4), the *c-jun c-fos* protein dimer enhances transcription of those genes regulated by an AP-I palyndrome consensus site. There are many genes that have palyndromic sites that bind this dimer or other related homo or hetero dimers. The transcriptional activation capability of *c-fos* protein depends on its level of phosphorylation and on the availability of the translation product of *c-jun* mRNA. The binding of this *c-fos c-jun* complex to the AP-I enhancer region of a gene, by providing additional contacts with the carbohydrate-phosphate backbone of the DNA palyndrome, enhances the activation of gene transcription that is primarily regulated by a specific promoter (Fig. 4).

The long-term goal of the present research on early inducible genes in mature neurons is to establish the syntax of the language that regulates the simultaneous expression of a set of genes that can express mRNA encoding for functionally related proteins. The rules of this language are spelled out by various post-translational modifications of early inducible gene translation products and their occupation of the different DNA palyndromes located on various genes. In fact, the occupation of these sites by dimers of various regulatory proteins of nuclear function that are post-translationally modified may spell out mechanisms whereby the transcription of a given gene may be included or excluded from the set of genes that are simultaneously transcriptionally activated to orchestrate either a specific morphological change or other specific functional changes of mature neurons. In the understanding of this interplay between genes and their nuclear third messenger regulatory proteins may reside our ability to create appropriate tools to interfere pharmacologically in the expression of high brain function such as memory consolidation or neuronal plasticity for which a cascade of multiple gene expression is required.

The mRNA for *c-fos* encodes a 62 kDa protein that can be phosphorylated and that forms a heterodimer with the translation products of *c-jun* mRNA. However, complexes of the regulatory proteins might be established with *c-fos* protein always using the leucine zipper for dimerization. These regulatory nuclear proteins can be identified as *fos*-related antigens. They represent a set of proteins that may result either from truncation of *c-fos* protein or from a set of proteins that can be induced in circumstances similar to those that induce *c-fos* protein formation. The possibility that a variety of highly homologous proteins can couple with *c-fos* protein to form and bind as a heterodimer to activate various DNA palyndromes in addition to AP-I binding site raises a number of unanswered questions concerning specificity and time constant of the multiple gene expression regulation. The leucine zipper involvement in heterodimer formation may generate a diversity of protein complexes which can regulate the expression of a variety of gene sets. In addition, the time constant of the inertia for a specific gene to be activated by the specific heterodimer might be codified in a manner still unknown. In fact, this inertia may be as important as the activation of a given gene in establishing the final expression

of the specificity of a given message. To investigate functional relationships between genes and regulatory nuclear proteins, the technology of gel retardation analysis of nuclear extracts has been devised. The binding of the nuclear proteins to synthetic oligonucleotide palyndromes analogues to the AP-I binding domain delays migration of the oligonucleotides in their gel electrophoresis analysis. The use of specific antibodies for nuclear third messengers or the competition assay with authentic nuclear regulatory proteins allows evaluation of the amount of third messenger present in nuclei before, during, and after sustained activation of synaptic receptors for neurotransmitters. We believe that progress of our understanding of this regulation of gene expression by nuclear protein functioning as third messengers in multigene expression may open new avenues for the study of transmitter-modulated neuronal plasticity.

## CONCLUSION

Second messenger biosynthesis in cytosol contributes to neuronal transcriptional activation of genes by activating and translocating to the nucleus specific PK(s). These enzymes regulate the induction of a specific set of early inducible genes whose translation products regulate rates of simultaneous expression of genes transcribing mRNA(s) encoding for functionally related proteins. It is believed that this process is of fundamental importance in reaching a better understanding of the synaptic coordination in the synthesis of a set of newly synthesized proteins in high brain function.

In this report, we have characterized two basic mechanisms for gene regulation by neurotransmitters, one exemplified by TH gene induction and the other exemplified by the transsynaptic transcriptional activation of a set of functionally related genes.

The TH model shows that multiple extracellular first messengers can increase cytosolic cyclic nucleotide content which, by activating a specific PKA and inducing its translocation to the nucleus, causes the post-translational phosphorylation of a specific regulatory nuclear protein (CREB), which thereby acquires the functional capability to form a homeodimer. This binds to CRE, a palyndrome regulating the expression of TH and probably any other genes that include CRE in their regulatory region. A mechanism that regulates the transsynaptic induction of multiple gene expression is mediated in cerebellar granule cells through NMDA-sensitive glutamate receptors. It includes the induction of immediate inducible genes brought about by an influx of  $\text{Ca}^{2+}$  that activates and translocates either a PKC or a phosphorylated transcription protein. This translocation, alone or in combination with other  $\text{Ca}^{2+}$  mediated mechanisms that are still unknown, causes the transcriptional activation of early inducible genes. Their translation products revert to the nucleus where they are post-translationally modified to bind as heterodimers to specific palyndromes inserted in the gene region upstream from the 5' end of the transcribed message to coordinate the expression of a set of genes

whose number and specificity is determined by a complex interaction between regulatory proteins and various palindromes which is still poorly understood.

## SUMMARY

The neurotransmitter-mediated regulation of gene expression is discussed in the context of the following two models:

1. In adrenal medulla the transsynaptic induction of tyrosinehydroxylose (TH) requires the first messenger acetylcholine (Ach) as well as the participation of another synaptic signal, perhaps a neuropeptide coexisting with Ach. In fact, in chromaffin cell cultures, a short pulse of Ach neither increases cyclic AMP (cAMP) nor induces TH. When cAMP is increased by the double signal acting as the first messenger, cytosolic protein kinases (PK) of type 1 and 2 are activated. However, only PK1 is translocated to the nucleus where it phosphorylates CREB (cAMP responsive element binding protein) that, as a homeodimer, acts as a third messenger and binds CRE (cAMP responsive element) to activate TH gene transcription (CRA = TGACGTCA-5').

2. In primary cultures of cerebellar granule cells, the first messenger operative in multigene transcription is glutamate acting on N-methyl-D-Aspartic Acid (NMDA)-sensitive glutamate receptors. The identity of the second messenger is not clear. Probably  $\text{Ca}^{2+}$  and PKC are operative in the activation of early inducible gene transcription. The nuclear transfer of the two translation products of *c-fos* and *jun* mRNAs and the formation through the "leucine zipper" of a phosphorylated heterodimer (third messenger) activates TGACTCA-5', the responsive element of those genes that are transcriptionally activated by the third messenger. We do not yet understand the details whereby the selection and synchronization of a set of gene transcription occurs.

## REFERENCES

1. Axelrod J. Noradrenaline: fate and control of its biosynthesis. *Science* 1971;173:598–606.
2. Chin R, Boyle WJ, Meek J, Smeal T, Hunter T, Karin M. The *c-fos* protein interacts with *c-jun* AP-I to stimulate transcription of AP-I responsive genes. *Cell* 1988;54:541–552.
3. Costa E, Guidotti A. Molecular mechanisms mediating the transsynaptic regulation of gene expression in adrenal medulla. In: *Psychopharmacology: a generation of progress*. New York: Raven Press 1978;235–246.
4. Curran T, Franzia Jr. BR. *Fos* and *Jun*: the AP-1 connection. *Cell* 1988;55:395–397.
5. Dragenow M, Robertson HA. Kindling stimulation induces *c-fos* protein in granule cells of the rat dentate gyrus. *Nature* 1988;129:441–442.

6. Gonzalez GA, Yamamoto KK, Fischer WH, Karr D, Menzel P, Biggs, III, W, Vale WW, Montminy MR. A cluster of phosphorylation sites on the cyclic AMP-regulated nuclear factor CREB predicted by its sequence. *Nature* 1989;337:749–752.
7. Greenberg ME, Greene LA, Ziff EB. Nerve growth factor and epidermal growth factor induced rapid transient changes in protooncogene transcription in PC12 cell. *J. Biol. Chem.* 1985;280:14101–14110.
8. Greenberg ME, Ziff EB, and Greene LA. Stimulation of neuronal acetylcholine receptors induces rapid gene transcription. *Science* 1986;234:80–83.
9. Guidotti A, Costa E. Involvement of adenosine 3', 5'-monophosphate in the activation of tyrosine hydroxylase elicited by drugs. *Science* 1973;179:902–904.
10. Guidotti A, Costa E. Transsynaptic regulation of tyrosine-3-monoxygenase biosynthesis in rat adrenal medulla. *Biochem. Pharmacol.* 1977;26:817–823.
11. Heldin CH, Westermark B, eds. Growth factors: mechanisms of action and relation to oncogenes. *Cell* 1984;37:9–20.
12. Hokfelt T, Fuxe KG, Pernow B. Coexistence of neuronal messenger: a new principle in chemical transmission. *Prog. Biochem. Res.*, vol. 68. Amsterdam: Elsevier, 1986.
13. Hokfelt T, Johansson O, Ljungdahl A, Lundberg JM, Schultzberg M. Peptidergic neurons. *Nature* 1980;284:515–521.
14. Hunt S, Pini A, Evan G. Induction of c-fos-like protein in spinal cord neurons following sensory stimulation. *Nature* 1987;328:632–634.
15. Kouzarides T, Ziff E. The role of leucine zipper in the *fos-jun* interaction. *Nature* 1988;336:646–651.
16. Kuma Kura K, Guidotti A, Costa E. Primary cultures of chromaffin cells: molecular mechanisms for the induction of tyrosine hydroxylase mediated by 8-Br-cyclic AMP. *Mol. Pharmacol.* 1979;16:865–879.
17. Lewis EJ, Harrington CA, Chikaraishi DM. Transcriptional regulation of the tyrosine hydroxylase gene by glucocorticoid and cyclic AMP. *Proc. Nat. Acad. Sci. USA.* 1987; 84:3550–3554.
18. Morgan JI, Cohen DR, Hempstead JL, Curran T. Mapping patterns of c-fos expression on the central nervous system after seizure. *Science* 1987;237:192–196.
19. Paul MI, Kvetnanski R, Cramer H, Silbergeld S, Kopin IJ. Immobilization stress induced changes in adrenocortical and medullary cyclic content in the rat. *Endocrinology* 1971;88:338–344.
20. Ramon y Cajal. 1894 Croonian Lecture to the British Royal Society. Reprinted in: *Trends Neurosci.* 1988;11:125.
21. Rauscher FJ, Cohne DR, Curran T, Bos TJ, Vogt PK, Bohrman D, Tjian R, Franze Jr. BR. Fos associated protein p 39 is the product of the *jun* proto-oncogene. *Science* 1988;240:1010–1016.
22. Sagar SM, Sharp FW, Curran T. Expression of c-fos protein in brain: metabolic mapping at the cellular level. *Science* 1988;240:1328–1331.
23. Szekely AM, Barbaccia ML, Alho H, Costa E. In primary cultures of cerebellar granule cells, the activation of N-methyl-D-aspartate-sensitive glutamate receptors induces c-fos mRNA expression. *Molec. Pharmacol.* 1989;35:401–408.
24. Szekely AM, Barbaccia ML, Costa E. Activation of specific glutamate receptor subtypes increases c-fos proto-oncogene expression on primary cultures of neonatal rat cerebellar granule cells. *Neuropharmacology* 1987;26:1779–1782.
25. Zivkovic B, Guidotti A, Costa E. Effects of neuroleptics on striated tyrosine hydroxylase: changes in affinity for pteridine cofactor. *Molec. Pharmacol.* 1974;10:727–735.

# Comparison of Exogenous and Endogenous Gangliosides as Modulators of Neuronal Differentiation

Robert W. Ledeen, Gusheng Wu, Kuldeep K. Vaswani and Michael S. Cannella

*Departments of Neurology and Biochemistry  
Albert Einstein College of Medicine, Bronx, NY 10461*

The ganglioside changes accompanying neuronal differentiation are noteworthy for the remarkable increases in gangliotetraose structures that occur during the course of neurite outgrowth and synaptogenesis. This has been well-demonstrated in analyses of embryonic and neonatal brain (35,41) as well as primary neuronal cultures (10,29,46). Recent study of growth cone membranes isolated from embryonic rat brain revealed this characteristic gangliotetraose pattern to be present in the leading edge of the growing neurite as well as other components of the differentiating neuron (27).

The specific functional roles of gangliosides in the developing and mature neuron are not well understood. Some studies have focused on the behavior of the cell's natural (i.e. endogenous) gangliosides during these various phases and much has been learned through use of specific cell surface probes and interventive agents. The discovery that exogenously administered gangliosides have neuritogenic and neuronotrophic properties capable of influencing neuronal differentiation and survival (for review, 17,18) has provided another general approach to this aspect of ganglioside function. As it turns out, the two models do not reflect identical phenomena and hence the results obtained with exogenous gangliosides must be interpreted with caution in relation to the cellular function of endogenous gangliosides. Nevertheless, the exogenous effects are worthy of investigation owing to the significant pharmacological properties that administered gangliosides exhibit in many *in vitro* and *in vivo* systems. In this presentation we shall attempt to summarize some of the insights gained from these two approaches to the study of ganglioside function in the neuron.

## EXOGENOUS GANGLIOSIDES

### Structural Requirements for Neuritogenesis

Beginning with reports that exogenous gangliosides added to the B104 neuronal cell line acted as differentiation and survival factors (22), many laboratories have demonstrated similar ganglioside effects with a variety of neuronal cell lines and primary neuronal cultures. The N2A mouse neuroblastoma cell line has proven especially useful for this purpose, owing to the prolific outgrowth of processes induced by exogenous gangliosides at concentrations in the micromolar range (26). The physiological significance of this phenomenon was suggested by the formation under favorable conditions of mature synapse-like contacts resembling the mature postsynaptic thickenings found in Gray Type I synapses (33).

To determine the structural requirements for neuritogenic activity of this kind, we first compared the effects of 11 different naturally occurring gangliosides on N2A cells (3). Despite wide variations in structure, ranging from GM<sub>4</sub> with two sugars to GQ<sub>1b</sub> with nine, all 11 substances proved to be active neuritogenic agents. This was true even in the presence of serum which normally inhibits neurite outgrowth. Subsequent studies with synthetic sialoglycolipids, including "epi-GM<sub>3</sub>" with a  $\beta$ -linked sialic acid (5) and a "glycero-ganglioside" containing sialic acid attached to a diglyceride-like backbone (6), revealed these also to be neuritogenic not only toward N2A cells but also dorsal root ganglia and PC12 cells. In the latter case, gangliosides or synthetic sialoglycolipids proved neuritogenic only in the presence of nerve growth factor, as previously described (12). Additional synthetic sialoglycolipids were shown to enhance neurite outgrowth of N2A cells (38).

The above studies demonstrated that a sphingosine-containing ceramide moiety is not necessary, nor is a complex oligosaccharide chain, since sialic acid was the sole carbohydrate attached to the lipid backbone in the case of the glycero-ganglioside. Furthermore, GM<sub>1</sub> derivatives with the negative charge removed were still capable of enhancing neurite outgrowth in N2A cells, dorsal root ganglia and PC12 cells (4). \* The broad spectrum of sialoglycolipid structures showing this kind of activity suggest a mechanism involving general membrane perturbation, possibly of a physical-chemical nature. This mechanism would be somewhat independent of carbohydrate structure, except for an apparent requirement of sialic acid or related moiety. The latter is inferred from the fact that asialo GM<sub>1</sub> and various other neutral glycolipids were without activity toward N2A cells (3) (although a few such substances along with other surfactants were recently claimed to exert trophic effects toward certain primary neurons in culture [39]). The effect appears

\* It is of some interest to note that these GM<sub>1</sub> derivatives lacking a negative charge proved inactive as trophic factors *in vivo*, despite their potency as neuritogenic agents *in vitro* (7).

specific for lipids since sialoproteins were without effect, at least toward N2A cells (19).

Physical-chemical perturbations of the above postulated type would be expected to require at least moderately high concentrations of sialolipids, which may explain why these substances have generally been used in the range 10-100  $\mu\text{M}$ . The mechanism of this effect could differ fundamentally from that of the "N-type" phenomenon which requires an order-of-magnitude lower concentration (23). Thus far two neuroblastoma lines of the N-type have been reported, GOTO and NB-1 (37). In addition to the lower concentration requirement, these cells showed great selectivity in responding only to GQ<sub>1b</sub>.

### Role of $\text{Ca}^{2+}$ with Exogenous Gangliosides

A recent study in our laboratory (45) demonstrated a requirement for exogenous  $\text{Ca}^{2+}$  in ganglioside-enhanced neurite outgrowth of N2A cells. This was indicated by blockage of such outgrowth through use of  $\text{Ca}^{2+}$ -depleted medium (modified DMEM lacking  $\text{Ca}^{2+}$ ) containing EGTA; the latter was necessary since medium lacking it ceased to inhibit neuritogenesis induced by added ganglioside. On the other hand,  $\text{Ca}^{2+}$ -depleted medium containing EGTA did not block neurite outgrowth of N2A cells stimulated by retinoic acid or dibutyryl cyclic AMP (cAMP).

This  $\text{Ca}^{2+}$  requirement was possibly related to the modest but statistically significant enhancement of  $^{45}\text{Ca}^{2+}$  influx induced by bovine brain ganglioside mixture (BBG). As shown in Table 1, this BBG-stimulated influx occurred in low

TABLE 1. Effect of BBG and serum on  $^{45}\text{Ca}^{2+}$  influx in N2A cells

	1% FBS-DMEM n = 12		
Time (min)	10	20	30
Control	515 $\pm$ 29	783 $\pm$ 43	919 $\pm$ 56
BBG	568 $\pm$ 37	843 $\pm$ 54	1030 $\pm$ 54
p <	0.001	0.01	0.01

	10% FBS-DMEM n = 6		
Time (min)	10	20	30
Control	614 $\pm$ 49	842 $\pm$ 24	890 $\pm$ 49
BBG	614 $\pm$ 48	848 $\pm$ 38	867 $\pm$ 64

N2A cells growing in DMEM with 1% or 10% fetal bovine serum (FBS) were treated simultaneously with 0.11 mM BBG and  $^{45}\text{Ca}^{2+}$  for the times shown ( $\text{Ca}^{2+}$  = 1.8 mM). Reaction was stopped by placing on ice and rapidly washing; radioactivity was measured in the harvested cells. Data are pmol  $^{45}\text{Ca}^{2+}$ /mg protein  $\pm$  SD. p refers to Student's t test. The differences with 10% FBS were not significant.

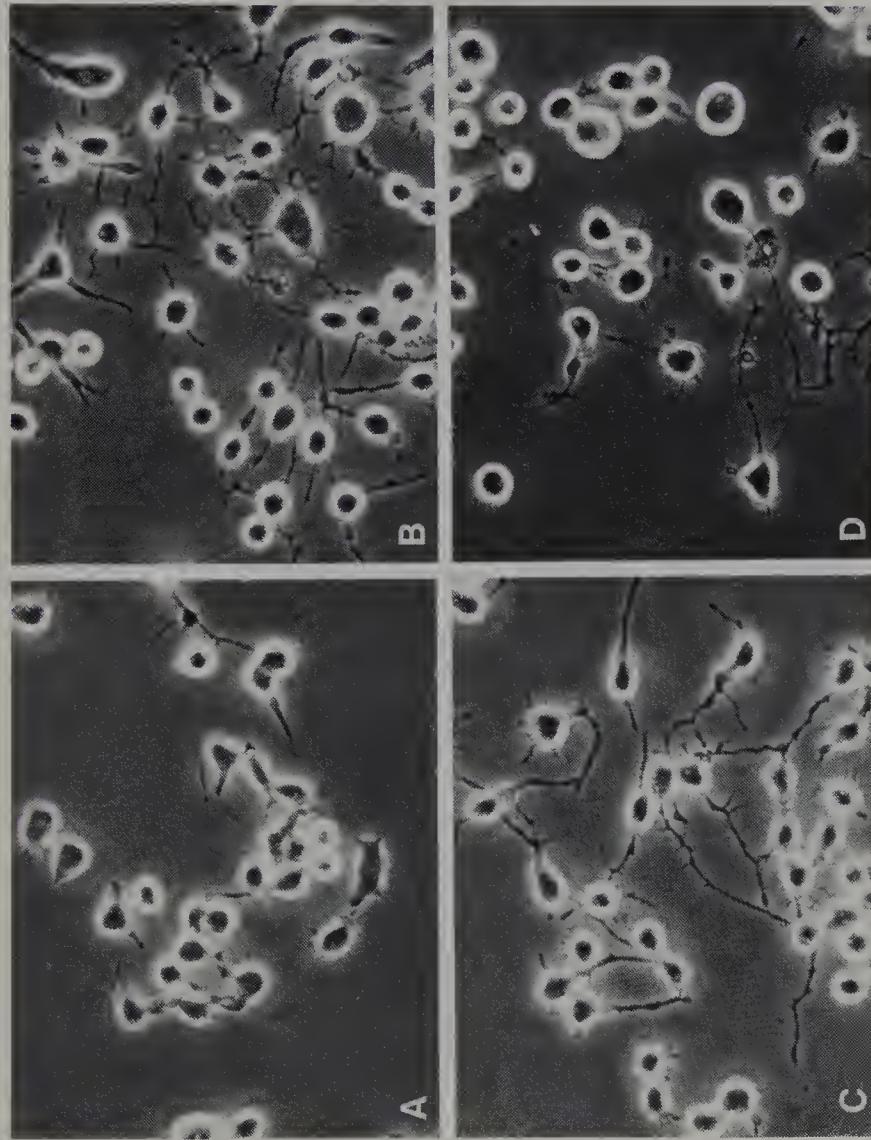
**TABLE 2.** Effect of BBG and serum on  $^{45}\text{Ca}^{2+}$  efflux in N2A cells

	1% FBS-DMEM n = 6		
Time (min)	10	20	30
Control	63 $\pm$ 8.4	71 $\pm$ 5.1	80 $\pm$ 5.6
BBG	57 $\pm$ 8.9	67 $\pm$ 9.3	75 $\pm$ 10
p <	NS	NS	NS
	10% FBS-DMEM n = 6		
Time (min)	10	20	30
Control	57 $\pm$ 3.1	64 $\pm$ 7.8	72 $\pm$ 7.4
BBG	76 $\pm$ 4.4	88 $\pm$ 6.7	96 $\pm$ 3.6
p <	0.001	0.001	0.001

N2A cells growing in DMEM with 1% or 10% FBS ( $\text{Ca}^{2+} = 1.8 \text{ mM}$ ) were labeled by incubating 1 hr at 37°C with 4  $\mu\text{Ci}$  of  $^{45}\text{Ca}^{2+}$  in 1 ml. The medium was removed, the attached cells were washed and treated with the same medium lacking label but containing BBG as described (45). The cells were then incubated for the periods indicated, aliquots being withdrawn for  $^{45}\text{Ca}^{2+}$  measurement. Efflux is expressed as % of  $^{45}\text{Ca}^{2+}$  detected in the sum of withdrawn aliquots relative to the total preloaded  $^{45}\text{Ca}^{2+}$ . Data are mean  $\pm$  SD. NS, not significant.

serum-containing medium but not when fetal bovine serum (FBS) was raised to 10%. Calcium efflux was also stimulated by BBG, this being favored by higher concentrations of FBS (Table 2). Enhanced  $\text{Ca}^{2+}$  cycling thus appears to have been triggered by exogenous BBG in a serum-dependent manner. It is not known whether total cytosolic  $\text{Ca}^{2+}$  increases significantly or which subcellular compartment receives extracellular  $\text{Ca}^{2+}$  upon BBG stimulation. Conceivably the membrane itself could be the critical locus of  $\text{Ca}^{2+}$  cycling, as recently described for other systems (1).

The fact that BBG-induced differentiation can be made to occur in 10% FBS suggests that  $\text{Ca}^{2+}$  flux may occur to some extent in this medium even though  $^{45}\text{Ca}^{2+}$  influx could not be detected under the chosen conditions (Table 1). It is possible, of course, that these conditions were not optimal for observing this effect. Enhancement of  $\text{Ca}^{2+}$  efflux by BBG is undoubtedly important in this process, as evidenced in the observation that BBG treatment of N2A cells in 1% FBS (where efflux is not facilitated—cf. Table 2) led to retraction of processes and eventual cell death (Fig. 1). For example, under these conditions only  $\frac{1}{3}$  as many N2A cells survived in the presence of 0.11 mM BBG as in its absence. These experiments also illustrated the essentiality of serum in facilitating the  $\text{Ca}^{2+}$  cycling enhancement promoted by BBG.



**FIG. 1.** Deleterious effect of gangliosides on N2A cells in low serum. Cells were reseeded after dissociation with 0.25% trypsin at a density of about  $10^4$  cells/cm $^2$ , and cultured in DMEM supplemented with 10% FBS, and 50 mg/L gentamicin;  $\text{Ca}^{2+} = 1.8$  mM. The incubator was equilibrated at 37°C with 5% CO<sub>2</sub>/95% humidified air. After establishing growth, the media were changed to contain the above elements with the following FBS/BBG contents: (A) 5% FBS, no BBG; (B) 5% FBS + 0.11 mM BBG; (C) 1% FBS, no BBG; (D) 1% FBS + 0.11 mM BBG. All incubations were for 48 hr. The neurite outgrowth stimulated by BBG in the presence of 5% FBS (B) or by serum reduction alone (C) was repressed by BBG in low serum (D). The number of viable cells also decreased by 2/3 in the latter.

## Effects of $\text{Ca}^{2+}$ Influx by Other Means: Protective Effect of Gangliosides

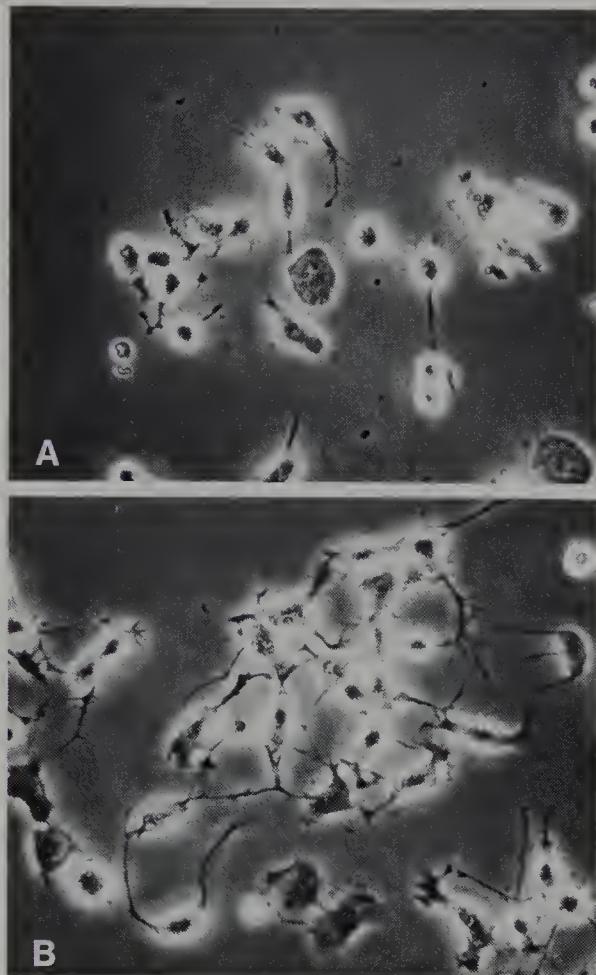
Calcium was shown to be an important intracellular messenger in neuronal differentiation (14) and, as noted above, to be a key factor in the assertion of neuritogenic effects by exogenous gangliosides. Neurite outgrowth of N2A cells was also enhanced by subjecting these cells to  $\text{Na}^+$ -free medium (Fig. 2). In this experiment, use of Hank's balanced salt solution in which choline chloride has replaced  $\text{NaCl}$  caused  $\text{Ca}^{2+}$  influx due to reversal of the  $\text{Na}^+/\text{Ca}^{2+}$  exchanger (8).

Additional evidence for a  $\text{Ca}^{2+}$  role in N2A cells comes from the effects of the  $\text{Ca}^{2+}$  ionophore A23187 which had an initial stimulatory effect analogous to BBG (Fig. 3). Neurite outgrowth was evident after 12 hr exposure to  $10^{-6}$  M ionophore and even longer neurites were evident after 24 hr. However, by 48 hr neurite contraction was observed (Fig. 3D) along with increasing cell mortality. It was interesting to note that exogenous ganglioside had a protective effect in this system (Fig. 3F), possibly due to its promotion of  $\text{Ca}^{2+}$  efflux. Similar effects were observed with  $\text{GM}_1$ ,  $\text{GD}_{1a}$ , and BBG (not shown). Quantification of this neuronotrophic effect revealed  $\text{GT}_{1b}$  to be the most effective, although even this was not fully protective in terms of cell mortality. Thus, it would appear that both the neuritogenic and neuronotrophic properties of exogenous gangliosides are mediated through their effect on  $\text{Ca}^{2+}$  cycling and/or the maintenance of  $\text{Ca}^{2+}$  homeostasis. These results may be compared with the protective effect of gangliosides toward glutamate and kainate neurotoxicity in primary neuronal cultures (11,40). In those systems exogenous gangliosides appeared to assert this effect through blocking translocation of protein kinase C to the membrane, while a recent report (13) has suggested that modulation of  $\text{Ca}^{2+}$  influx may also be a factor. It is interesting to note that, as with our ionophore model,  $\text{GT}_{1b}$  proved the most effective individual ganglioside.

## ENDOGENOUS GANGLIOSIDES

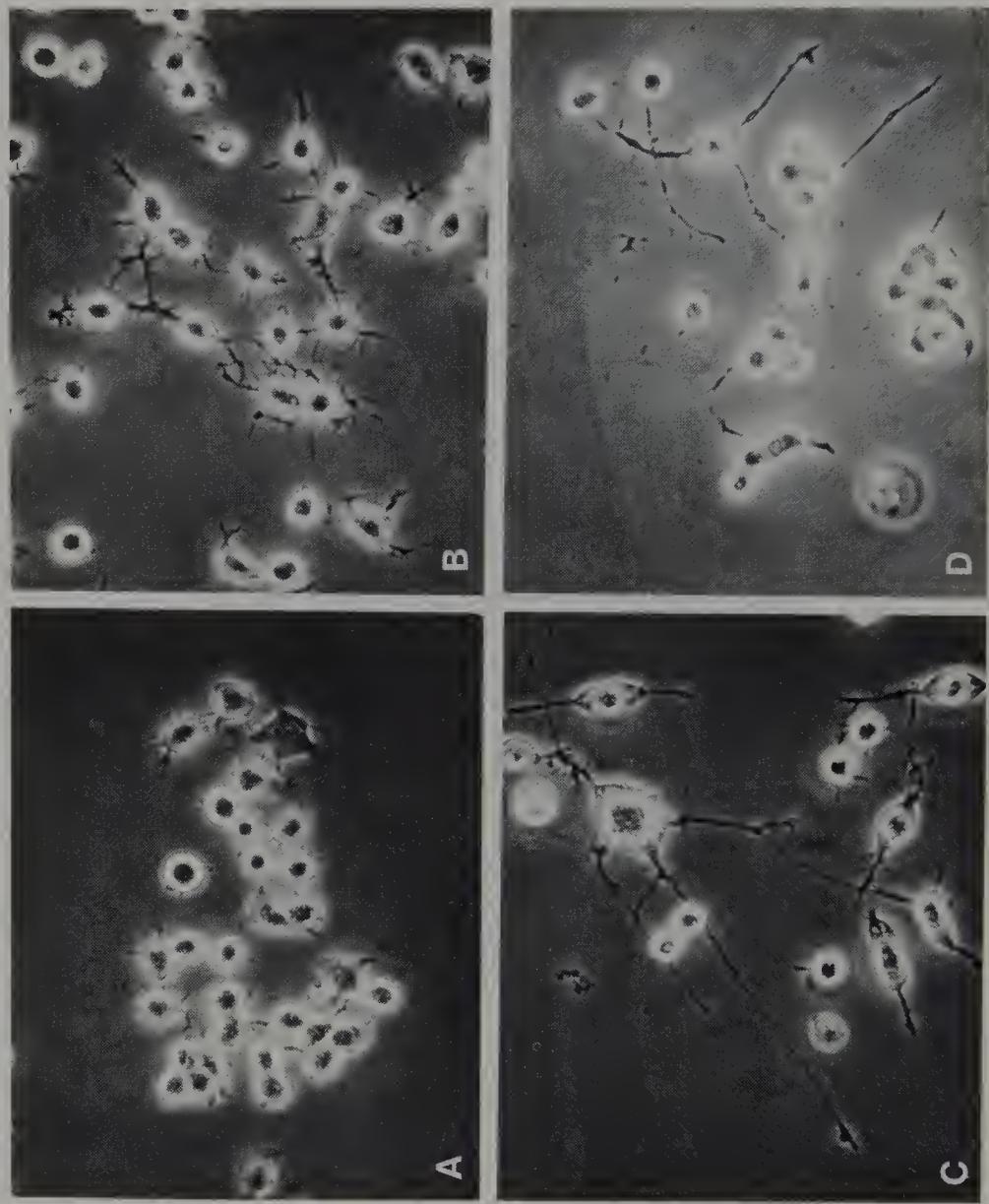
### Correlation of $\text{GM}_1$ with Neurite Outgrowth

Ganglioside  $\text{GM}_1$  was proposed as a marker for neuronal differentiation in the mouse cerebellum (42) and use of anti- $\text{GM}_1$  antibody as interventive agent caused inhibition of neurite outgrowth both *in vitro* (28) and *in vivo* (32). Conditioned media-enhanced neuritogenesis of dorsal root ganglia was blocked by several monoclonal antibodies to  $\text{GM}_1$ , though the potency of this blockage was variable (34). Our own study of several neuroblastoma cell lines with varying neurite-forming potential revealed that those cells most responsive to neuritogenic stimuli had the highest levels of gangliotetraose gangliosides (43). There was no correlation with respect to other ganglioside families or total ganglioside content. More recent work



**FIG. 2.** Neuritogenesis of N2A cells in  $\text{Na}^+$ -free medium. Cells were grown without serum in regular Hank's balanced salt solution (A) or the latter with choline in place of  $\text{Na}^+$  (B). Stimulated neurite outgrowth is believed due to reversal of the  $\text{Na}^+/\text{Ca}^{2+}$  antiporter.

FIG. 3.



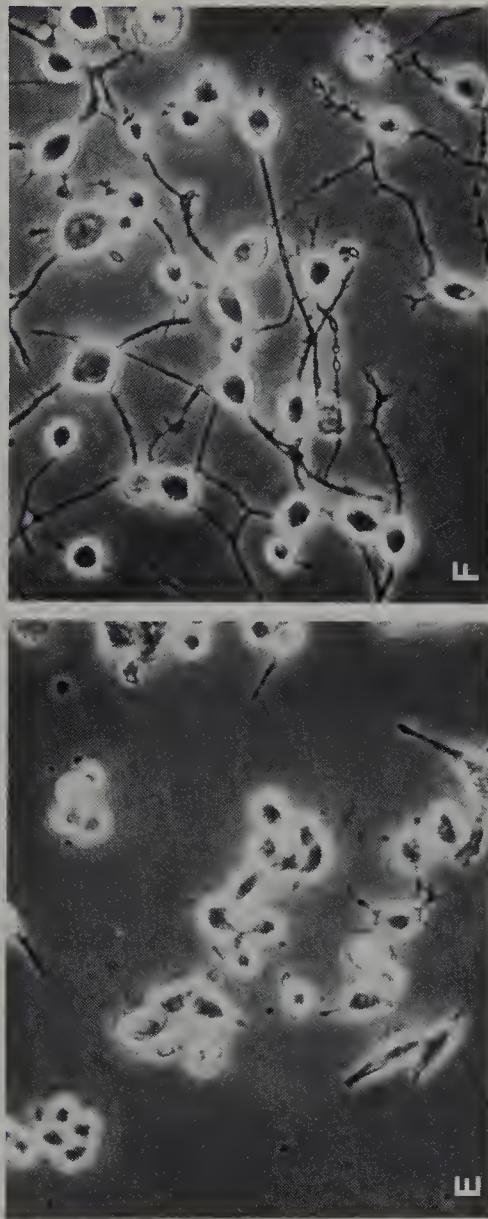
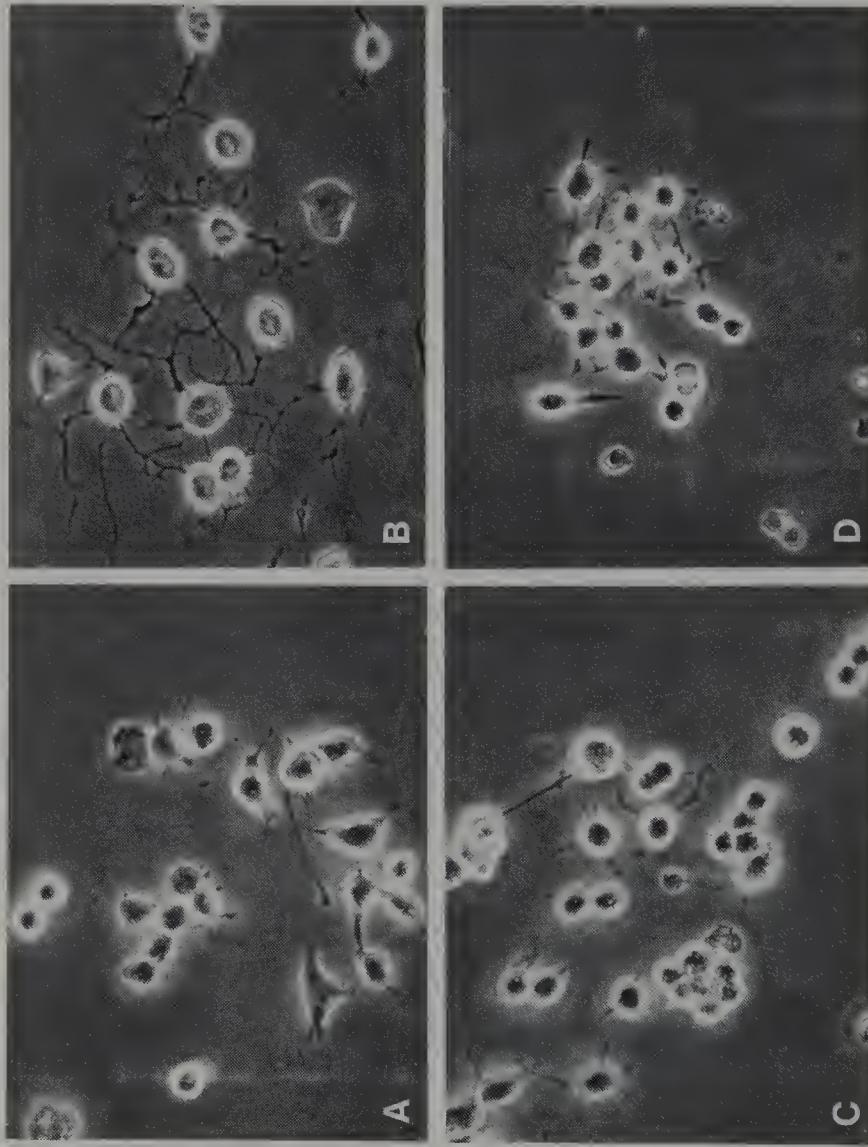


FIG. 3. Stimulation of N2A cells by  $\text{Ca}^{2+}$  ionophore and neurotrophic effect of ganglioside. Cells were grown similar to method in Fig. 1 with the following changes: (A) 10% FBS, 12 hr; (B)  $10^{-6}\text{M}$  A23187 (10 $^{-6}\text{M}$ ), 12 hr; (C) same as B, 24 hr; (D) and (E) same as B, 48 hr; (F) same as D and E + GT<sub>1b</sub> (200  $\mu\text{g}/\text{ml}$ ). The initial neuritogenic effect of ionophore A23187 (B,C) becomes inhibitory and lethal by 48 hr (D,E). Some protection is offered by GT<sub>1b</sub> (and other gangliosides, not shown).



**FIG. 4.** Stimulation of neurite outgrowth with neuraminidase and inhibition with cholera B and anti-GM<sub>1</sub> antibody. N2A cells were cultured as in Fig. 1 in the presence of 5% FBS for 24 hr without (A) or with (B) neuraminidase (Sigma C. perfringens Type V, 1 unit/ml, pH 7.2). To B was added cholera B, 10 µg/ml (C) or anti-GM<sub>1</sub> antibody, affinity purified and diluted 1:100 (D). The prolific neurite outgrowth generated by N'ase was effectively blocked by both cholera B and anti-GM<sub>1</sub> antibody.

has indicated GM<sub>1</sub> to be the most important of the gangliotetraose species. The potential for neurite recruitment thus appears to depend on the presence of a threshold level of GM<sub>1</sub> (or related species) contributed by the cell to its own membrane. In that sense the structural requirements related to neuronal differentiation appear much more stringent for endogenous as opposed to exogenous ganglioside.

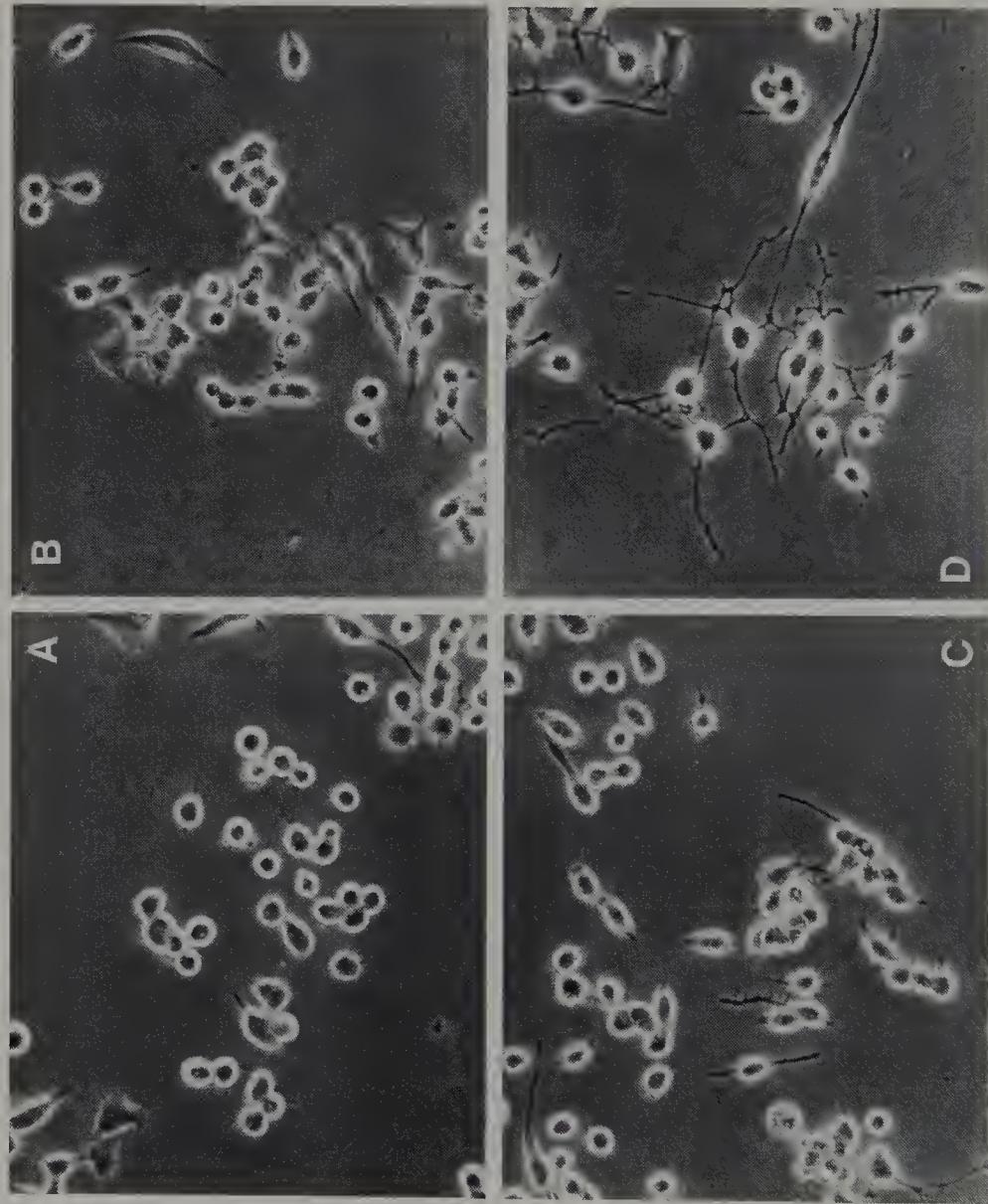
### Enhancement of Neurite Outgrowth with Neuraminidase

If, as discussed above, the presence of GM<sub>1</sub> is critical to the process of neurite outgrowth, it was of interest to determine the effect of increasing the level of this endogenous ganglioside on the cell surface. This was accomplished in a recent study of our group (44) by growing N2A neuroblastoma cells in the presence of neuraminidase (N'ase), the increased GM<sub>1</sub> being demonstrated by fluorescent staining of the cells with the B subunit of cholera toxin (cholera B) conjugated to FITC. The result was prolific outgrowth of neurites over a period of several hours (Fig. 4). The same result was achieved with the B104 and B50 rat neuroblastoma lines, but not the N1A-103 line (2) described as "neurite minus" owing to its intrinsic inability to differentiate (not shown).

This experiment must be interpreted with caution owing to the fact that treatment of living cells with N'ase causes other changes in the cell surface glycoconjugates besides the desired conversion of oligosialogangliosides of the gangliotetraose series to GM<sub>1</sub>: e.g., removal of sialic acid from glycoproteins, reduction of gangliosides belonging to other series (e.g., hematoside, neolacto, globo), and increase of neutral glycosphingolipids derived from the latter. The gangliotetraose series is somewhat unique in retaining one sialic acid (of GM<sub>1</sub>) following reaction with N'ase under nonforcing conditions. The increase of cell surface GM<sub>1</sub> would appear to be the critical event in the above experiment, based on the observation that neurite outgrowth was blocked by both cholera B and anti-GM<sub>1</sub> antibody (Fig. 4). However, other possibilities involving other glycoconjugate changes have not been rigorously excluded. That the blocking effect of cholera B could have resulted from cAMP production due to residual cholera A subunit contamination was ruled out by the observation that whole cholera toxin treatment of N2A cells caused enhancement rather than retardation of neurite outgrowth (not shown).

### Dependence of the Neuritogenic Effect of N'ase on Ca<sup>2+</sup>

A requirement for Ca<sup>2+</sup> was demonstrated by the fact that reduction of extracellular Ca<sup>2+</sup> blocked the neuritogenic effect of N'ase (Fig. 5). In this experiment it was not necessary to use EGTA to see the effect. At the same time, N'ase treatment caused a modest but statistically significant increase in the influx of



**FIG. 5.** Blockage of neuraminidase-stimulated neuritogenesis with reduced  $\text{Ca}^{2+}$ . (A) N2A cells were grown as in Fig. 1 with 5% FBS but using  $\text{Ca}^{2+}$ -free DMEM (low  $\text{Ca}^{2+}$  medium, without EGTA); (B) same as A with addition of N'ase as in Fig. 4, 24 hr; (C) same as B, 48 hr; (D) cells grown with N'ase in normal  $\text{Ca}^{2+}$  medium (1.8 mM), as in Fig. 4. Note that reduced  $\text{Ca}^{2+}$  medium contained a small amount of  $\text{Ca}^{2+}$  from FBS, but the reduction in  $\text{Ca}^{2+}$  was sufficient to block N'ase-simulated neurite formation.

**TABLE 3.**  $^{45}\text{Ca}^{2+}$  uptake in N2A cells stimulated by N'ase

Time (min)	Control	N'ase	p <
60	858 ± 44	1020 ± 49	0.05
90	906 ± 31	1005 ± 31	0.05
120	835 ± 41	996 ± 34	0.01
180	829 ± 44	965 ± 35	0.05
240	801 ± 41	958 ± 45	0.02

Cells in T-25 Falcon flasks were incubated with 4 ml DMEM (plus 5% FBS) containing 5 units of N'ase for times up to 180 min; the cultures were then treated with 3  $\mu\text{Ci}$  of  $^{45}\text{Ca}^{2+}$  in 1 ml of same medium (total vol, 5 ml) for an additional 60 min. Thus, cells were exposed to N'ase for a total time indicated in first column. After washing, the cells were harvested and counted. Data are expressed as pmol/mg protein ± SEM. n = 10 in all experiments.

$^{45}\text{Ca}^{2+}$  (Table 3). As with neurite outgrowth, this effect was blocked by cholera B. The increase in  $^{45}\text{Ca}^{2+}$  influx was maintained over 4 hr with no indication of return to control level, suggesting that  $\text{Ca}^{2+}$  was persistently elevated throughout the period of N'ase treatment. However, measurement of intracellular  $\text{Ca}^{2+}$  will be needed to obtain a complete picture of  $\text{Ca}^{2+}$  flux and relocation under these conditions.

## DISCUSSION

To explain the above phenomena and correlate our findings with the known behavior of neurons during differentiation, we have proposed as a working hypothesis that neurons (and neuroblastoma cells capable of differentiation) must possess a threshold level of GM<sub>1</sub> gangliosides in their membrane at the critical phase of neurite outgrowth. This is in reference to the endogenous pool, although conceivably exogenous GM<sub>1</sub> might also contribute under appropriate circumstances. This model is supported by the blocking effects of cholera B and anti-GM<sub>1</sub> antibody in N2A cells as well as in the other *in vitro* and *in vivo* systems mentioned above. Our results further suggest that membrane localized GM<sub>1</sub> functions in some manner through modulation of  $\text{Ca}^{2+}$  flux. Possibly relevant to this idea are recent reports indicating enhanced influx of  $\text{Ca}^{2+}$  into cultured heart cells accompanying N'ase treatment (15,24,47). Among a number of possibilities, consideration is being given a possible modulatory role of GM<sub>1</sub> on specific  $\text{Ca}^{2+}$  channels.

Several reports have emphasized a potential functional connection between gangliosides and  $\text{Ca}^{2+}$  (21,25). Although the electrostatic interaction of these substances at the membrane level has been characterized as rather weak (16), gangliosides and  $\text{Ca}^{2+}$  have been shown to interact in various ways including joint

modulation of kinase activities in neural membranes (48). It is of some interest that cholera B added to lymphocytes (9) or quiescent 3T3 cells (31) caused pronounced increase in intracellular  $\text{Ca}^{2+}$  while acting as a mitogenic agent. In that model the binding of cholera B would have the effect of reducing "free GM<sub>1</sub>" on the cell surface, opposite to the action of N'ase. However, it was noted that cholera B inhibited mitogenesis when applied to 3T3 cells that were transformed or in a state of rapid growth (30), thus suggesting that endogenous GM<sub>1</sub> can function as a bimodal regulator of positive or negative signals depending on the metabolic state of the cell.

These comparative studies have served to emphasize an important difference between the mechanism of action of endogenous as opposed to exogenous gangliosides. While it is true that the latter insert themselves spontaneously into the cell membrane, it is not yet clear that such incorporation is a prerequisite for neuritogenesis. Only a very small portion of added ganglioside is incorporated in this manner, a much larger pool remaining loosely attached to the cell surface with potential for affecting neuronal behavior. Even for that portion which does incorporate, it is not certain that it behaves the same as endogenous ganglioside, which likely exists in close association with membrane proteins (20). The broad spectrum of sialoglycolipids having exogenous biological activity would seem to argue in favor of a relatively nonspecific physical-chemical perturbation of the neuronal membrane, resulting in alterations of  $\text{Ca}^{2+}$  flux which perhaps provides the signal for differentiation. While a common linkage appears to exist between endogenous GM<sub>1</sub> and exogenous sialoglycolipids in relation to their modulatory effect on  $\text{Ca}^{2+}$ , there is a fundamental difference in the nature of this modulation and hence of underlying mechanism. Clearly much remains to be learned about both mechanisms in elucidating the neuronotrophic and neuritogenic properties of gangliosides.

## SUMMARY

During the normal course of neuronal differentiation, gangliosides undergo marked increase in concentration with appearance of the gangliotetraose family. In addition to these naturally occurring (endogenous) manifestations, exogenously administered gangliosides have been observed to exert neuritogenic and/or neuronotrophic effects on a variety of neuroblastoma cell lines and primary neuronal culture systems. Unlike the endogenous effects, which appear to require gangliotetraose structures, the structural specificity of the exogenous effect is quite broad. These results point to general perturbation of the membrane (a physico-chemical effect) in a manner which triggers events leading to differentiation. The associated events have been found to include enhanced  $\text{Ca}^{2+}$  influx and efflux, that is, acceleration of  $\text{Ca}^{2+}$  cycling. Study of the endogenous ganglioside content of several neuroblastoma cell lines has revealed a correlation between capacity for neurite recruitment and the content of gangliotetraose species—especially GM<sub>1</sub>.

When neuroblastoma cells were cultured in the presence of neuraminidase (causing a rise in GM<sub>1</sub>), neurite outgrowth proceeded more readily. Neurite outgrowth was blocked by anti-GM<sub>1</sub> antibody and cholera B subunit. Another effect of neuraminidase treatment was to increase the influx of Ca<sup>2+</sup>. Thus, Ca<sup>2+</sup> effects appear to operate in both cases although the mechanisms likely differ since the N'ase-induced response was more readily inhibited by Ca<sup>2+</sup> reduction. The apparently special role for GM<sub>1</sub> in the latter is contrasted with the diverse sialoglycolipid structures able to elicit neurite outgrowth as exogenous agents.

## ACKNOWLEDGMENT

This work is supported by USPHS, NIH grants NS 04834 and NS 24172.

## REFERENCES

1. Alkon DL, Rasmussen H. A spatial-temporal model of cell activation. *Science* 1988;239:998–1004.
2. Amano T, Richelson E, Nirenberg M. Neurotransmitter synthesis by neuroblastoma clones. *Proc. Nat. Acad. Sci. USA* 1972;69:258–263.
3. Byrne MC, Ledeen RW, Roisen FJ, Yorke G, Sclafani JR. Ganglioside-induced neuritogenesis: Verification that gangliosides are the active agents, and comparison of molecular species. *J. Neurochem.* 1983;41:1214–1222.
4. Cannella MS, Wu G, Vaswani KK, Ledeen RW. Neuritogenic effects of exogenous gangliosides and synthetic sialoglycolipids: Comparison to endogenous ganglioside requirements. In: Ledeen RW, Hogan EL, Tettamanti G, Yates AJ, Yu RK, eds. *New Trends in Ganglioside Research: Neurochemical and Neurogenerative Aspects*. Padova: Liviana Press, 1988; 379–390.
5. Cannella MS, Roisen FJ, Ogawa T, Sugimoto M, Ledeen RW. Comparison of epi-GM3 with GM3 and GM<sub>1</sub> as stimulators of neurite outgrowth. *Dev. Brain Res.* 1988;39:137–143.
6. Cannella MS, Acher AJ, Ledeen RW. Stimulation of neurite outgrowth *in vitro* by a glyceroganglioside. *Int. J. Dev. Neurosci.* 1988;6:319–326.
7. Cannella MS, Oderfeld-Nowak B, Gradkowska M, Skup M, Garofalo L, Cuello AC, Ledeen RW. Derivatives of ganglioside GM<sub>1</sub> as neuronotrophic agents: Comparison of *in vivo* and *in vitro* effects. *Brain Res.* 1990; in press.
8. DiPolo R, Beauge L. Characterization of the reverse Na/Ca exchange in squid axons and its modulation by Ca, and ATP. *J. Gen. Physiol.* 1987;90:505–525.
9. Dixon SJ, Stewart D, Grinstein S, Spiegel S. Transmembrane signaling by the beta-subunit of cholera toxin increases cytoplasmic free calcium in rat thymocytes. *J. Cell Biol.* 1987;105:1153–1161.
10. Dreyfus H, Louis JC, Harth S, Mandel P. Gangliosides in cultured neurons. *Neuroscience* 1980;5:1647–1655.
11. Favaron M, Manev H, Alho H, Bertolino M, Ferret B, Guidotti A, Costa E. Gangliosides prevent glutamate and kainate neurotoxicity in primary neuronal cultures of neonatal rat cerebellum and cortex. *Proc. Natl. Acad. Sci. USA* 1988;85:7351–7355.

12. Ferrari G, Fabris M, Gorio A. Gangliosides enhance neurite outgrowth in PC12 cells. *Dev. Brain Res.* 1983;8:215–222.
13. Guidotti A, Manev H, Favaron M, Costa E. Gangliosides prevent excitatory amino acid-induced cell death in primary neuronal cultures. In: *Symposium on Cell Culture as a Tool for Studying Metabolism, Function and Signaling of Glycolipids*, Vittel, France. 1989;14 (Abstr.).
14. Kater SB, Mattson MP, Cohan C, Connor J. Calcium regulation of the neuronal growth cone. *Trends Neurosci.* 1988;11:315–321.
15. Langer GA, Frank JS, Nudd LM, Seraydarian K. Sialic acid: Effect of removal on calcium exchangeability of cultured heart cells. *Science* 1976;193:1013–1015.
16. Langer M, Winiski A, Eisenberg M, McLaughlin A, McLaughlin S. The electrostatic potential adjacent to bilayer membranes containing either charged phospholipids or gangliosides. In: Ledeen RW, Hogan EL, Tettamanti G, Yates AJ, Yu RK, eds. *New Trends in Ganglioside Research: Neurochemical and Neuroregenerative Aspects*, Padova: Liviana Press, 1988;121–131.
17. Ledeen RW. Gangliosides. In: Lajtha A, ed. *Handbook of Neurochemistry*, Vol. 3. 2nd edition. New York: Plenum Press, 1983;41–90.
18. Ledeen RW. Biology of gangliosides: Neuritogenic and neuronotrophic properties. *J. Neurosci. Res.* 1984;12:147–159.
19. Ledeen RW, Cannella MS. The neuritogenic effect of gangliosides in cell cultures. In: Rahmann H, ed. *Gangliosides and Modulation of Neuronal Functions*, Berlin: Springer-Verlag, 1987;491–500.
20. Ledeen RW. Biosynthesis, metabolism, and biological effects of gangliosides. In: Margolis RU, Margolis RK, eds. *Neurobiology of Glycoconjugates*, New York: Plenum Press, 1989;43–83.
21. Maggio B, Sturtevant JM, Yu RK. Effect of calcium ion on the thermotropic behavior of neutral and anionic glycosphingolipids. *Biochim. Biophys. Acta*. 1987;901:173–182.
22. Morgan JI, Seifert W. Growth factors and gangliosides: A possible new perspective in neuronal growth control. *J. Supramol. Struct.* 1979;10:111–124.
23. Nagai Y, Tsuji S. Cell biological significance of gangliosides in neural differentiation and development: Critique and proposals. In: Ledeen RW, Hogan EL, Tettamanti G, Yates AJ, Yu RK, eds. *New Trends in Ganglioside Research: Neurochemical and Neuroregenerative Aspects*. Padova: Liviana Press, 1988;329–350.
24. Nathan RD, Fung SJ, Stocco DM, Barron EA, Markwald RR. Sialic acid: Regulation of electrogenesis in cultured heart cells. *Am. J. Physiol.* 1980;239:C197–C207.
25. Rahmann H, Probst W. Calcium, gangliosides and brain function. In: Tucek S, Strípek S, Stastný F, Krivanek J, eds. *Molecular Basis of Neural Activity*, Prague: European Society Neurochemistry, 1986;54.
26. Roisen FJ, Bartfeld H, Nagele R, Yorke G. Ganglioside stimulation of axonal sprouting *in vitro*. *Science* 1981;214:577–578.
27. Sbaschnig-Agler M, Pfenninger KH, Ledeen RW. Gangliosides and other lipids of the growth cone membrane. *J. Neurochem.* 1988;51:212–220.
28. Schwartz M, Spirman N. Sprouting from chicken embryo dorsal root ganglia induced by nerve growth factor is specifically inhibited by affinity-purified antiganglioside antibodies. *Proc. Natl. Acad. Sci. USA* 1982;79:6080–6083.
29. Seifert W, Fink H-J. *In vitro* and *in vivo* studies on gangliosides in the developing and regenerating hippocampus of the rat. In: Ledeen RW, Yu RK, Rapport MM, Suzuki

- K, eds. *Ganglioside Structure, Function, and Biomedical Potential*, New York: Plenum Press, 1984;535–545.
- 30. Spiegel S, Fishman PH. Gangliosides as bimodal regulators of cell growth. *Proc. Natl. Acad. Sci. USA* 1987;84:141–145.
  - 31. Spiegel S, Panagiotopoulos C. Mitogenesis of 3T3 fibroblasts induced by endogenous ganglioside is not mediated by cAMP, protein kinase C, or phosphoinositides turnover. *Exp. Cell Res.* 1988;177:414–427.
  - 32. Spirman N, Sela B-A, Gilter C, Calef E, Schwartz M. Regenerative capacity of the goldfish visual system is affected by antibodies specific to gangliosides injected intraocularly. *J. Neuroimmunol.* 1984;6:197–207.
  - 33. Spoerri PE. Effects of gangliosides on the *in vitro* development of neuroblastoma cells: An ultrastructural study. *Int. J. Dev. Neurosci.* 1983;1:383–391.
  - 34. Spoerri PE, Rapport MM, Mahadik SP, Roisen FJ. Inhibition of conditioned media-mediated neuritogenesis of sensory ganglia by monoclonal antibodies to GM<sub>1</sub> ganglioside. *Dev. Brain Res.* 1988;41:71–77.
  - 35. Suzuki K. The pattern of mammalian brain gangliosides. III. Regional and developmental differences. *J. Neurochem.* 1965;12:969–979.
  - 36. Svennerholm L. Chromatographic separation of human brain ganglioside. *J. Neurochem.* 1963;10:613–623.
  - 37. Tsuji S, Arita M, Nagai Y. GQ<sub>1b</sub>, a bioactive ganglioside that exhibits novel nerve growth factor (NGF)-like activities in the two neuroblastoma cell lines. *J. Biochem.* 1983;94:303–306.
  - 38. Tsuji S, Yamashita T, Tanaka M, Nagai Y. Synthetic sialyl compounds as well as natural gangliosides induce neuritogenesis in a mouse neuroblastoma cell line (Neuro 2A). *J. Neurochem.* 1988;50:414–423.
  - 39. Unsicker K, Wiegandt H. Promotion of survival and neurite outgrowth of cultured peripheral neurons by exogenous lipids and detergents. *Exp. Cell Res.* 1988;178:377–389.
  - 40. Vaccarino F, Guidotti A, Costa E. Ganglioside inhibition of glutamate-mediated protein kinase C translocation in primary cultures of cerebellar neurons. *Proc. Natl. Acad. Sci. USA* 1987;84:8707–8711.
  - 41. Vanier MT, Holm M, Mansson JE, Svennerholm L. The distribution of lipids in the human nervous system. V. Gangliosides and allied neutral glycolipids of infant brain. *J. Neurochem.* 1973;21:1375–1384.
  - 42. Willinger M, Schachner M. GM<sub>1</sub> ganglioside as a marker for neuronal differentiation in mouse cerebellum. *Develop. Biol.* 1980;74:101–117.
  - 43. Wu G, Ledeen RW. Quantification of gangliotetraose type gangliosides in several neuroblastoma lines and correlation with neuritogenic potential. *Trans. Am. Soc. Neurochem.* 1988;19:217.
  - 44. Wu G, Ledeen RW. Stimulation of neurite outgrowth in neuroblastoma lines by neuraminidase: Putative role of GM<sub>1</sub> ganglioside in calcium-mediated differentiation. *J. Neurochem.* 1990; accepted.
  - 45. Wu G, Vaswani KK, Lu Z-H, Ledeen RW. Gangliosides stimulate calcium flux in Neuro-2A cells and require exogenous calcium for neuritogenesis. *J. Neurochem.* 1990; in press.
  - 46. Yavin E, Yavin Z. Ganglioside profiles during neural tissue development. Acquisition in the prenatal rat brain and cerebral cell cultures. *Dev. Neurosci.* 1979;2:25–37.

47. Yee HF, Jr., Weiss JN, Langer GA. Neuraminidase selectively enhances transient  $\text{Ca}^{2+}$  current in cardiac myocytes. *Am. J. Physiol.* 1989;256:C1267–C1272.
48. Yu RK. Regulation of protein phosphorylation by gangliosides. In: Ledeen RW, Hogan EL, Tettamanti G, Yates AJ, Yu RK, eds. *New Trends in Ganglioside Research: Neurochemical and Neuroregenerative Aspects*, Padova: Liviana Press, 1988;461–471.

# Platelet-derived Growth Factor: Role in Gliogenesis and in the Development of Glioblastoma

Christer Betsholtz<sup>1</sup>, Monica Nistér<sup>1</sup>, Carl-Henrik Heldin<sup>2</sup> and  
Bengt Westermark<sup>2</sup>

<sup>1</sup> Department of Pathology, University Hospital, S-751 85 Uppsala, Sweden

<sup>2</sup> Ludwig Institute for Cancer Research, Biomedical Center, S-751 23 Uppsala,  
Sweden.

**P**latelet-derived growth factor (PDGF), a 30 kDa polypeptide mitogen, was originally purified from human platelets, but has subsequently been shown to be produced by a number of different cell types (29,31,66). PDGF acts primarily on connective tissue-derived cells and glia cells and it has been speculated that PDGF released from platelets at sites of vascular damage plays an important role in wound healing processes. There are also reasons to believe that PDGF operate in fundamental growth processes during embryogenesis (20,69). It was recently demonstrated that frog oocytes contain maternally derived transcripts for one of the PDGF subunits, the A chain (51). It is therefore possible that the PDGF A chain protein performs important functions during the first cell divisions after the fertilization of the egg. It has subsequently been shown that early mouse embryos express the same gene (61). Members of the PDGF family may thus have different functions in different parts of development; normal growth regulatory functions during embryogenesis, and tissue repair processes in the adult organism.

Aberrant effects of PDGF have also been implicated in various pathological conditions involving connective tissue, e.g., rheumatoid arthritis (67,68), but also in neoplasia. The finding that the simian sarcoma virus oncogene was a transduced version of the PDGF B chain gene provided the first direct example that oncogenes may act by subverting the mitogenic pathways of growth factors (17,73). This was followed by numerous reports stating that PDGF is expressed and may have a role as an autocrine mitogen in various transformed cells including human (31).

## THE PDGF FAMILY OF LIGANDS AND RECEPTORS

The mature PDGF molecule is a dimer involving two different polypeptide chains, denoted A and B (37). All three theoretically possible configurations, AA,

AB, and BB, have been identified in platelets and as products from various transformed cells (22,23,30,63).

The cDNA sequences for the two chains show that both are synthesized as precursor molecules and mature through proteolytic processing of the aminoterminals and, in the case of the B chain, also in the carboxyterminus (4,38,42). The resulting polypeptides, approximately 100 amino acid residues in size, show about 60% amino acid sequence identity. All eight cysteine residues are conserved indicating a similar tertiary structure.

Both the A and B chain precursors have hydrophobic leader sequences, indicating that they are transported into the secretory apparatus. *In vivo* labeling experiments show that the AA and AB molecules rapidly become secreted from the cells (2,8,56). However, PDGF-BB occur in two forms, a 30 kDa molecule which also is rapidly secreted and a 24 kDa dimer which stays associated with the cells (63,8,1, Östman, A., Rall, L., Hammacher, A., Wormsted, M.A., Coit, D., Valenzuela, P., Betsholtz, C., Westermark, B., Heldin, C.-H., unpublished data). The reason for the behaviour of the latter is unknown. Maybe processing to the 24 kDa form exposes a determinant (e.g., hydrophobic) that associates the molecule with membrane components.

The A- and B chain genes are located on human chromosomes 7 and 22, respectively (4,13,71,72). The exon/intron organization of the two genes is similar and correlated to functional domains of the proteins (7,9,38,42,65). Thus, exon 1 encodes the signal sequence, exons 2 and 3 the aminoterminal propeptides and exons 4 and 5 encode the major parts of the mature chains. The A chain occurs in two variants as a result of differential splicing. In cells that express PDGF A chain transcripts, a small proportion, approximately 5–10% of the mRNA molecules contain an extra 69 base pairs that derive from a separate exon (no. 6) in the gene (4,7,65). As a result, the three most carboxyterminal amino acids in the common form of PDGF-A are replaced by 18 different amino acids. These contain a high number of basic residues, giving this C-terminal extension unusual properties. The role for this alternative form of the A chain is not known, but a specific function is indicated by a high degree of conservation of the exon 6 sequence in mammals (47) and amphibians (51).

Whereas the B chain transcript has been identified as a single 3.5 kb species (19,60), the A chain transcript occurs as three major species ranging from 1.9 to 2.8 kb in size (4). This is most likely due to an alternative use of polyadenylation signals, several of which are found in the 3' end of the A chain gene (65).

It has recently become evident that the PDGF isoforms interact with different affinities with two different cell surface receptors (27,34). The first receptor to be purified and cloned (75) is now referred to as the B type PDGF receptor (10,21). It binds PDGF-BB with high affinity, PDGF-AB with lower affinity, and lacks affinity for PDGF-AA (27,32). The recently identified (11) and cloned (12,48) PDGF A type receptor binds all three ligands with high affinities. Both receptors are associated with highly homologous intracellular tyrosine kinase domains and possess ligand-stimulated tyrosine kinase activity (18,25).

The biological effects of PDGF-AA differ in several respects from those elicited by PDGF-BB. PDGR-AA lacks completely the ability to induce actin filament reorganization and lacks chemotactic properties on cells with endogenous expression of both receptor types (53). These effects are most probably mediated via the B type receptor as indicated by the ability of cells lacking endogenous PDGF receptors but expressing a transfected B type receptor cDNA to respond to PDGF with actin reorganization and chemotaxis (Claesson-Welsh, L., Heldin, C.-H. and Westermark, B. in preparation). PDGF-AA has also a relatively low mitogenic activity on human fibroblasts compared with PDGF-BB and -AB (53). This, however, could be because of the relatively lower numbers of A type receptors on such cells. PDGF-AA and -BB are more similar in mitogenic efficiency when assayed on other cells such as NIH- and Swiss 3T3 (43). Taken together, the results indicate that the two PDGF receptor types might be associated with different signal transduction pathways. The two activated receptors may for example phosphorylate different intracellular substrates.

Several recent observations indicate that PDGF receptors dimerize upon ligand binding. Since the PDGF molecule itself is a dimer, one could speculate that each chain has a receptor binding surface and that the ligand itself is the receptor crosslinking force. Direct demonstration of B type receptor dimerization has been demonstrated *in vitro* by using purified receptor (33). Interestingly, dimerization correlates with and appears to be a prerequisite for receptor autophosphorylation (33). If ability to dimerize is a general property of PDGF receptors, one would predict that PDGF-AB had the ability to dimerize one A type with one B type receptor. Indirect evidence in favor of this has been obtained through the study of PDGF-induced actin reorganization (ruffling) in human fibroblasts (24). In these, and some other cells, PDGF-BB and -AB (but not -AA or any other tested growth factor) induce the formation of circular ruffles on the dorsal cell surfaces (49,50,53). This rapid cellular response is seen with PDGF-AB only if both A- and B-type receptors are present. If available A-type receptors are downregulated by exposure to PDGF-AA prior to the addition of PDGF-AB, the ruffling is inhibited (24).

## SIMIAN SARCOMA VIRUS AND ITS TRANSFORMING GENE *v-sis*

Simian sarcoma virus (SSV) is an acutely transforming retrovirus isolated from a woolly monkey fibrosarcoma (reviewed in 14). Its oncogene, *v-sis*, encodes a 28 kDa protein closely resembling the PDGF B-chain precursor (15,17,73). The few amino acid substitutions found, may constitute the difference between human and woolly monkey PDGF B-chain. In *v-sis*, the complete coding sequence for the B chain precursor is present, except for the N-terminal signal sequence. This is, however, substituted for by a hydrophobic sequence derived from the retroviral *env*-gene.

A large body of evidence suggests that SSV-transformation is effectuated by the PDGF-like growth factor encoded by the *v-sis* gene. Firstly, the *v-sis* gene product is processed and has a subcellular localization indistinguishable from a PDGF B chain homodimer (1,8,63,64). Secondly, SSV-transformed cells release a growth factor functionally identical to PDGF-BB (it binds to and activates PDGF receptors) (16,36,39). Thirdly, acute transformation by SSV *in vitro* can be reverted by high concentrations of neutralizing PDGF antibodies (40) or suramin, a compound which dissociates PDGF from its receptors (5). Finally, the cellular effects of SSV could not be distinguished from those elicited by continuous PDGF-stimulation only (41).

In 1984, we suggested that autocrine growth stimulation may occur as an entirely intracellular event provided that ligand and receptor meet in the intracellular compartment formed by, e.g., the endoplasmic reticulum, the Golgi apparatus, and secretory vesicles (3). If so, the receptor activation might escape normal feedback negative regulatory events, such as receptor downregulation, and for this reason constitute a transforming rather than a normal growth stimulatory event. Since then, this question has been addressed in several other studies concerning PDGF as well as other growth factors (26,44,45). Evidence has been presented that the *v-sis* product can induce autophosphorylation of the PDGF (B type) receptor kinase already inside the cell (26,44). However, the question of whether this phenomenon could constitute a mitogenic signal was elegantly approached in the study by Hannink and Donoghue (26) who used cells expressing the *v-sis* gene under the control of an inducible heat shock gene promoter. The drug monensin, which blocks transport of secretory proteins from the Golgi apparatus, resulted in the accumulation of an autophosphorylated 160 kDa (immature) PDGF (B type) receptor protein. However, induction of the *c-fos* gene, which is believed to be an obligatory event in transmission of a mitogenic signal, was blocked. In combination with the data showing that SSV-transformation can be reverted by exogenously added PDGF antibodies, this suggests that although activation of the receptor kinase evidently can occur inside the cell, the ligand-receptor complex has to reach the cell surface in order to transmit signals necessary for mitogenesis. It is not unlikely that crucial molecules that interact with the PDGF receptor (e.g., substrates for the receptor), may be associated with the inner leaflet of the plasma membrane.

SSV induces malignant gliomas at high frequency in experimental animals (74). However, characteristics associated with malignant transformation other than growth stimulation, such as immortalization and genomic instability could not be induced by SSV *in vitro* (41). Thus, to explain the oncogenic properties of SSV *in vivo*, one has to assume that the SSV-driven autocrine loop is complemented by additional genetic changes. The development of these might depend on a specific cellular phenotype (for example, that of a stem cell) present only in the newborn primate brain. Alternatively, the random integration of the SSV and its helper virus genome into the host may cause mutations that are favorably selected *in vivo* but not *in vitro*.

## EXPRESSION OF PDGF AND PDGF RECEPTORS IN HUMAN MALIGNANT GLIOMA

One of the first cell lines shown to produce a growth factor of the PDGF family was derived from a human glioblastoma (2,52). Even before the homology between the PDGF B chain and the *v-sis* product was recognized, it was shown that human cell lines derived from glioblastomas in addition to those derived from sarcomas expressed a *v-sis* homologous gene at high frequency (19). Subsequently, numerous reports have stated the expression of either or both of the PDGF genes in a variety of tumors including others than sarcomas and gliomas (reviewed in 31). It has been suggested that the expression of PDGF in sarcomas and gliomas may drive proliferation of these tumors in an autocrine manner, assuming that the transformed cells, like their normal counterparts, carry PDGF receptors. The expression in certain other tumors, e.g., carcinomas (6,57,70), may promote tumor growth indirectly through its effects on stroma cells. However, access to tools necessary to obtain a true estimate of PDGF receptors on cells, e.g., cDNA clones corresponding to both receptor types and radiolabeled versions of all three PDGF isoforms, has recently made it possible to investigate the PDGF receptor occurrence on different tumor cells in detail. It has been found that many sarcomas (Leeven P., Betsholtz C., Nistér M., Claesson-Welsh L., Heldin C.-H., Westermark B., *unpublished*) and gliomas (Nistér M., Claesson-Welsh L., Heldin C.-H., Westermark B., *unpublished*) lack either or both of the PDGF receptors and that aberrant PDGF receptor expression sometimes occurs in carcinomas derived from the thyroid (Heldin et al., 1988) and lung (Söderdahl G., Bergh J., Betsholtz C., *unpublished*). Members of the PDGF family might thus exert mainly paracrine functions in some sarcomas and glioblastomas and autocrine functions in some carcinomas. Finally, it should also be remembered that the coexpression of a normally growth promoting ligand and its corresponding receptor in the same cell does not prove the existence of autocrine growth stimulation. The osteosarcoma cell line U-2 OS coexpresses both PDGF genes with both PDGF receptor genes and has been analyzed extensively regarding autocrine loops involving PDGF (3, Betsholtz, C., *unpublished*). Although autocrine downregulation of PDGF receptors has been demonstrated, this does not appear to affect the cellular growth rate *in vitro*, even under growth-limiting conditions (3).

A second problem in evaluating the role of PDGF in the growth of malignant brain tumors regards the site of growth factor expression. When 50% or so of the established glioblastoma cell lines expressed the PDGF B chain gene, it came as a surprise that this mRNA was readily detected in 100% of the primary tumors. Analysis using *in situ* hybridization showed, however, that the main site of PDGF B chain expression in brain tumors was in the reactive endothelium (35). Moreover, these cells expressed PDGF B type receptor mRNA at high levels indicating that autocrine loops also may operate in stroma formation (35). Because several other growth factors than PDGF, e.g., transforming growth factor type - $\alpha$  (TGF- $\alpha$ )

(54) and acidic fibroblast growth factor (FGF) (46), are also expressed at high frequency in malignant gliomas, the network of intercellular growth signals in this tumor may be very complex and difficult to analyze *in vitro*.

Another question regards to what extent growth factor production seen in tumor cells reflects phenomena participating directly in the transforming process, occurring as a result thereof or normally in the state of differentiation to which the tumor phenotype corresponds. In relation to glioblastoma, one has to ask what, if any, normal functions PDGF performs during the development of the central nervous system. These are questions addressed only recently and are summarized below.

## ROLE OF PDGF IN GLIOGENESIS

It has been suggested that during the development of the rat optical nerve, the differentiation of O-2A progenitor cells into oligodendrocytes and type-2 astrocytes requires a specific signal(s) provided by the type-1 astrocytes. A series of reports recently stated that for this process, the presence of type-1 astrocytes can be substituted for by PDGF, which by itself induces a normal timing of differentiation of the O-2A progenitor cells (55,59,62). At the same time, the type-1 astrocytes were shown to express PDGF A chain mRNA and release a PDGF-like protein likely to be the PDGF-AA homodimer (62). Subsequent studies have shown that O-2A progenitor cells carry PDGF receptors resembling the A type receptors identified on fibroblasts and respond better to PDGF-AA than to PDGF-BB (28,58). All in all, these results strongly suggest a role for PDGF in the development of the glia compartment of the central nervous system, and interestingly, that the molecule operating is PDGF-AA. Thus, this is the first example of a potential specific function for this isoform of PDGF.

## REFERENCES

1. Beckmann MP, Betsholtz C, Heldin C-H, Westermark B, Di Marco E, Di Fiore PP, Robbins KC, Aaronson SA. Human PDGF-A and PDGF-B chains differ in their biological properties and transforming potential. *Science* 1988;241:1346–1349.
2. Betsholtz C, Heldin C-H, Nistér M, Ek B, Wasteson A, Westermark B. Synthesis of a PDGF-like growth factor in human glioma and sarcoma cells suggests the expression of the cellular homologue to the transforming protein of simian sarcoma virus. *Biochem. Biophys. Res. Commun.* 1983;117:176–182.
3. Betsholtz C, Westermark B, Ek B, Heldin C-H. Coexpression of a PDGF-like growth factor and PDGF receptors in a human osteosarcoma cell line: implications for autocrine receptor activation. *Cell* 1984;39:447–457.
4. Betsholtz C, Johnsson A, Heldin C-H, Westermark B, Lind P, Urdea MS, Shows TB, Philpott K, Mellor A, Knott TJ, Scott J. cDNA sequence and chromosomal localization of human platelet-derived growth factor A-chain and its expression in tumor cell lines. *Nature* 1986a;320:695–699.

5. Betsholtz C, Johnsson A, Heldin C-H, Westermark B. Efficient reversion of SSV-transformation and inhibition of growth factor-induced mitogenesis by suramin. *Proc. Natl. Acad. Sci. USA* 1986b;83:6440–6444.
6. Betsholtz C, Bergh J, Bywater M, Pettersson M, Johnsson A, Heldin C-H, Ohlsson R, Knott TJ, Scott J, Bell G, Westermark B. Expression of multiple growth factors in a human lung cancer cell line. *Int. J. Cancer* 1987;39:502–507.
7. Bonthron DT, Morton CC, Orkin SH, Collins T. Platelet-derived growth factor A chain: gene structure, chromosomal localization and basis for alternative mRNA splicing. *Proc. Natl. Acad. Sci. USA* 1988;85:1492–1496.
8. Bywater M, Rorsman F, Bongcam-Rudloff E, Mark G, Heldin C-H, Westermark B, Betsholtz C. Expression of recombinant PDGF-B but not PDGF-A homodimers result in phenotypical transformation of Rat-1 cells and human fibroblasts. *Mol. Cell. Biol.* 1988;8:2753–2762.
9. Chiu I-M, Reddy EP, Givol D, Robbins KC, Tronick SR, Aaronson SA. Nucleotide sequence analysis identifies the human c-sis protooncogene as a structural gene for platelet-derived growth factor. *Cell* 1984;37:123–129.
10. Claesson-Welsh L, Eriksson A, Morén A, Severinsson L, Ek B, Östman A, Betsholtz C, Heldin C-H. cDNA cloning and expression of a human PDGF receptor specific for B chain containing PDGF molecules. *Mol. Cell. Biol.* 1988;8:3476–3486.
11. Claesson-Welsh L, Hammacher A, Westermark B, Heldin C-H, Nister M. Identification and structural analysis of the A type receptor for PDGF: similarities with the B type receptor. *J. Biol. Chem.* 1989a;264:1742–1747.
12. Claesson-Welsh L, Eriksson A, Westermark B, Heldin C-H. cDNA cloning and expression of the human A type PDGF receptor establishes structural similarity to the B type receptor. *Proc. Natl. Acad. Sci. USA* 1989b;86:4917–4921.
13. Dalla Favera R, Gallo RC, Giallongo A, Croce CM. Chromosomal localization of the human homolog (c-sis) of the simian sarcoma virus onc gene. *Science* 1982;218:686–688.
14. Deinhart, F. The biology of primate retroviruses. In: Klein G, ed. *Oncology*. New York: Raven Press, 1980;357–398.
15. Devare SG, Reddy EP, Law JD, Robbins KC, Aaronson SA. Nucleotide sequence of the simian sarcoma virus genome: demonstration that its acquired cellular sequences encode the transforming gene product p28<sup>sis</sup>. *Proc. Natl. Acad. Sci. USA* 1983;80:731–735.
16. Deuel TF, Huang JS, Huang SS, Stroobant P, Waterfield MD. Expression of a platelet-derived growth factor-like protein in simian sarcoma virus transformed cells. *Science* 1983;221:1348–1350.
17. Doolittle RF, Hunkapiller MW, Hood LE, Devare SG, Robbins KC, Aaronson SA, Antoniades HN. Simian sarcoma virus onc gene, v-sis, is derived from the gene (or genes) encoding a platelet-derived growth factor. *Science* 1983;221:275–277.
18. Ek B, Westermark B, Wasteson Å, Heldin C-H. Stimulation of tyrosine-specific phosphorylation by platelet-derived growth factor. *Nature* 1982;295:419–420.
19. Eva A, Robbins KC, Andersen PR, Srinivasan A, Tronick SR, Reddy EP, Ellmore NW, Galen AT, Lautenberg JA, Papas TS, Westin EH, Wong-Staal F, Gallo RC, Aaronson SA. Cellular genes analogous to retroviral onc genes are transcribed in human tumor cells. *Nature* 1982;295:116–119.
20. Goustin AS, Betsholtz C, Pfeifer-Ohlsson S, Persson H, Rydnert J, Bywater M, Holmgren G, Heldin C-H, Westermark B, Ohlsson R. Coexpression of the sis and myc

- proto-oncogenes in human placenta suggest autocrine control of trophoblast growth. *Cell* 1985;41:301–312.
21. Gronwald RGK, Grant FJ, Haldeman BA, Hart CE, O'Hara PJ, Hagen FS, Ross R, Bowen-Pope DF, Murray MJ. Cloning and expression of a cDNA coding for the human platelet-derived growth factor receptor: evidence for more than one receptor class. *Proc. Natl. Acad. Sci. USA* 1988;85:3435–3439.
  22. Hammacher A, Hellman U, Johnsson A, Gunnarsson K, Östman A, Westermark B, Wasteson Å, Heldin C-H. A major part of PDGF purified from human platelets is a heterodimer of one A and one B chain. *J. Biol. Chem.* 1988a;263:16493–16498.
  23. Hammacher A, Nistér M, Westermark B, Heldin C-H. A human glioma cell line secretes three structurally and functionally different dimeric forms of PDGF. *Eur. J. Biochem.* 1988b;176:179–186.
  24. Hammacher A, Mellström K, Heldin C-H, Westermark B. Isoform-specific induction of actin reorganization by platelet-derived growth factor suggests that the functionally active receptor is a dimer. *EMBO J.* 1989;8:2489–2495.
  25. Hammacher A, Nistér M, Heldin C-H. The A type receptor for platelet-derived growth factor mediates protein tyrosine phosphorylation receptor transmodulation and a mitogenic response. *Biochem. J.* 1989;265:15–20.
  26. Hannink M, Donoghue DJ. Autocrine stimulation by the v-sis gene product requires a ligand-receptor interaction at the cell surface. *J. Cell Biol.* 1988;107:287–298.
  27. Hart C, Forstrom JW, Kelly JD, Seifert RA, Smith RA, Ross R, Murray MJ, Bowen-Pope DF. Two classes of PDGF receptor recognize different isoforms of PDGF. *Science* 1988;240:1529–1531.
  28. Hart IK, Richardson WD, Heldin C-H, Westermark B, Raff MC. PDGF receptors on cells of the oligodendrocyte-type-2 astrocyte (O-2A) cell lineage. *Development* 1989;105:595–603.
  29. Heldin C-H, Wasteson Å, Westermark B. Platelet-derived growth factor. *Mol. Cell. Endocrinol.* 1985;39:169–187.
  30. Heldin C-H, Johnsson A, Wennergren S, Wernstedt C, Betsholtz C, Westermark B. A human osteosarcoma cell line secretes a growth factor structurally related to a homodimer of PDGF A-chains. *Nature* 1986;319:511–514.
  31. Heldin C-H, Betsholtz C, Claesson-Welsh L, Westermark B. Subversion of growth regulatory pathways in malignant transformation. *Biochim. Biophys. Acta* 1987;907:219–244.
  32. Heldin C-H, Bäckström G, Östman A, Hammacher A, Rönnstrand L, Rubin K, Nistér M, Westermark B. Binding of different dimeric forms of PDGF to human fibroblasts: evidence for two separate receptor types. *EMBO J.* 1988;7:1387–1393.
  33. Heldin C-H, Ernlund A, Rorsman C, Rönnstrand L. Dimerization of B type PDGF receptors occurs after ligand binding and is closely associated with receptor kinase activation. *J. Biol. Chem.* 1989;264:8905–8912.
  34. Heldin N-E, Gustavsson B, Claesson-Welsh L, Hammacher A, Mark J, Heldin C-H, Westermark B. Aberrant expression of receptors for platelet-derived growth factor in an anaplastic thyroid carcinoma cell line. *Proc. Natl. Acad. Sci. USA* 1988;85:9302–9306.
  35. Hermansson M, Nistér M, Betsholtz C, Heldin C-H, Westermark B, Funakoshi K. Endothelial cell hyperplasia in human glioblastoma: coexpression of mRNA for platelet-derived growth factor (PDGF) and PDGF receptor suggests autocrine growth stimulation. *Proc. Natl. Acad. Sci. USA* 1988;85:7748–7752.

36. Huang JS, Huang SS, Deuel TF. Transforming protein of simian sarcoma virus stimulates autocrine growth of SSV-transformed cells through PDGF cell-surface receptors. *Cell* 1984;39:79–87.
37. Johnsson A, Heldin C-H, Westermark B, Wasteson Å. Platelet-derived growth factor: identification of constituent polypeptide chains. *Biochem. Biophys. Res. Commun.* 1982;104:66–74.
38. Johnsson A, Heldin C-H, Wasteson Å, Westermark B, Deuel TF, Huang JS, Seeburg PH, Gray A, Ullrich A, Scrace G, Stroobant P, Waterfield MD. The c-sis gene encodes a precursor of the B-chain of platelet-derived growth factor. *EMBO J.* 1984;3:921–928.
39. Johnsson A, Betsholtz C, von der Helm K, Heldin C-H, Westermark B. Platelet-derived growth factor agonist activity of a secreted form of the v-sis oncogene product. *Proc. Natl. Acad. Sci. USA* 1985a;82:1721–1725.
40. Johnsson A, Betsholtz C, Heldin C-H, Westermark B. Antibodies against platelet-derived growth factor inhibit acute transformation by simian sarcoma virus. *Nature* 1985b;317:438–440.
41. Johnsson A, Betsholtz C, Heldin C-H, Westermark B. The phenotypic characteristics of simian sarcoma virus-transformed human fibroblasts suggest that the v-sis product solely acts as a PDGF receptor agonist in cell transformation. *EMBO J.* 1986;5:1535–1541.
42. Josephs SF, Guo C, Ratner L, Wong-Staal F. Human proto-oncogene nucleotide sequences corresponding to the transforming region of simian sarcoma virus. *Science* 1984;223:487–491.
43. Kazlauskas A, Bowen-Pope DF, Seifert R, Hart CE, Cooper JA. Different effects of homo- and heterodimers of platelet-derived growth factor A and B chains on human and mouse fibroblasts. *EMBO J.* 1988;7:3727–3735.
44. Keating MT, Williams LT. Autocrine stimulation of intracellular PDGF receptors in sis-transformed cells. *Science* 1988;239:914–916.
45. Lang RA, Metcalf D, Gough NM, Dunn AR, Gonda TJ. Expression of a hemopoietic growth factor cDNA in a factor-dependent cell line results in autonomous growth and tumorigenicity. *Cell* 1985;43:531–542.
46. Libermann TA, Friesel R, Jaye M, Lyall RM, Westermark B, Drohan W, Schmidt A, Maciag T, Schlessinger J. An angiogenic factor is expressed in human glioma cells. *EMBO J.* 1987;6:1627–1632.
47. Matoskova B, Rorsman F, Svensson V, Betsholtz C. Alternative splicing of the platelet-derived growth factor A-chain transcript occurs in normal as well as tumor cells and is conserved among mammalian species. *Mol. Cell. Biol.* 1989;9:3148–3150.
48. Matsui T, Heidaran M, Miki T, Popescu N, La Rochelle W, Kraus M, Pierce J, Aaronson SA. Isolation of a novel receptor cDNA establishes the existence of two PDGF receptor genes. *Science* 1989;243:800–803.
49. Mellström K, Höglund A-S, Nistér M, Heldin C-H, Westermark B, Lindberg U. The effect of platelet-derived growth factor on morphology and motility of human glial cells. *J. Musc. Res. Cell. Motil.* 1983;4:589–609.
50. Mellström K, Heldin C-H, Westermark B. Induction of circular membrane ruffling on human fibroblasts by platelet-derived growth factor. *Exp. Cell Res.* 1988;177:347–359.
51. Mercola M, Melton DA, Stiles CD. Platelet-derived growth factor A chain is maternally encoded in *Xenopus* embryos. *Science* 1988;241:1223–1225.

52. Nistér M, Heldin C-H, Wasteson Å, Westermark B. A glioma-derived analog to platelet-derived growth factor: demonstration of receptor-competing activity and immunological crossreactivity. *Proc. Natl. Acad. Sci. USA* 1984;81:926–930.
53. Nistér M, Hammacher A, Mellström K, Siegbahn A, Rönstrand L, Westermark B, Heldin C-H. A glioma-derived PDGF A chain homodimer has different functional activities from a PDGF AB heterodimer purified from human platelets. *Cell* 1988a;52:791–799.
54. Nistér M, Liberman TA, Betsholtz C, Pettersson M, Claesson-Welsh L, Heldin C-H, Schlessinger J, Westermark B. Possible autocrine loops involving PDGF/PDGF receptors and TGF- $\alpha$ /EGF receptors in human malignant glioma cell lines. *Cancer Res.* 1988b;48:3910–3918.
55. Noble M, Murray K, Stroobant P, Waterfield M, and Riddle P. Platelet-derived growth factor promotes division and mobility and inhibits premature differentiation of the oligodendrocyte/type-1 astrocyte progenitor cell. *Nature* 1988;333:560–562.
56. Östman A, Rall L, Hammacher A, Wormsted MA, Coit D, Valenzuela P, Betsholtz C, Westermark B, Heldin C-H. Synthesis and assembly of a functionally active recombinant platelet-derived growth factor AB heterodimer. *J. Biol. Chem.* 1988;263:16202–16208.
57. Perez R, Betsholtz C, Westermark B, Heldin C-H. Frequent expression of growth factors for mesenchymal cells in human mammary carcinoma cell lines. *Cancer Res.* 1987;47:3425–3429.
58. Pringle N, Collarini EJ, Mosley MJ, Heldin C-H, Westermark B, Richardson WD. PDGF A-chain homodimers drive proliferation of oligodendrocyte/type-2 astrocyte (O-2A) progenitor cells in the developing rat optic nerve. *EMBO J.* 1989;8:1049–1056.
59. Raff MC, Lillien LE, Richardson WD, Burne JF, Noble MD. Platelet-derived growth factor from astrocytes drives the clock that times oligodendrocyte development in culture. *Nature* 1988;333:562–565.
60. Rao CD, Igarashi H, Chiu I-M, Robbins KC, Aaronson SA. Structure and sequence of the human c-sis/platelet-derived growth factor 2 (SIS/PDGF2) transcriptional unit. *Proc. Natl. Acad. Sci. USA* 1986;83:2392–2396.
61. Rappolee DA, Brenner CA, Schultz R, Mark D, Werb Z. Developmental expression of PDGF, TGF- $\alpha$  and TGF- $\beta$  genes in preimplantation mouse embryos. *Science* 1988;241:1823–1825.
62. Richardson WD, Pringle N, Mosley MJ, Westermark B, Dubois-Dalcq M. A role for platelet-derived growth factor in normal gliogenesis in the central nervous system. *Cell* 1988;53:309–319.
63. Robbins KC, Antoniades HN, Devare SG, Hunkapiller MW, Aaronson SA. Structural and immunological similarities between simian sarcoma virus gene product(s) and human platelet-derived growth factor. *Nature* 1983;305:605–608.
64. Robbins KC, Leal F, Pierce JH, Aaronson SA. The v-sis/PDGF-2 transforming gene product localizes to cell membranes but is not a secretory protein. *EMBO J.* 1985;4:1783–1792.
65. Rorsman F, Bywater M, Knott TJ, Scott J, Betsholtz C. Structural characterization of the human platelet-derived growth factor A-chain cDNA and gene: alternative exon usage predicts two different precursor proteins. *Mol. Cell. Biol.* 1988;8:571–577.
66. Ross R, Raines EW, Bowen-Pope DF. The biology of platelet-derived growth factor. *Cell* 1986;46:155–169.

67. Rubin K, Terracio L, Rönnstrand L, Heldin C-H, Klareskog L. Expression of platelet-derived growth factor receptor is induced on connective tissue cells during chronic synovial inflammation. *Scand. J. Immunol.* 1988a;27:285–294.
68. Rubin K, Tingström A., Hansson K, Larsson E, Rönnstrand L, Klareskog L, Claesson-Welsh L, Heldin C-H, Fellström B, Terracio L. Induction of PDGF B-type receptors in vascular inflammation: possible implications for the development of vascular proliferative lesions. *Lancet* 1988b;1:1353–1356.
69. Seifert RA, Schwartz SM, Bowen-Pope DF. Developmentally regulated production of platelet-derived growth factor-like molecules. *Nature* 1984;311:669–671.
70. Söderdahl G, Betsholtz C, Johansson A, Nilsson K, Bergh J. Differential expression of platelet-derived growth factor and transforming growth factor genes in small- and non-small cell human lung cancer cell lines. *Int. J. Cancer* 1988;41:636–641.
71. Stenman G, Rorsman F, Betsholtz C. Sublocalization of the human PDGF A-chain gene to chromosome 7, band q11.23 by *in situ* hybridization. *Exp. Cell Res.* 1988;178:180–184.
72. Swan DC, McBride OW, Robbins KC, Keithley DA, Reddy EP, Aaronson SA. Chromosomal mapping of the simian sarcoma onc gene analogue in human cells. *Proc. Natl. Acad. Sci. USA* 1982;79:4691–4695.
73. Waterfield MD, Scrace GT, Whittle N, Stroobant P, Johnsson A, Wasteson Å, Westermark B, Heldin C-H, Huang JS, Deuel TF. Platelet-derived growth factor is structurally related to the putative transforming protein p28<sup>sis</sup> of simian sarcoma virus. *Nature* 1983;304:35–39.
74. Wolfe LG, Deinhart F, Thiele GH, Rabin H, Kawakami T, Bustad LK. Induction of tumors in marmoset monkeys by simian sarcoma virus, Type 1 (*Lagothrix*): A preliminary report. *J. Natl. Cancer Inst.* 1971;47:1115–1120.
75. Yarden Y, Escobedo JA, Kuang W-J, Yang-Feng TL, Daniel TO, Tremble PM, Chen EY, Ando ME, Harkins RN, Francke U, Fried VA, Ullrich A, Williams LT. Structure of the receptor for platelet-derived growth factor helps define a family of closely related growth factor receptors. *Nature* 1986;323:226–232.



# Growth-associated Triggering Factors and Central Nervous System Regeneration

M. Schwartz<sup>1</sup>, V. Lavie<sup>1</sup>, A. Cohen<sup>1</sup>, A. Harel<sup>1</sup>, A. Solomon<sup>2</sup>  
and M. Belkin<sup>2</sup>

<sup>1</sup> Department of Neurobiology, Weizmann Institute of Science, Rehovot, Israel

<sup>2</sup> Goldschleger Eye Research Institute, Tel Aviv University Sackler School of Medicine, Sheba Medical Center, Tel Hashomer, Israel

**N**euronal regeneration involves regrowth of injured axons, followed by restoration of their synaptic connections and, finally, recovery of the original physiological functions. Different classes of neurons, and neurons from different species, differ in their ability to regenerate. Thus, for example, neurons of lower vertebrates ordinarily regenerate. This also holds for neurons of the peripheral nervous system (PNS) in mammals. Distinctively, most neurons of the central nervous system (CNS) in mammals seldom regenerate (17,38). Studies on regeneration have therefore focused on identifying the factors (neuronal or glial) of lower vertebrates that allow these neurons to regenerate, and on attempting to devise ways to circumvent the impediments or the deficiencies of regeneration in nonregenerative systems.

In intact nerves, there is a mutual relationship between the axon and the surrounding nonneuronal cells. This relationship is presumably required for the maintenance and functional activity of the axon and the fully differentiated nonneuronal cells. As a result of an injury, the neuron is deprived of target-derived substances. In addition, the mutual relationship of the axons with the environment is disrupted consequently. The state of growth of the surrounding nonneuronal cells is altered (31,32). This may lead to formation of an environment that is inhospitable to regeneration (26,39) or to formation of a growth-supportive environment (25,43,46,47). It seems that the formation of both the hostile and the supportive environments may occur at different time periods after injury, at least in lower vertebrates' CNS (32). It is the net outcome of these opposing contributions which may determine the nature of the response to the injury, i.e., regeneration or degeneration.

Attempts were made to attribute the regenerative failure of axons in the adult mammalian CNS to the scar tissue formed by the astrocytes or to growth inhibitors associated with oligodendrocytes (41,42). Surgical manipulations aimed at determining whether a dense glial scar, which is formed by the proliferating glial cells,

interferes with outgrowth of neurites in regeneration showed that such a scar does not represent a major mechanical obstacle (39). Instead, the scar tissue might be a biochemical obstacle resulting from its deficiency in supportive elements that the cells making up the scar tissue can provide to the external milieu. Among needed components are neurotrophic factors (14), extracellular matrix proteins (48), and cell surface molecules (e.g., cell adhesion molecules) (33). It is therefore conceivable that the lack of regeneration may stem from the inability of the reactive glial cells, forming the scar tissue, to provide the appropriate elements needed for growth and from the nonpermissive nature of the mature oligodendrocytes. Both these types of glial cells, however, may be developed into growth-supportive and permissive cells by an appropriate intervention at the right time. Such an intervention might be in the form of soluble substances, putative glial factors which can provide a control mechanism to the glial cells so as to allow them to reach a state whereby they can produce these aforementioned components.

Several putative modulators have been identified lately, including: interleukin-1 (IL-1) (23) and platelet-derived growth factor (PDGF) (40). The cellular origin of such factors might be infiltrating macrophages, resident glial cells (macro- or microglia), or the neurons. The inability of the glial cells of mammalian CNS after injury to acquire the state of growth described above, needed for regeneration, may stem from absence of glial growth modulators or their appearance at an inappropriate time or amount, or presence of neutralizing or masking factors. Alternatively, it is possible that inhibitory factor(s) are present in a regenerating system but are absent in a nonregenerating system.

It appears that growing nerves (regenerating or developing nerves) have the ability to provide substances that can trigger a regenerative response in a nonregenerative system. Such factors, named collectively by us as growth-associated triggering factors (GATFs), presumably include several factors working in synchrony or in concert (11,43).

## A CORRELATION BETWEEN GATFs AND REGENERATION

Axonal injury causes changes in the activity of soluble substances originating from the environment which surround the injured nerve, or from the target organ. Thus, the growth of the proximal stump of a transected PNS nerve is facilitated by diffusible proteinaceous molecules possibly anchored in the basal lamina and released from the distal stump of the transected nerve (24). Similarly, axonal injury induces increased neurite-promoting activity in extracts of the target organ (13,34,35,36). Extracts prepared from denervated adult skeletal muscle contain an increased amount of neurotrophic activity, which promotes survival of dissociated motor neurons and outgrowth of neurites from explants of spinal cord maintained in serum-free defined media (36). This injury-induced increase in activity has also been observed in the brain. For example, the activity of a soluble substance that is collected from the site of a brain lesion, and which affects the survival of

chick sensory neurons in culture, is increased with time after the lesion (25,34). Injury-induced increased activity is also manifested by the better survival of brain grafts in wound cavities several days after the injury (34,35).

In both the visual system of fish and the sciatic nerve of mammals, two regenerative systems, injury causes changes in the appearance of nonneuronal-derived polypeptides (37,44). The changes in the fish optic nerve were circumstantially correlated with the increased GATF activity. Thus, soluble substances in the form of medium conditioned by regenerating optic nerves of fish are more active than those of intact nerves in triggering a regenerative response when applied to injured optic nerves of adult rabbits (a nonregenerative system) (43). Media conditioned by nerve segments of neurons that are deficient with respect to their ability to regenerate lack this regeneration-triggering activity. This lack of activity may be a cause of the poor regeneration ability of the nerves. During ontogeny in mammals, however, optic neurons do possess such an activity (11). A similar correlation between the regenerative stage and the ability of media conditioned by the corresponding nerves to produce active substances was found *in vitro*, with respect to the ability of the conditioned media to promote neurite outgrowth from dissociated embryonic rat cerebrocortical and hindbrain neurons (8). In the fish visual system, injury was also found to cause an increase in target-derived activity manifested by the promotion of glial proliferation *in vitro* (9). Thus, while brain of intact fish optic nerves contains peptides that accelerate the growth of glial cells (10), injury causes an increase in such activity (9).

The increased activity after injury in a regenerative system could evolve from an increased production and release of active molecules, from activation of preexisting molecules, from deactivation of or a reduction in substances that inhibit the activity, or from increased production of inhibitors that neutralize or prevent creation of potential growth suppressors.

## GAFTs AND EXTRACELLULAR MATRIX PROTEINS: PUTATIVE GLIAL CELL MODULATORS

As a result of application of media conditioned by regenerating fish optic nerves (i.e., application of GATFs), morphological regeneration was observed, manifested by the appearance of growth cones embedded in astrocytic processes (18). Although the growth was limited, it suggested to us that what might be hostile for growth is amenable to treatment to acquire features supportive for growth. Such a treatment might cause the cells forming the scar to acquire growth-supportive features such as expression of matrix or surface proteins or production of neurotrophic factors. Our initial experiments were focused on finding out whether the GATFs-treated injured nonregenerative nerves (rabbit optic nerves) show any changes in the expression of extracellular matrix proteins such as laminin, a matrix component that, in a regenerative system, contributes to the formation of a growth supportive milieu (15,47).

Our results revealed that in cryosections of intact adult rabbit optic nerve, laminin-immunoreactive sites appeared in the epineurium and around the sparsely distributed blood vessels. After injury, laminin immunoreactivity could be detected in the epineurium, in connective tissue surrounding blood vessels, and in coarse bundles of scar tissue. However, after injury and application of GATFs, the pattern of laminin immunoreactivity was changed, and additional laminin reactive sites could be detected. These appeared as a fine network coursing throughout the matrix of the nerve at the actual site of injury as well as at sites proximal, but not distal, to it. The more widespread distribution of laminin may be directly induced by the GATFs or may result from a cascade of events initiated by the GATFs and could involve increased production or secretion of laminin or from metabolic changes that affect its accumulation (48).

More recently, we have shown that, indeed, among the GATFs there is a factor(s) which directly modulates the level of laminin in C-6 glioma cells, a cell line which resembles more closely type-1 astrocytes. The identity of the laminin was confirmed by metabolic labeling of the treated cells with [<sup>35</sup>S]methionine and by subsequent immunoprecipitation and gel electrophoresis (5).

This GATFs-induced elevation of laminin was not unique to the pool of membrane-associated proteins. Its level increased also in the medium in which the cells were grown and thus indicated that the conditioned medium (CM) also caused an increase in the level of laminin secreted into the media. Fibronectin, an additional matrix protein, exhibited a similar pattern of CM-induced increased labeling within the high salt extractable proteins (containing extracellular matrix proteins) and the proteins secreted into the media (5).

It has been proposed that the continuous expression of laminin by astrocytes is a prerequisite for axonal growth and regeneration in adult CNS (20). After optic nerve crush in goldfish, laminin distribution becomes more widespread (or diffuse) and intense, especially in regions distal to the crush site (15). In the adult brain, laminin has been detected only in association with capillary walls and meningeal structures, in contrast to its widespread distribution in the PNS (4,6). Thus, while *in vivo* adult astrocytes do not produce laminin, astrocytes of developing rat, like Schwann cells, were shown in primary cultures to produce and deposit laminin into the extracellular matrix (21). However, *in vivo* mature astrocytes do produce laminin for short periods after injury in some instances (2,22) but not in all tested nerves (27).

In view of the results presented above, and the fact that laminin can be considered as a marker for premature astrocytes, it is possible that the GATFs affect maturation of type-1 astrocytes. Type-1 astrocytes, which during development are supportive for growth, lose their supportive elements upon maturation and become the growth impeding cells after injury, as they are the scar forming cells (29). Accordingly, one may speculate that the GATFs modulate and/or activate the potentially scar forming cells, so as to make them acquire or maintain some of the features of the premature state, as are marked in our studies by laminin. This issue is currently being investigated.

## GATFs AND APOLIPOPROTEINS

In the course of research towards identifying GATFs with potential activity in regeneration, a 28 kDa polypeptide was found to display regeneration-associated changes in its level (37). Such a polypeptide was identified as apolipoprotein-A-I (apo-A-I) (12). Similarly, in the PNS, a polypeptide of 37 kDa, which also showed a regeneration associated level, was identified as apolipoprotein-E (apo-E) (16,45). In both systems, these apolipoproteins accumulate after injury in contrast to their nonregenerative counterparts (rat and avian optic nerves) in which no accumulation was observed in spite of the increased synthesis (7,30).

It is suggested that in regenerating systems, apolipoproteins play a role at the early posttraumatic stages of degeneration as scavengers which remove lipid degradation products, thereby (a) eliminating putative growth inhibitors, (b) providing a mechanism for storage of lipids which are reused upon need when regrowth is taken place.

## GATFs IN COMBINATION WITH OTHER GROWTH MODALITY

As described above, implantation of GATFs in injured adult rabbit optic nerve resulted in growth which was limited, probably because the treatment was not sufficient to counteract the environmental impediments. We have considered the possibility that temporal factors are critical for regeneration to proceed to a successful outcome, mainly in view of the fact that environmental cells are likely to be affected by any treatment prior to their maturation. Therefore, it was suggested that implanted GATFs would be more effective if some aspects of the normal response to injury could be delayed. The best treatment that caused, in our hands, a delay in morphological and functional features of the process of degeneration was irradiation with a low energy He-Ne laser (1). We have therefore used these two treatment modalities in combination, i.e., application of GATFs, and daily laser irradiation for 14 consecutive days. The combined treatment seems to counteract the above impediments and thereby allowed CNS axons to grow into their own degenerating CNS tissue.

The growing axons under such treatment modalities were abundant and traversed the site of the injury while penetrating the astrocytic environment, which otherwise would form a scar tissue (Fig. 1). In transected nerves, which were treated with this combination, about 16,000 axons were counted 2 mm after the site of the injury 8 weeks after the operation (19). Several criteria were used to examine and to prove that the observed axons were newly growing axons, and that they originated from the retina: (a) the abundancy of unmyelinated axons, (b) the abundance of growth cones, (c) the thin myelin of the myelinated axons, and (d) anterograde transport of horseradish peroxidase, which was injected intraocularly in the treated injured nerves, in axons which traversed the site of the injury. Some

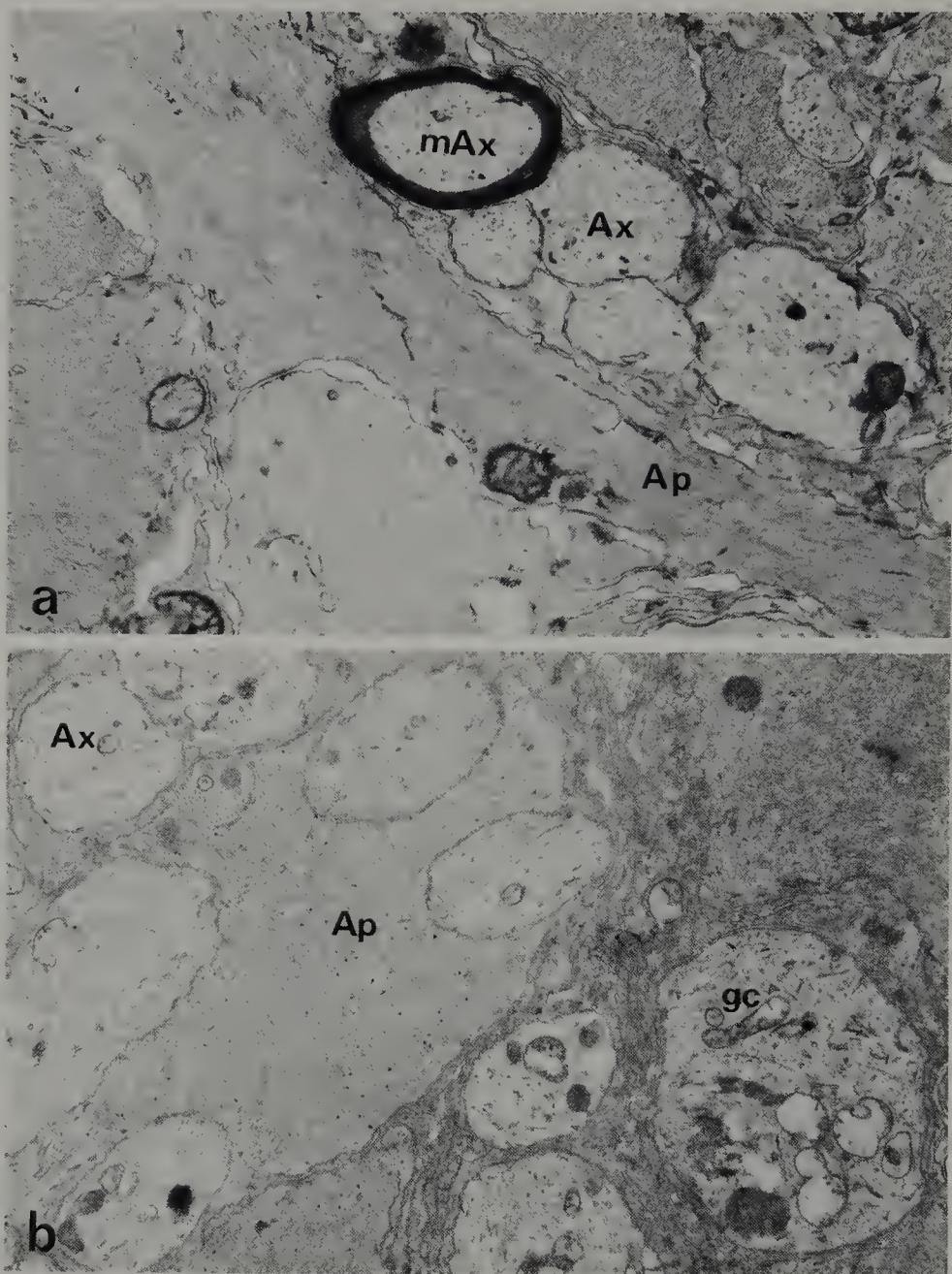


FIG. 1. Electron micrographs of injured nerves treated with GATFs and low energy He-Ne laser. A piece of nitrocellulose soaked with GATFs ( $100 \mu\text{g}$  protein/ml), derived from regenerating fish optic nerves, was placed in the cavity formed by transection in adult rabbit optic nerves. Beginning 30 min after surgery, the rabbit was irradiated transocularly for 14 days with low energy He-Ne laser irradiation (630 nm, 5 min, 35 mW). Six weeks after injury, the nerves were dissected out and fixed with 1% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4. The excised nerves were divided in 1 mm segments, processed for electron microscopy, and embedded in Polybed (Polyscience). Micrograph (a) shows an area of a cross-section of the nerve taken 2 mm distal to the site of the injury. Note the abundant unmyelinated axons (Ax) embedded in astrocytic processes (Ap). Micrograph (b) shows a similar area stained by a postembedding technique with antibodies directed to glial fibrillary acidic protein (GFAP). Note the labeling on astrocytes in which the unmyelinated axons and growth cones (gc) are embedded. (a) X18000; (b) X12400.

of the growing axons were in a process of myelination by oligodendrocytes. It was therefore conceivable that treatments somehow created an hospitable environment for growth manifested by supportive astrocytes (Fig. 1) and permissive oligodendrocytes. Figure 1b shows unmyelinated axons embedded in astrocytes labeled with antibodies specific to glial fibrillary acidic protein (GFAP).

Current studies are devoted to finding out whether the effect of the GATFs on astrocytes and oligodendrocytes are mediated *in vitro* by two distinct or common factor(s).

## SUMMARY

Central nervous system axons of adult mammals, such as those of rodent optic nerves, have only a poor regenerative capability after axonal injury. We have shown that this can be circumvented, at least anatomically, by application of soluble substances derived from media conditioned by injured fish optic nerves. These soluble substances were named by us as growth-associated triggering factors (GATFs), as their level or activity seems to be correlated with the growth activity of the nerves from which they are derived. Early attempts to identify active components within such conditioned media have shown that it contains several substances, which are likely to work in synchrony or in concert in the process of axonal regeneration. Application of GATFs into the injured adult rabbit optic nerve resulted in growth of axons embedded by astrocytes. The latter cells otherwise create a nonsupportive glial scar. This growth was accompanied by an increased and widespread distribution of laminin. *In vitro* studies using a line of glial cells (C-6 glioma) reproduced these results and showed that GATFs caused elevations in the synthesis and accumulation of laminin and fibronectin. As laminin might be considered a marker for premature glial cells, changes in laminin levels might represent a change of the growth state of glial cells, possibly from nonsupportive into supportive.

In addition to these triggering factors, we have identified in these media conditioned by injured fish optic nerves substances such as apolipoprotein-A-I, which are presumably participating in the early posttraumatic events as scavengers of lipid degradation and thereby paving the way for subsequent regeneration.

Recently, treatment of adult injured rabbit optic nerves with a combination of GATFs and a treatment modality which delays the onset of the degeneration has resulted in abundant growth of axons embedded in astrocytic processes traversing the site of the injury and extending up to 6 mm distally to it, by 8 weeks after injury. Some of the axons were in a process of remyelination. This treatment thus represents a way whereby adult injured CNS axons are growing within their own glial environment. Otherwise, when treatment is omitted, their growth is impeded.

## ACKNOWLEDGMENTS

This work was supported by grants of the National Council for Research and Development at the Israel Ministry of Science, the Israel Academy of Science, and the U.S. Army Medical Research and Development Command, given to M.S.

## REFERENCES

1. Assia E, Rosner M, Belkin M, Solomon A, Schwartz M. Temporal parameters of low energy laser irradiation for optimal delay of posttraumatic degeneration of rat optic nerves. *Brain Res.* 1989;476:205–212.
2. Bernstein JJ, Getz R, Jefferson M, Kelemen M. Astrocytes secrete basal lamina after hemisection of rat spinal cord. *Brain Res.* 1985;327:135–141.
3. Blaugrund E, Hernandez M, Martini R, Barch U, Schachner M, Schwartz M. Neuronal cell adhesion molecule L<sub>1</sub> and regeneration of the fish optic nerve. *Int. J. Dev. Neurosci.* 1988;6:83.
4. Bunge RP, Bunge MB. Interrelationship between Schwann cell function and extracellular matrix production. *Trends Neurosci.* 1983;6:499–505.
5. Cohen A, Schwartz M. Conditioned media of regenerating fish optic nerves modulate laminin levels in glial cells. *J. Neurosci. Res.* 1989;22:269–273.
6. Cornbrooks CJ, Carey DJ, McDonald JA, Timpl R, Bunge RP. *In vivo* and *in vitro* observations on laminin production by Schwann cells. *Proc. Natl. Acad. Sci. U.S.A.* 1983;80:3850–3854.
7. Dawson PA, Schechter N, Williams DL. Induction of rat E and chicken A-I apolipoproteins and mRNAs during optic nerve degeneration. *J. Biol. Chem.* 1986;261:5681–5684.
8. Finkelstein SP, Benowitz L, Olsson AJ, Perrone-Bizzozero NI, Majocha RE, Apostolidis PJ. Conditioned media from injured lower vertebrate CNS promote neurite outgrowth from mammalian brain neurons *in vitro*. *Brain Res.* 1987;413:267–274.
9. Giulian D, Allen RL, Baker TJ, Tomozawa Y. Brain peptides and glial growth. I. Glia-promoting factors as regulator of gliogenesis in the developing and injured central nervous system. *J. Cell Biol.* 1986;102:803.
10. Giulian D, Tomozawa Y, Hindman H, Allen R. Peptides from regenerating central nervous system promotes specific population of macroglia. *Proc. Natl. Acad. Sci. U.S.A.* 1985;82:4287.
11. Hadani M, Harel A, Solomon A, Belkin M, Lavie V, Schwartz M. Substances originating from optic nerve of neonatal rabbit induce regeneration associated response in the injured nerve of adult rabbit. *Proc. Natl. Acad. Sci. U.S.A.* 1984;81:7965–7969.
12. Harel A, Fainaru M, Shafer Z, Schwartz M. Optic nerve regeneration in adult fish is associated with accumulation of apolipoprotein-A-I. *J. Neurochem.* 1989;52:1218–1228.
13. Henderson CE, Huchet M, Changeux J-P. Denervation increases a neurite-promoting activity in extracts of skeletal muscle. *Nature* 1983;302:609–611.

14. Heumann R, Lindholm D, Bandtlow C, Meyer M, Radeke MJ, Misko TP, Shooter E, Thoenen H. Differential regulation of mRNA encoding nerve growth factor and its receptor in rat sciatic nerve during development, degeneration and regeneration: role of macrophages. *Proc. Natl. Acad. Sci. U.S.A.* 1987;84:8735–8739.
15. Hopkins JM, Ford-Holevinski TS, McCoy JP, Agranoff BW. Laminin and optic nerve regeneration in the goldfish. *J. Neurosci.* 1985;5:3030–3038.
16. Ignatius MJ, Gebicke-Harter PJ, Skene JHP, Schilling JW, Weisgraber KH, Mahley RW, Shooter EM. Expression of apolipoprotein-E during nerve degeneration and regeneration. *Proc. Natl. Acad. Sci. U.S.A.* 1986;83:1125–1129.
17. Kiernan JA. Hypotheses concerned with axonal regeneration in the mammalian nervous system. *Biol. Rev.* 1979;54:155–197.
18. Lavie V, Harel A, Doron A, Solomon A, Lobel D, Belkin M, Ben-Bassat S, Sharma S, Schwartz M. Morphological responses of injured adult rabbit optic nerve to implants containing media conditioned by growing optic nerves. *Brain Res.* 1987;419:166–172.
19. Lavie V, Murray M, Solomon A, Ben-Bassat S, Rumlet S, Belkin M, Schwartz M. Growth of injured CNS axons within their own degenerative environment. *J. Comp. Neurol.*; in press.
20. Liesi P. Laminin immunoreactive glial cells distinguish regenerative adult CNS systems from nonregenerative ones. *EMBO J.* 1985;4:2505–2511.
21. Liesi P, Dahl D, Vaheri A. Laminin is produced by early rat astrocytes in primary culture. *J. Cell Biol.* 1983;96:920–924.
22. Liesi P, Kaakkola S, Dahl D, Vaheri A. Laminin is induced in astrocytes of adult brain by injury. *EMBO J.* 1984;3:683–686.
23. Lindholm D, Heumann R, Meyer M, Thoenen H. Interleukin-1 regulates synthesis of nerve growth factor in nonneuronal cells of rat sciatic nerve. *Nature* 1987;330:658–659.
24. Longo FM, Skaper SD, Manthorpe M, Williams LR, Lundborg G, Varon S. Temporal changes in neuronotrophic activities accumulating *in vivo* within nerve regeneration chambers. *Exp. Neurol.* 1983;81:756–769.
25. Manthorpe M, Nieto-Sampedro M, Skaper SD, Lewis ER, Longo FM, Cotman CW, Varon S. Neurotrophic activity in brain wounds of the developing rat: correlation with implant survival in the wound cavity. *Brain Res.* 1983;267:47–56.
26. McConnell P, Berry M. Regeneration of ganglion cell axons in the adult mouse retina. *Brain Res.* 1982;241:362–366.
27. McLoon SC. Response of astrocytes in the visual system to Wallerian degeneration: an immunohistochemical analysis of laminin and glial fibrillary acid protein (GFAP). *Exp. Neurol.* 1986;91:613–621.
28. McLoon SC, McLoon LK, Palm SL, Furcht LT. Transient expression of laminin in the optic nerve of the developing rat. *J. Neurosci.* 1988;8:1981–1990.
29. Miller RH, Abney ER, David S, Ffrench-Constant C, Lindsay R, Patel R, Stone J, Raff M. Is reactive gliosis a property of a distinct subpopulation of astrocytes? *J. Neurosci.* 1986;6:22–29.
30. Muller HW, Gebicke-Harter PJ, Hangen DH, Shooter EM. A specific 37000-dalton protein that accumulates in regenerating but not in nonregenerating mammalian nerves. *Science* 1985;228:499–501.
31. Nathaniel EJH, Pease DR. Collagen and basement membrane formation by Schwann cells during nerve regeneration. *J. Ultrastruct. Res.* 1963;9:550.
32. Neuman D, Yerushalmi A, Schwartz M. Inhibition of nonneuronal cell proliferation

- in the goldfish visual pathway affects the regenerative capacity of the retina. *Brain Res.* 1983;272:237–245.
33. Nieke J, Schachner M. Expression of the neural cell adhesion molecules L<sub>1</sub> and N-CAM and their common carbohydrate epitope L<sub>2</sub>/HNK-1 during development after transection of the mouse sciatic nerve. *Differentiation* 1985;30:141–151.
34. Nieto-Sampedro M, Manthorpe M, Barbin G, Varon S, Cotman CW. Injury-induced neuronotrophic activity in adult rat brain: correlation with survival of delayed implants in the wound cavity. *J. Neurosci.* 1983;3:2219–2289.
35. Nieto-Sampedro M, Whittemore SR, Needels DL, Larson J, Cotman CW. The survival of brain transplants is enhanced by extracts from injured brain. *Proc. Natl. Acad. Sci. USA* 1984;81:6250–6254.
36. Nurcombe V, Hill MA, Eagleson KL, Bennett MR. Motor neuron survival and neuritic extension from spinal cord explants induced by factors released from denervated muscle. *Brain Res.* 1984;291:19–28.
37. Rachailovich I, Schwartz M. Molecular events associated with increased regenerative capacity of the goldfish retinal ganglion cells following x-irradiation: decreased level of axonal growth inhibitors. *Brain Res.* 1984;306:149–155.
38. Ramon y Cajal S. *Degeneration and Regeneration of the Nervous System* Vol. 1, translated by R.M. May. New York: Hafner, 1959.
39. Reier PJ, Stensaas LJ, Guth L. The astrocytes scar as an impediment to regeneration in central nervous system. In: Kan CG, Bunge RP, Reier PJ, eds. *Spinal Cord Reconstruction*, New York: Raven Press, 1983;163–195.
40. Richardson WD, Pringle N, Mosley MJ, Westermark B, Dubois-Dalcq M. A role for platelet derived growth factor in normal gliogenesis in the central nervous system. *Cell* 1988;53:309–319.
41. Schwab ME, Caroni P. Oligodendrocytes and CNS myelin are nonpermissive substrates for neurite growth and fibroblast spreading *in vitro*. *J. Neurosci.* 1988;8:2381–2393.
42. Schwab ME, Thoenen H. Dissociated neurons regenerate into sciatic but not optic nerve explants in culture irrespective of neurotrophic factors. *J. Neurosci.* 1985;6:2415–2423.
43. Schwartz M, Belkin M, Harel A, Solomon A, Lavie V, Hadani M, Rachailovich I, Stein-Izsak C. Regenerating fish optic nerves and regeneration-like response in injured optic nerve of adult rabbits. *Science* 1985;228:600–603.
44. Skene JHP, Shooter EM. Denervated sheath cells secrete a new protein after nerve injury. *Proc. Natl. Acad. Sci. USA* 1983;80:4173–4178.
45. Snipes GJ, McGuire CB, Norden JJ, Freeman JA. Nerve injury stimulates the secretion of apolipoprotein-E by nonneuronal cells. *Proc. Natl. Acad. Sci. USA* 1986;83:1130–1134.
46. Varon S, Manthorpe M, Longo FM, Williams LR. Growth factors in regeneration of neural tissues. In: Seil FY, ed. *Nerve, Organ and Tissue Regeneration: Research Perspectives*, New York: Academic Press, 1983;127.
47. Williams LR, Longo FM, Powell HC, Lundborg G, Varon S. Spatial-temporal progress of peripheral nerve regeneration within a silicone chamber: parameters for bioassay. *J. Comp. Neurol.* 1983;218:460–470.
48. Zak NB, Harel A, Bawnik Y, Ben-Bassat S, Vogel Z, Schwartz M. Laminin immunoreactive sites are induced by growth-associated triggering factors in injured rabbit optic nerve. *Brain Res.* 1987;408:263–266.

# Brain-derived Heparin-binding Growth Factors and their Oncogenic Homologs

Ing-Ming Chiu, Per Sandberg and Wen-Pin Wang

Department of Internal Medicine  
The Ohio State University  
Davis Medical Research Center  
Columbus, OH 43210

**B**rain extracts have been used for half a century as supplements to grow tissue culture cells (75). The continual search for the biological response modifiers for mammalian cell growth has led to the discovery of polypeptide growth factors (42). Certain ingredients from the brain extracts turned out to be essential for the growth of endothelial cells *in vitro* (32). Endothelial cells are the innermost layering cells in blood vessels. Thus, the formation of new blood vessels, or neovascularization, will involve the migration, proliferation, and differentiation of the endothelial cells. The turnover rate of normal endothelial cells *in vivo* is measured in years except during embryogenesis, ovulation, the growth of solid tumors, or wound healing (17). The process of blood vessel formation is termed angiogenesis and involves the controlled outgrowth of new capillaries. Angiogenesis also occurs in a variety of other pathological processes such as chronic inflammation (62), immune reactions (2), and diabetic retinopathies (49).

One prominent example of neovascularization appears during the progression of tumor as first observed by Folkman (24). He suggested that once tumor take has occurred, every increase in tumor cell population must be preceded by an increase in new capillaries that converge on the tumor. In other words, the tumors induce new capillary growth as a pre-requisite for tumor growth. Progressive tumor growth depends on the continuous buildup of a new network of vascular structure. This factor is consequently named tumor angiogenesis factor (25).

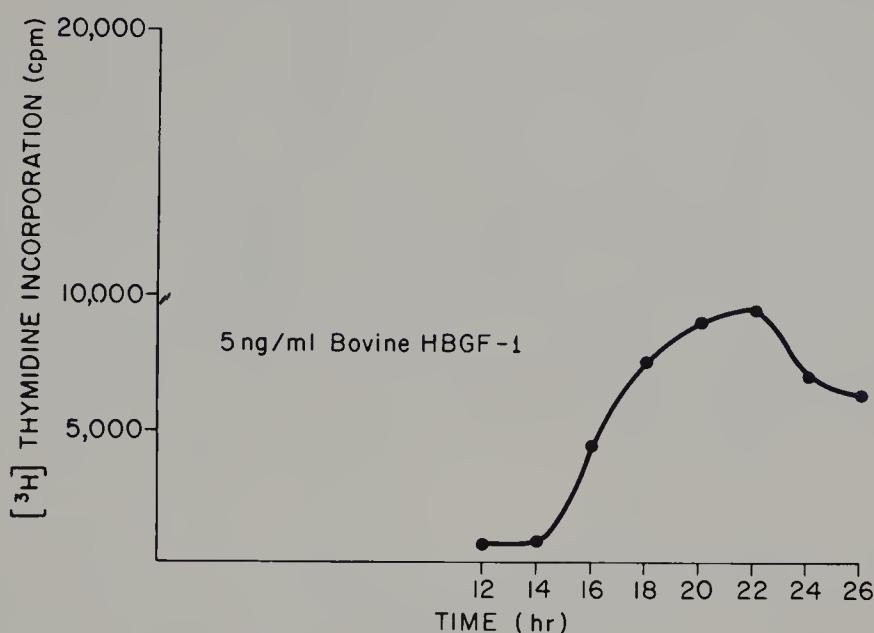
## BIOCHEMISTRY AND BIOLOGY OF HEPARIN-BINDING GROWTH FACTOR (HBGF)

Biochemists have been looking for the angiogenic factors from brain and other sources. The biological activities, in terms of stimulation of endothelial cell growth,

could be detected from the brain extracts and from other sources. However, further biochemical characterization of the factors proved to be difficult. Thus, a variety of names, including fibroblast growth factor (FGF), endothelial cell growth factor, and astroglial growth factor (according to the assay system) were documented in the literature. They are also known by the names derived from the sources from which they were purified (32,46). Most of the above mitogens appear to have very similar biochemical properties. Further comparison was virtually impossible without further purification. In 1984, Shing et al. discovered the affinity of chondrosarcoma-derived growth factor to heparin-Sepharose (69). This property greatly simplified the purification of these mitogens (33,50,69). Within a couple of years, most of the endothelial cell mitogens were purified to homogeneity and were shown to be either class 1 or class 2 heparin-binding growth factors (46,48). Other angiogenic factors include angiogenin, transforming growth factor  $\alpha$ , transforming growth factor  $\beta$ , tumor necrosis factor  $\alpha$ , and platelet-derived endothelial cell growth factor (25).

Class 1 HBGF (HBGF-1) is a protein of mol. wt. 16,000–18,000 with a pI of 5.6, whereas class 2 HBGF (HBGF-2) is of similar size molecular weight with a pI of 9.6. HBGF-2 has been isolated from a variety of tissues and tumor cell lines (32,48) while the sources of HBGF-1 have been limited to the neural tissues such as brain, hypothalamus, and retina (46,48). Recently, HBGF-1 was also isolated from kidney (28), smooth muscle cells (80), several glioblastoma cell lines (43), and two other tumor cell lines (47). Sequence analysis of cDNA and genomic clones of the human HBGF-2 predicts a single 18 kDa (155 amino acid) protein (1). However, higher molecular weight species of HBGF-2 have been described (54,63,71). It was shown that the higher molecular weight forms of HBGF-2 are initiated at idiosyncratic CUG codons while the 18 kDa protein is initiated at the traditional AUG codons (63; A. Sommer and D. Hirsh, *personal communication*). These unusual start codons might be a means for down-regulation of protein synthesis as well as an important feature of alternative initiation of protein synthesis.

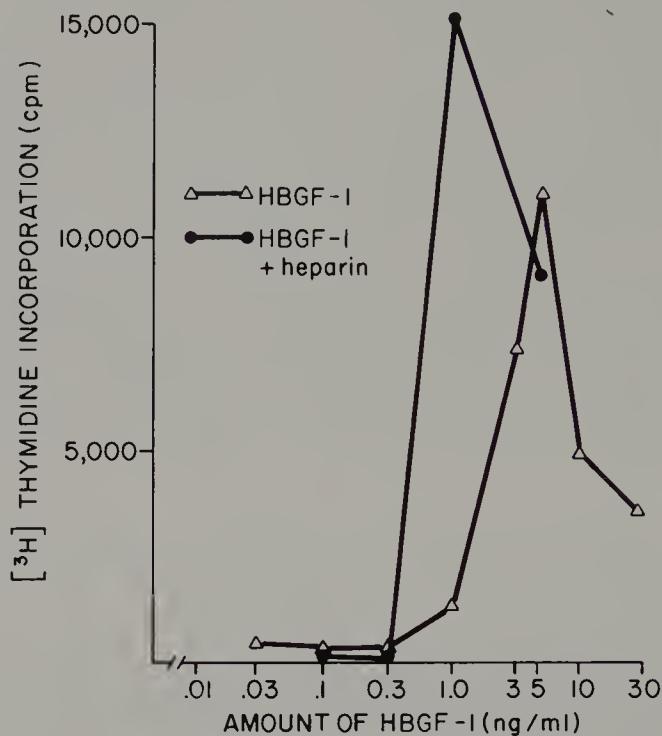
The amino acid sequences of both HBGF-1 (29,30,35) and HBGF-2 (21,71) have been determined and show a 55% sequence similarity. HBGF can induce the growth of most cultured cells having mesoderm or neuroectoderm origin (34,72) as well as prostatic epithelial cells (13). The mitogenic effect was usually measured by the uptake of [ $^3$ H]thymidine into DNA. Mouse Swiss/3T3 fibroblast cells responded to the mitogenic stimulation of bovine HBGF-1 at 5 ng/ml and peaked at 22–24 hr after addition of the mitogen (Fig. 1). The mitogenic activity of HBGF-1 can be potentiated by heparin while that of HBGF-2 is not. Thus, at 1 ng/ml of HBGF-1, heparin can increase the mitogenic activity of HBGF-1 by more than ten fold (Fig. 2). In their purified forms, both HBGFs are capable of inducing angiogenesis *in vivo* (25,45,73). Furthermore, HBGFs have other non-mitogenic activities such as the stimulation of thyrotropin and prolactin secretion in rat pituitary cells (5). It was also shown that HBGF-2 can expedite cartilage repair *in vivo* by stimulating chondrocyte proliferation and the formation of extracellular matrix (14).



**FIG. 1.** Mitogenic effect of bovine HBGF-1 on mouse Swiss/3T3 cells. Swiss/3T3 cells were grown to confluence and changed to Dulbecco's modified Eagle medium containing 0.5% calf serum to starve for two days. HBGF-1 (5 ng/ml) was then added and at each specific time thereafter, [<sup>3</sup>H]thymidine was added. Cells were incubated in the presence of [<sup>3</sup>H]thymidine for two hours. The amount of radioactivity incorporated into DNA as TCA-precipitable materials was measured as the mitogenic activity.

HBGF can also elicit neurite outgrowth of PC12 pheochromocytoma cells (56,74). In the case of HBGF-1, induction of neurite outgrowth in PC12 cells is augmented by heparin (76). In addition, HBGF-2 substantially promotes both cell survival and process extension in primary cultures of fetal rat hippocampal and cortical neurons (53,57). In contrast, the outgrowth of processes by rat retinal ganglion cells *in vitro* was found to be promoted to a far greater degree by HBGF-1 than by HBGF-2 (44). The action of HBGF-1 on process outgrowth was markedly potentiated by the addition of heparin to the medium (44). These findings suggest that HBGF-1 and HBGF-2 may work on separate populations of central neurons, which in turn suggests that their effects may be mediated through distinct receptors. Thus, next to nerve growth factor, HBGFs have become the best-characterized neurotrophic polypeptide growth factors for neuronal differentiation (27).

Molecular cloning of the cDNA for both HBGF-1 (38, I.-M. Chiu, W.-P. Wang, K. Lehtoma, *manuscript in preparation*) and HBGF-2 (1,41,63,71) allowed direct comparison of the predicted amino acid sequences with the HBGF protein se-



**FIG. 2.** Dosage effect of bovine HBGF-1 on Swiss/3T3 cells. Swiss/3T3 Cells were starved in Dulbecco's modified Eagle medium containing 0.5% calf serum for two days. The cells were pulsed with [<sup>3</sup>H]thymidine from 18–24 hr after the addition of the specified amount of bovine HBGF-1 and DNA synthesis rate was determined. Alternatively, 5 units/ml of heparin in addition to the specified amount of HBGF-1 were added to the media.

quences. The amino-terminal residues predicted from the human HBGF-1 DNA sequence are relatively nonhydrophobic, suggesting that the protein is synthesized without an amino-terminal signal peptide (38,78). The lack of a signal peptide in HBGF-1, a polypeptide for which high affinity cell surface receptors have been characterized (26,36,55,60), invoked the obvious question of how it is released from the site of biosynthesis. The lack of a signal peptide or an internal hydrophobic domain in HBGF-1 (9,13,38) is shared by another extracellular protein, interleukin-1 (IL-1) (3). IL-1 has approximately 30% sequence identity with HBGF (31,73). It has been postulated that IL-1 is released from stimulated monocytes or macrophages by leakage, perhaps due to cell damage (3). If release of HBGF-1 is also due to leakage from cells, it is likely that neural tissue is not the only site of biosynthesis.

It was demonstrated that HBGF-2 is present in extracts of stimulated peritoneal macrophages (4). Delivery, by the stimulated macrophages, of endothelial mitogens

to sites of injury may be the underlying mechanism by which these polypeptides play a role in the regulation and maintenance of homeostasis *in vivo*. This may also be relevant to the high affinity binding of monocytes to endothelial cells (61), which appears to be an early event in atherogenesis (22). It is postulated that the active growth factors could be released by heparinases secreted from adjacent normal or tumor cells, although it is not known if HBGFs are active when bound to heparan sulfates or only when they dissociate from the proteoglycans. At any rate, the release of HBGF to the extracellular environment is not likely to be through the membrane of the rough endoplasmic reticulum by co-translational transport (7). Alternatively, mRNA coding for HBGF with signal peptide may be present in tissues where HBGF is secreted. A definitive answer to these questions awaits systematic analysis of cDNA clones and genomic DNA clones coding for HBGF.

## GENE STRUCTURE OF HBGF-1

Since brain and other neural tissues are major sources of the heparin-binding growth factors, the system may provide a unique opportunity to study the control of normal growth and oncogenesis of cells in the nervous system. We have isolated several cDNA clones from a human brain stem cDNA library (I.-M. Chiu, W.-P. Wang, K. Lehtoma, *manuscript in preparation*). A partial AluI-HaeIII human genomic DNA library was screened with the HBGF-1 cDNA probe. The DNA sequences identical to the first and the third exons were identified by Southern hybridization and then confirmed by nucleotide sequence analysis. The alignment of common restriction enzyme sites between the genomic DNA and cDNA suggested the third exon contains a very long, contiguous, 3'-untranslated sequence (78).

By aligning the DNA sequence of the first and the third exons with the cDNA sequence, we found a gap of 104 bp which could represent at least one exon. As the previous isolates did not contain DNA sequence coding for the middle portion of the protein, another library constructed by partial EcoRI digestion and cloned into Charon 4A was used for screening. A single clone was isolated and shown to contain sequences hybridizing to the middle exon-specific oligonucleotide probe. It also contained sequences hybridizing to the DNA probes derived from the third exon. The restriction enzyme maps of all the genomic DNA clones were established and aligned based on the common restriction enzyme sites (Fig. 3). The DNA sequence analysis showed that these clones contain the entire protein coding sequence as compared with the published amino acid sequences (29,30,35). The coding regions were comprised of three exons, with 169, 104, and 192 bp of protein coding sequence for each exon. By using the genomic DNA as a probe, we were able to show that HBGF-1 gene is a single copy gene (78). Therefore, the different forms of HBGF-1 protein are probably encoded by the same gene. The gene has been mapped to chromosome 5q31.3-33.2 (38), the distal breakpoint of 5q<sup>-</sup> chromosome (59).

### HBGF-1 LOCUS

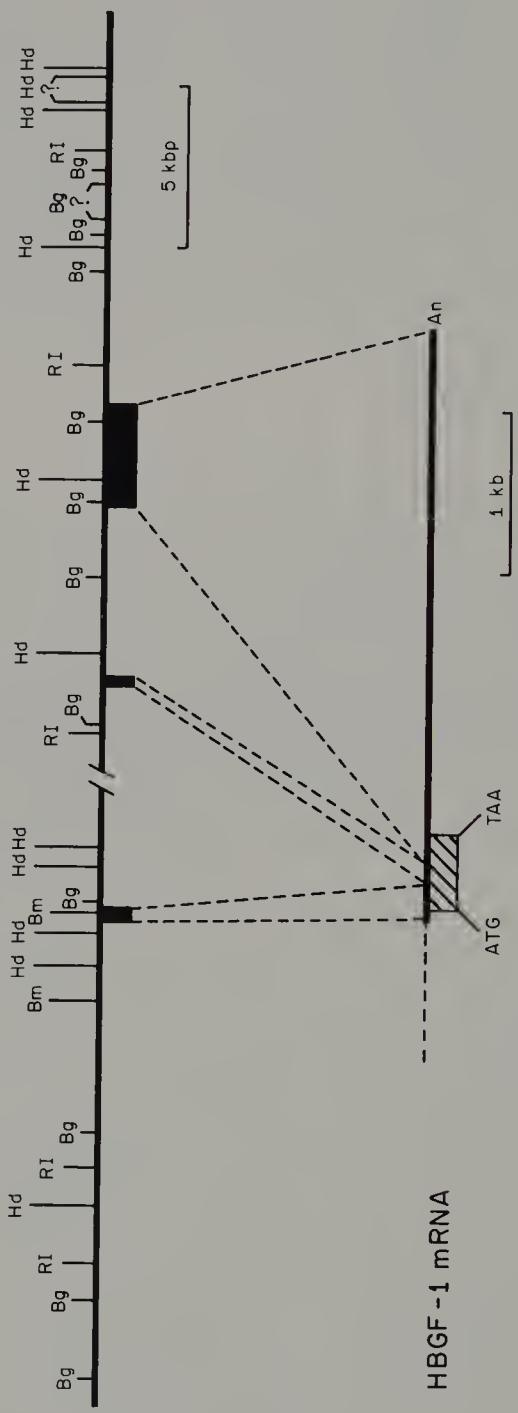


FIG. 3. Exon-intron organization of the gene coding for human HBGF-1. The solid line represents the regions cloned while the dashed line represents uncharacterized region. Closed boxes represent exons and the hatched box represents the protein-coding region. The distance between the first and the second exons is 13.6 kbp and the distance between the second and the third exons is 5.3 kbp. Both the location(s) of the 5'-end exon(s) and the transcription initiation site(s) are not mapped. RI, EcoRI; Bm, BamHI; Bg, BglII; H<sub>d</sub>, HindIII.

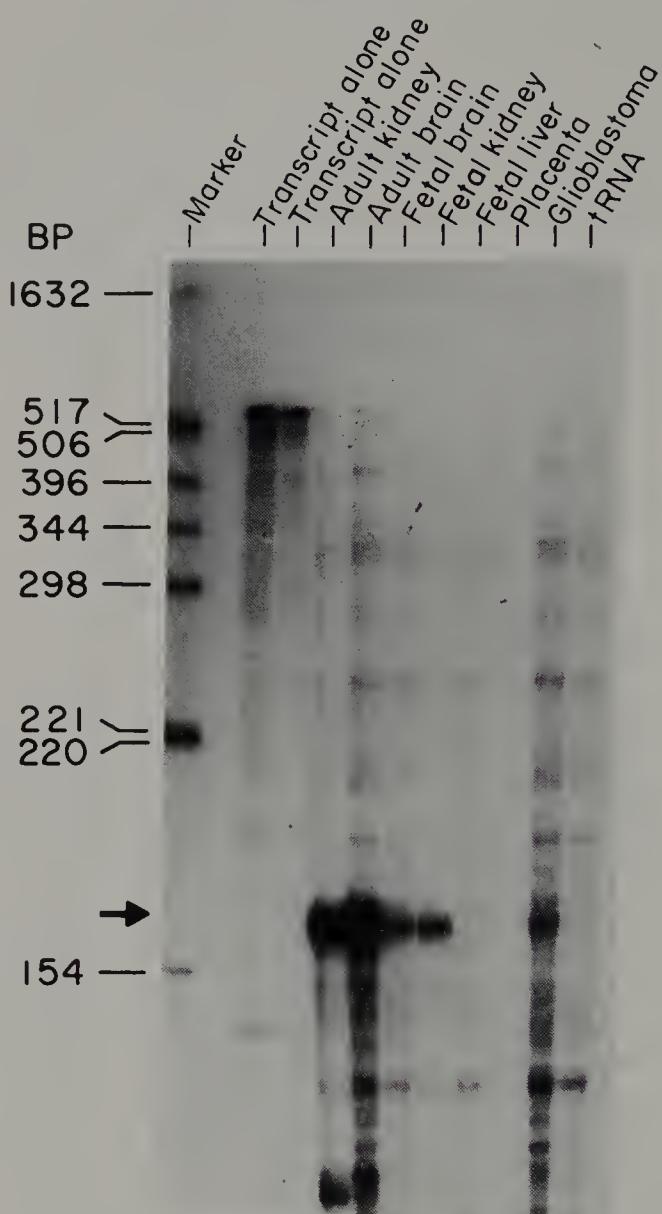
A major 4.5 kb HBGF-1 mRNA species was detected in RNA isolated from both human brain and kidney by Northern hybridization. Furthermore, two additional faint bands with the sizes of 3.4 kb and 2.0 kb were also detected in both kidney and brain RNA (78). To pursue the possibility of HBGF-1 expression in human fetal tissues, a more sensitive method based on the RNase protection assay was developed. With this technique, we were able to show that both the fetal brain and fetal kidney (from a 13-week-old fetus) expressed HBGF-1 mRNA while fetal liver and term placenta did not express a detectable level of HBGF-1 mRNA (Fig. 4). This supports the previous reports that both HBGF-1 and HBGF-2 are involved in the induction of mesoderm during development and act as morphogens in early embryogenesis (40,70).

### Oncogenic Members of the HBGF Family

Recently, three oncogenes have been shown to encode proteins related to HBGF. Oncogene *int-2* was identified by investigating the common integration sites of mouse mammary tumor virus in the DNA of virus-induced mammary carcinoma (19). It was subsequently shown that disruption of the locus by a provirus had activated the transcription of the gene (52). Recently, the human homolog of mouse *int-2* has been cloned (10) and completely sequenced (8). Comparison of the predicted amino acid sequence of human *int-2* with HBGF-1 and -2 showed a significant homology (18; Fig. 5) among them. The *int-2* gene was expressed only in early gestation stages of development (37,79). These data together with the evidence described in the previous section suggest the possibility that members of the HBGF gene family may, like the globin gene family, undergo switching from embryonic genes to the adult genes. If this is the case, the HBGF gene family will present an interesting system to study gene expression switching.

The other two oncogenes, *hst/KS3* and FGF-5, were detected as transforming genes in human tumor DNA which could transform nontumorigenic cells upon transfection. The transforming gene *hst/KS3* was originally detected in a human stomach tumor (81) and Kaposi's sarcoma (16) by two independent investigators. It was subsequently found that the *hst/KS3* genes isolated from a noncancerous portion of stomach mucosa, a variety of other tumors, and the normal human leukocytes all have transforming activity. It was reasoned that the expression of the gene is normally suppressed by a *cis*-acting element, and that rearrangement of these sequences during transfection might activate the transcription of the gene. The *hst/KS3* protein elutes from the heparin-Sepharose column at a salt concentration similar to that of HBGF-1 (16). It was also shown that the human *int-2* and *hst/KS3* gene loci were within 40 kbp of each other on chromosome 11q13 and they are co-amplified in human cancer (23,58,82).

FGF-5 oncogene was originally isolated from a human bladder carcinoma by selection for transformed cells using a defined medium lacking HBGFs or platelet-



**FIG. 4.** RNase protection assays and expression of HBGF-1 RNA in human tissues. Plasmid DNA, pHBg1.0, containing the first protein-coding exon of HBGF-1 gene was digested with NcoI and RNA was transcribed *in vitro* using T7 RNA polymerase. Lanes 1 and 2, transcript alone without RNase digestion; Lane 3, adult kidney; Lane 4, adult brain; Lane 5, fetal brain; Lane 6, fetal kidney; Lane 7, fetal liver; Lane 8, placenta; Lane 9, glioblastoma cell line, A2781; and Lane 10, tRNA. Total cellular RNA was isolated from various human tissues and the glioblastoma cells using the guanidinium thiocyanate method. The RNA transcript was hybridized to 20 µg of cellular RNA and then digested with RNase A and RNase T<sub>1</sub>. The protected fragments were separated on a 6% polyacrylamide gel under denaturing conditions. The RNase-resistant fragments were indicated by arrows. The marker used was pBR322 DNA digested with HinfI. (Modified from ref. 78.)

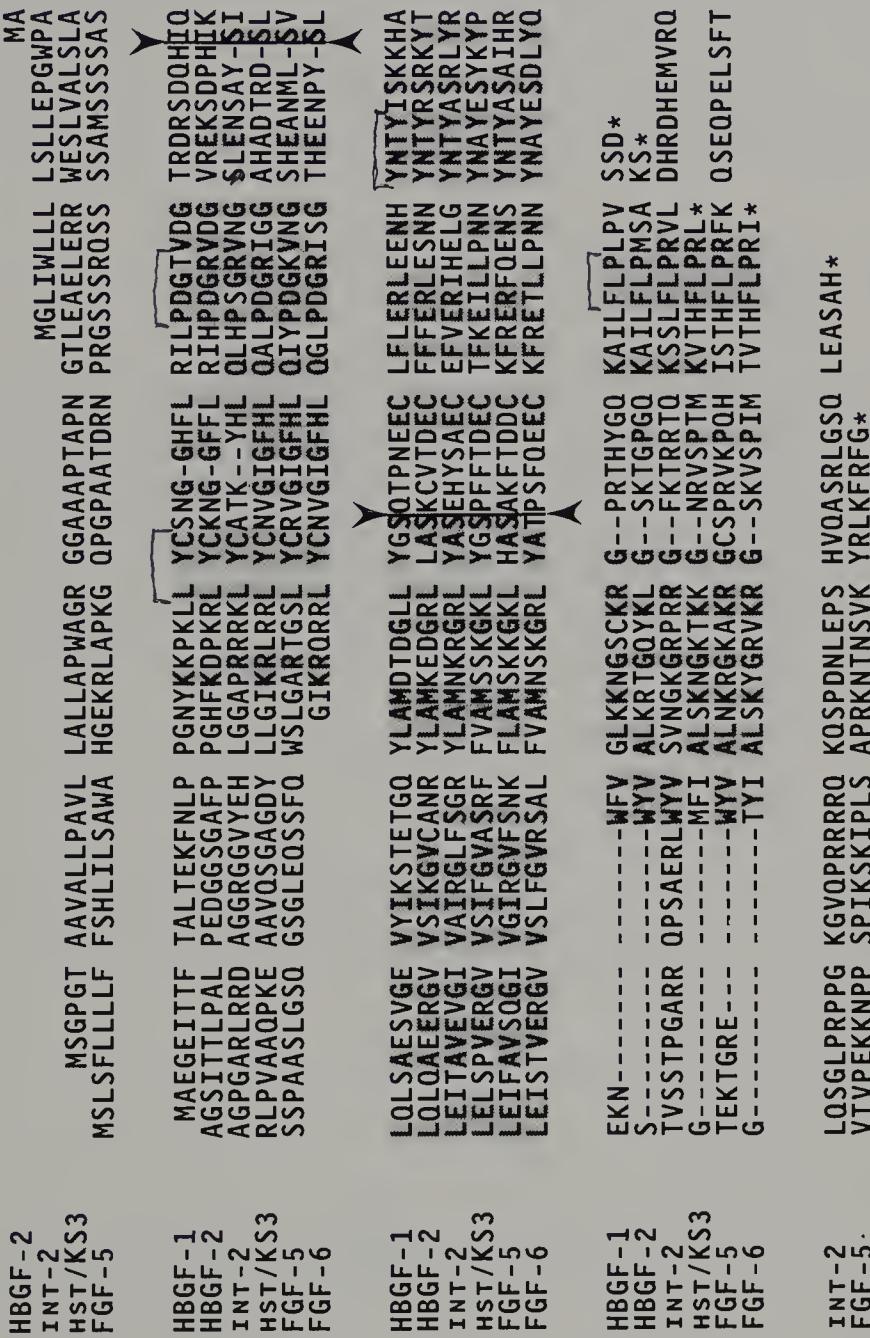


FIG. 5. Homology of protein sequence among members of HBGF family. Sequences shared by four or more proteins are shaded. The arrowheads indicate the junctions of exons. The middle exons of the genes coding for each of the six proteins all have 104 nucleotides with exactly the same boundary.

derived growth factor (84). This oncogene was activated by the juxtaposition of a murine retrovirus transcriptional enhancer next to the native promoter of the FGF-5 gene (83). The gene is mapped to chromosome 4q21, the same chromosome to which HBGF-2 is localized. The FGF-5 gene product, unlike HBGF-1 or -2, but similar to *hst/KS3*, was secreted to the medium. As expected, the cDNA sequences of FGF-5 and *hst/KS3* predict hydrophobic sequences at their respective amino-termini. Whether *int-2* encodes a secretory protein has not been firmly established (Peters G., *personal communication*). FGF-5 is also a mitogen for both fibroblasts and endothelial cells and elutes from heparin-Sepharose at 1.0 M NaCl concentration (83).

### Other HBGF Related Genes: FGF-6 and KGF

FGF-6 gene was isolated by screening genomic DNA libraries with a *hst/KS3* probe in reduced conditions of stringency (51). This gene presents strong sequence homology (70%, Table 1) with *hst/KS3*. Unlike *hst/KS3*, FGF-6 has never been identified from human tumor DNA as a transforming gene in NIH/3T3 assays. However, the cloned normal human FGF-6 gene is able to transform NIH/3T3 cells using both focus and tumorigenicity assays (51). The gene was mapped to human chromosome 12p13 by *in situ* hybridization. The 5' half of the first exon of FGF-6 did not show any similarity with *hst/KS3*, thus definition of the amino-terminus must await the cloning and sequencing of the full length cDNA. The biochemical and biological properties of the FGF-6 gene product also remain to be characterized. Nevertheless, focus assays in low serum concentration promise to identify more HBGF-related sequences in NIH/3T3 transformants (51,84).

Comparison of the structures of these six HBGF-related genes reveals that they all consist of three exons in the protein-coding sequences. Furthermore, the intervening sequences are present in the same positions with respect to the coding sequences, although they are of different lengths. The distance between the first

TABLE 1. Sequence similarity among the members of HBGF family

	HBGF-1	HBGF-2	<i>int-2</i>	<i>hst/KS3</i>	*FGF-5	FGF-6
HBGF-1	100.0% (155)	54.1% (157)	33.6% (143)	37.8% (127)	38.3% (128)	33.1% (127)
HBGF-2		100.0% (155)	40.0% (135)	43.0% (135)	41.4% (128)	43.2% (125)
<i>int-2</i>			100.0% (239)	32.4% (145)	43.0% (142)	37.1% (143)
<i>hst/KS3</i>				100.0% (206)	48.4% (128)	70.1% (127)
FGF-5					100.0% (233)	47.7% (132)
FGF-6						100.0% (128)

The numbers in parentheses are the numbers of amino acids in each protein or the numbers of amino acids for comparison between each pair of proteins. Gaps are introduced to each amino acid sequence to achieve maximum similarity using the Needleman-Wunsch method.

and the second exons of HBGF-1 gene is 13.6 kbp and the distance between the second and the third exons is 5.3 kbp (78, I.-M. Chiu et al., *unpublished results*). The sizes of the two introns of human *hst/KS3* are 617 and 638 bp, respectively (81), whereas those of human *int-2* are 2,289 and 5,648 bp, respectively (8). The sizes of the two introns for FGF-6 are approximately 1.0 and 8.2 kbp, respectively (51). The intron sizes of HBGF-2 and FGF-5 genes have not been determined precisely. The most prominent feature is that the six genes all have a 104 bp middle exon with identical 5' and 3' boundaries. Thus, these six genes are likely to have a common evolutionary origin, and the introduction of intervening sequences must have preceded gene duplication.

Recently, Rubin et al. (66) have identified a growth factor specific for epithelial cells but lacking mitogenic activity on either NIH/3T3 fibroblasts or human saphenous vein endothelial cells. This keratinocyte growth factor (KGF) was both acid and heat labile and consisted of a single polypeptide chain of ca. 28 kDa. In a chemically defined medium, KGF complemented the insulin-like growth factor I requirement of keratinocytes and was shown to act through a signal-transduction pathway shared with epidermal growth factor, transforming growth factor  $\alpha$ , and the HBGFs. KGF has less affinity to heparin-Sepharose than HBGFs (eluted at 0.6 M NaCl). However, sequence analysis of the cDNA coding for KGF reveals that it has significant structural homology to the other six known members of the HBGF family (S.A. Aaronson, *personal communication*). The gene structure of KGF is not available as yet for comparison.

## Transforming Potentials of HBGF-1 and HBGF-2

The notion of the relationship between growth factors and oncogenes was first vindicated by our report and others that showed the human *c-sis* proto-oncogene as the structural gene for the B chain of platelet-derived growth factor (12, for a review see ref. 11). Subsequently, it was shown that proto-oncogenes *erbB* and *fms* code for the receptors for epidermal growth factor (20) and colony-stimulating factor-1 (68), respectively. Together, they provide a compelling reason for testing the transforming potential of HBGF-1 and -2.

It was shown that NIH/3T3 cells transfected with bovine HBGF-2 cDNA and expressing the native 18 kDa protein appear to have only slightly greater saturation density and serum independence in culture than parental cells and have a low frequency of tumorigenicity in syngeneic mice (65). In contrast, the cells transfected with the HBGF-2 cDNA preceded by coding sequence for mouse immunoglobulin signal peptide (Ig-bFGF) are focus-forming, have transformed morphology, and are consistently tumorigenic in syngeneic mice (65). Similar results were obtained by Aaronson and his colleagues (6) who used the secretion signal sequence of growth hormone. Contradiction remains in that the former report showed both the wild type HBGF-2 and the chimeric Ig-bFGF are cell-associated,

while the latter reported the signal peptide-fused HBGF-2 is secreted to the conditioned medium. Nonetheless, the mitogenic activity of the extracellular HBGF-2 is significantly reduced (6). These data suggest that interaction of HBGF-2 with its cognate receptors likely occurs while the fusion protein with the artificial leader is being processed along the secretory pathway. In contrast, two other laboratories reported cells transformed with wild-type HBGF-2 cDNA grow autonomously on soft agar and have detectable levels of HBGF-2 in their conditioned media (57,67). In at least one case, the transformed phenotype could be reversed by the addition of neutralizing antibodies to the medium (67). The discrepancies among the four laboratories could result from the different cell lines or different expression vector systems used.

Thus far, there is only one report on the expression of a truncated form of HBGF-1. Cells expressing the truncated HBGF-1 are unable to support autocrine growth on soft agar and produce only small nonprogressive tumors in nude mice (39). Production of full length HBGF-1 in mammalian tissue culture cells is tenuous (P. Bunnag, P. Sandberg, I.-M. Chiu, *manuscript in preparation*; T. Maciag, *personal communication*). It is possible that high level production of HBGF-1 per se is toxic to the responsive cells. This cytoidal phenomenon has been well-documented for the *ras* oncogene (64) and was also reported for the *abl* oncogene (15) and the Ig-bFGF cDNA (65). Nevertheless, in the very few cases in which cells do express full length HBGF-1 protein, the transfectants are capable of supporting their own growth on soft agar (P. Bunnag, P. Sandberg, I.-M. Chiu, *manuscript in preparation*). Thus, there may be an intrinsic difference between the full length HBGF-1 and the truncated one. In this regard, our system will provide a tool to study such properties. It is also of interest to determine if nature would employ signal peptide translocation as an alternative route for carcinogenesis. Through the characterization of the brain-derived heparin-binding growth factors as well as their oncogenic homologs, we will be able to improve our understanding of the cellular response to these mitogens. The diversity of the HBGF genes and their functions support the emerging evidence that mitogenic and neurotrophic growth factors are not necessarily two separate entities.

## SUMMARY

Heparin-binding growth factors (HBGF) are a family of mitogens that have high-affinity to the proteoglycan, heparin. Their biological activities, including stimulation of endothelial cell growth *in vitro*, can be potentiated by heparin. The family consists of HBGF-1, HBGF-2, int-2, hst/KS3, fibroblast growth factor-5 (FGF-5), and FGF-6. All these proteins share 30–50% amino acid sequence identity. Recently, a keratinocyte growth factor (KGF) was also found to be homologous to the other members of the HBGF family and have affinity to heparin. In this paper, we describe the biochemical and biological properties of the HBGF as ex-

emplified by HBGF-1. The structure and expression of the gene coding for HBGF-1 was also discussed in greater detail.

## ACKNOWLEDGMENTS

We wish to thank Drs. T. Maciag, S. Harris, G. Peters, O. de Lapeyrier, and S.A. Aaronson for sharing unpublished information, Kirsten Lehtoma and Lee Varban for technical assistance, Barbara Mazzotta for secretarial help, M.-C. Chiu for preparation of the homology figure and Table 1, and Dr. M.-C. Hung for critical comments of the manuscript. The project was supported in part by the grants from the National Institutes of Health (CA-45611), the Ohio Cancer Research Associates, Inc. (88-90-01-01), and the March of Dimes Birth Defects Foundation (#6-549).

## REFERENCES

1. Abraham JA, Whang JL, Tumolo A, Mergia A, Friedman J, Gospodarowicz D, Fiddes JC. Human basic fibroblast growth factor: nucleotide sequence and genomic organization. *EMBO J.* 1986;5:2523–2528.
2. Auerbach R. Angiogenesis inducing factors. In: Pick E, ed. *Lymphokines*, vol. 4. New York: Academic Press, 1981;69–88.
3. Auron PE, Webb AC, Rosenwasser LJ, Mucci SF, Rich A, Wolff SM, Dinarello CA. Nucleotide sequence of human monocyte interleukin-1 precursor cDNA. *Proc. Natl. Acad. Sci. USA* 1984;81:7907–7911.
4. Baird A, Mormeda P, Beohlen P. Immunoreactive fibroblast growth factor in cells of peritoneal exudate suggests its identity with macrophage-derived growth factor. *Biochem. Biophys. Res. Commun.* 1985;126:358–364.
5. Baird A, Mormeda P, Ying S-Y, Wehrenberg WB, Ueno N, Ling N, Guillemin R. A nonmitogenic pituitary function of fibroblast growth factor: regulation of thyrotropin and prolactin secretion. *Proc. Natl. Acad. Sci. USA* 1985;82:5545–5549.
6. Blam SB, Mitchell R, Tischer E, Rubin JS, Silva M, Silver S, Fiddes JC, Abraham JA, Aaronson SA. Addition of growth hormone secretion signal to basic fibroblast growth factor results in cell transformation and secretion of aberrant forms of the protein. *Oncogene* 1988;3:129–136.
7. Blobel G, Water P, Change GN, Goldman BM, Erickson AH, Lingappa VR. Translocation of proteins across membranes: the signal hypothesis and beyond. *Symp. Soc. Exp. Biol.* 1979;33:9–36.
8. Brookes S, Smith R, Casey G, Dickson C, Peters G. Sequence organization of the human *int-2* gene and its expression in teratocarcinoma cells. *Oncogene* 1989;4:429–436.
9. Burgess WH, Mehlman T, Marshak DR, Fraser BA, Maciag T. Structural evidence that endothelial cell growth factor  $\beta$  is the precursor of both endothelial cell growth factor  $\alpha$  and acidic fibroblast growth factor. *Proc. Natl. Acad. Sci. USA* 1986;83:7216–7220.

10. Casey G, Smith R, McGillivray D, Peters G, Dickson C. Characterization and chromosome assignment of the human homolog of *int-2*, a potential proto-oncogene. *Mol. Cell. Biol.* 1986;6:502–510.
11. Chiu I-M. Growth factor genes as oncogenes. *Mol. Chem. Neuropathol.* 1989;10:37–52.
12. Chiu I-M, Reddy EP, Givol D, Robbins KC, Tronick SR, Aaronson SA. Nucleotide sequence analysis identifies the human *c-sis* proto-oncogene as a structural gene for platelet-derived growth factor. *Cell* 1984;37:123–129.
13. Crabb JW, Armes LG, Carr SA, Johnson CM, Roberts GD, Bordoli RS, McKeehan WL. Complete primary structure of prostatropin, a prostate epithelial cell growth factor. *Biochemistry* 1986;25:4988–4993.
14. Cuevas P, Burgos J, Baird A. Basic fibroblast growth factor (FGF) promotes cartilage repair *in vivo*. *Biochem. Biophys. Res. Commun.* 1988;156:611–618.
15. Daley GQ, McLaughlin J, Witte ON, Baltimore D. The CML-specific P210 *bcrabl* protein, unlike *v-abl*, does not transform NIH/3T3 fibroblasts. *Science* 1987;237:532–535.
16. Delli Bovi P, Curatola AM, Kern FG, Greco A, Ittmann M, Basilico C. An oncogene isolated by transfection of Kaposi's sarcoma DNA encodes a growth factor that is a member of the FGF family. *Cell* 1987;50:729–737.
17. Denekamp J. Vasculature as a target for tumor therapy. *Prog. Appl. Microcirc.* 1984; 4:28–38.
18. Dickson C, Peters G. Potential oncogene product related to growth factors. *Nature* 1987;326:833.
19. Dickson C, Smith R, Brookes S, Peters G. Tumorigenesis by mouse mammary tumor virus: proviral activation of a cellular gene in the common integration region *int-2*. *Cell* 1984;37:529–536.
20. Downward J, Yarden Y, Mayes E, Scrace G, Totty N, Stockwell P, Ullrich A, Schlesinger J, Waterfield MD. Close similarity of epidermal growth factor receptor and *v-erb-B* oncogene protein sequence. *Nature* 1984;307:521–527.
21. Esch F, Baird A, Ling N, Ueno N, Hill F, Denoroy L, Klepper R, Gospodarowicz D, Bohlen P, Guillemin R. Primary structure of bovine pituitary basic fibroblast growth factor (FGF) and comparison with the amino-terminal sequence of bovine brain acidic FGF. *Proc. Natl. Acad. Sci. USA* 1985;82:6507–6511.
22. Faggioto A, Ross R, Harker L. Studies of hypercholesterolemia in the nonhuman primate. *Arteriosclerosis* 1984;4:323–340.
23. Fantl V, Brookes S, Smith R, Casey G, Barnes D, Johnstone G, Peters G, Dickson C. Characterization of the proto-oncogene *int-2* and its potential for the diagnosis of human breast cancers. *Cancer Cells* 1989;7:283–287.
24. Folkman J. Toward an understanding of angiogenesis: search and discovery. *Perspect. Biol. Med.* 1985;29:10–36.
25. Folkman J, Klagsbrun M. Angiogenic factors. *Science* 1987;235:442–447.
26. Friesel R, Burgess WH, Mehlman T, Maciag T. The characterization of the receptor for endothelial cell growth factor by covalent ligand attachment. *J. Biol. Chem.* 1986; 261:7581–7584.
27. Fujita K, Lazarovici P, Guroff G. Regulation of the differentiation of PC12 pheochromocytoma cells. *Environ. Health Perspect.* 1989;80:127–142.
28. Gautschi-Sova P, Jiang Z-P, Frater-Schröder M, Bohlen P. Acidic fibroblast growth factor is present in nonneuronal tissue: isolation and chemical characterization from bovine kidney. *Biochemistry* 1987;26:5844–5847.

29. Gautschi-Sova P, Muller T, Bohlen P. Amino acid sequence of human acidic fibroblast growth factor. *Biochem. Biophys. Res. Commun.* 1986;140:874–880.
30. Gimenez-Gallego G, Conn G, Hatcher VB, Thomas KA. The complete amino acid sequence of human brain-derived acidic fibroblast growth factor. *Biochem. Biophys. Res. Commun.* 1986;138:611–617.
31. Gimenez-Gallego G, Rodkey J, Bennet C, Rios-Candelore M, DiSalvo J, Thomas KA. Brain-derived acidic fibroblast growth factor: complete amino acid sequence and homologies. *Science* 1985;230:1385–1388.
32. Gospodarowicz D. Isolation and characterization of acidic and basic fibroblast growth factor. *Methods Enzymol.* 1987;147:106–119.
33. Gospodarowicz D, Cheng J, Lui G-M, Baird A, Bohlen P. Isolation of brain fibroblast growth factor by heparin-Sepharose affinity chromatography: identity with pituitary fibroblast growth factor. *Proc. Natl. Acad. Sci. USA* 1984;81:6963–6967.
34. Gospodarowicz D, Neufeld G, Schweigerer L. Molecular and biological characterization of fibroblast growth factor, an angiogenic factor which also controls the proliferation and differentiation of mesoderm and neuroectoderm-derived cells. *Cell Differ.* 1986;19:1–17.
35. Harper JW, Strydom DJ, Lobb RR. Human class 1 heparin-binding growth factor: structure and homology to bovine acidic brain fibroblast growth factor. *Biochemistry* 1986;25:4097–4103.
36. Huang JS, Huang SS, Kue M-D. Bovine brain-derived growth factor: purification and characterization of its interaction with responsive cells. *J. Biol. Chem.* 1986;261:11600–11607.
37. Jakobovits A, Shackleford GM, Varmus HE, Martin GR. Two protooncogenes implicated in mammary carcinogenesis, *int-1* and *int-2*, are independently regulated during mouse development. *Proc. Natl. Acad. Sci. USA* 1986;83:7806–7810.
38. Jaye M, Howk R, Burgess W, Ricca GA, Chiu I-M, Ravera MW, O'Brien SJ, Modi WS, Maciag T, Drohan WN. Human endothelial cell growth factor: cloning, nucleotide sequence, and chromosomal localization. *Science* 1986;233:541–545.
39. Jaye M, Lyall RM, Mudd R, Schlessinger J, Sarver N. Expression of acidic fibroblast growth factor cDNA confers growth advantage and tumorigenesis to Swiss 3T3 cells. *EMBO J.* 1988;7:963–969.
40. Kimelman D, Abraham JA, Haaparanta T, Palisi TM, Kirschern MW. The presence of fibroblast growth factor in the frog egg: its role as a natural mesoderm inducer. *Science* 1988;242:1053–1056.
41. Kurokawa T, Sasada R, Iwane M, Igarashi K. Cloning and expression of cDNA encoding human basic fibroblast growth factor. *FEBS Lett.* 1987;213:189–194.
42. Levi-Montalcini R. The nerve growth factor 35 years later. *Science* 1987;237:1154–1162.
43. Libermann TA, Friesel R, Jaye M, Lyall RM, Westermark B, Drohan W, Schmidt A, Maciag T, Schlessinger J. An angiogenic growth factor is expressed in human glioma cells. *EMBO J.* 1987;6:1627–1632.
44. Lipton SA, Wagner JA, Madison RD, D'Amore PA. Acidic fibroblast growth factor enhances regeneration of processes by postnatal mammalian retinal ganglion cells in culture. *Proc. Natl. Acad. Sci. USA* 1988;85:2388–2392.
45. Lobb RR, Alderman EM, Fett JW. Induction of angiogenesis by bovine brain class 1 heparin-binding growth factor. *Biochemistry* 1985;24:4969–4973.
46. Lobb RR, Harper JW, Fett JW. Purification of heparin-binding growth factors. *Anal. Biochem.* 1986;154:1–14.

47. Lobb RR, Rybak SM, St. Clair DK, Fett JW. Lysates of two established human tumor lines contain heparin-binding growth factors related to bovine acidic fibroblast growth factor. *Biochem. Biophys. Res. Commun.* 1986;139:861–867.
48. Lobb R, Sasse J, Sullivan R, Shing Y, D'Amore P, Jacobs J, Klagsbrun M. Purification and characterization of heparin-binding endothelial cell growth factors. *J. Biol. Chem.* 1986;261:1924–1928.
49. Lutty GA, Chandler C, Bennett A, Fait C, Patz A. Presence of endothelial cell growth factor activity in normal and diabetic eyes. *Curr. Eye Res.* 1986;5:9–17.
50. Maciag T, Mehlman T, Freisel R, Schreiber AB. Heparin binds endothelial cell growth factor, the principal endothelial cell mitogen in bovine brain. *Science* 1984;225:932–935.
51. Marics I, Adelaide J, Raybaud F, Mattei MG, Coulier F, de Planche J, Lapeyriere O, Birnbaum D. Characterization of the *hst*-related FGF-6 gene, a new member of the fibroblast growth factor gene family. *Oncogene* 1989;4:335–340.
52. Moore R, Casey G, Brookes S, Dixon M, Peters G, Dickson C. Sequence, topography, and protein coding potential of mouse *int-2*: a putative oncogene activated by mouse mammary tumour virus. *EMBO J.* 1986;5:919–924.
53. Morrison RS, Sharma A, de Vellis J, Bradshaw RA. Basic fibroblast growth factor supports the survival of cerebral cortical neurons in primary culture. *Proc. Natl. Acad. Sci. USA* 1986;83:7537–7541.
54. Moscatelli D, Joseph-Silverstein J, Manejas R, Rifkin DB. Mr 25,000 heparin-binding protein from guinea pig brain is a high molecular weight form of basic fibroblast growth factor. *Proc. Natl. Acad. Sci. USA* 1987;84:5778–5782.
55. Neufeld G, Gospodarowicz D. Basic and acidic fibroblast growth factors interact with the same cell surface receptors. *J. Biol. Chem.* 1986;261:5631–5637.
56. Neufeld G, Gospodarowicz D, Dodge L, Fujii DK. Heparin modulation of the neurotropic effects of acidic and basic fibroblast growth factors and nerve growth factor on PC12 cells. *J. Cell. Physiol.* 1987;131:131–140.
57. Neufeld G, Mitchell R, Ponte P, Gospodarowicz D. Expression of human basic fibroblast growth factor cDNA in baby hamster kidney-derived cells results in autonomous cell growth. *J. Cell. Biol.* 1988;106:1385–1394.
58. Nguyen C, Roux D, Mattei M-G, de Lapeyriere O, Goldfarb M, Birnbaum D, Jordan BR. The FGF-related oncogenes *hst* and *int-2*, and the *bcl-1* locus are contained within one megabase in band q13 of chromosome 11, while the *fgf-5* oncogene maps to 4q21. *Oncogene* 1988;3:703–708.
59. Nimer SD, Golde DW. The 5q<sup>−</sup> abnormality. *Blood* 1987;70:1705–1712.
60. Olwin BB, Hauschka SD. Identification of the fibroblast growth factor receptor of Swiss 3T3 cells and mouse skeletal muscle myoblasts. *Biochemistry* 1986;25:3487–3492.
61. Pawlowski NA, Abraham EL, Pontier S, Scott WA, Cohn ZA. Human monocyte-endothelial cell interaction *in vitro*. *Proc. Natl. Acad. Sci. USA* 1985;82:8208–8212.
62. Polverini PJ, Cotran RS, Gimbrone MA Jr, Unanue ER. Activated macrophages induce vascular proliferation. *Nature* 1977;269:804–806.
63. Prats H, Kaghad M, Prats AC, Klagsbrun M, Lelias JM, Liauzun PL, Chalon P, Tauber JP, Amalric F, Smith JA, Caput D. High molecular mass forms of basic fibroblast growth factor are initiated by alternative CUG codons. *Proc. Natl. Acad. Sci. USA* 1989;86:1836–1840.
64. Ricketts MH, Levinson AD. High level expression of c-H-ras 1 fails to fully transform Rat-1 cells. *Mol. Cell. Biol.* 1988;8:1460–1468.

65. Rogelj S, Weinberg RA, Fanning P, Klagsbrun M. Basic fibroblast growth factor fused to a signal peptide transforms cells. *Nature* 1988;331:173–175.
66. Rubin JS, Osada H, Finch PW, Taylor WG, Rudikoff S, Aaronson SA. Purification and characterization of a newly identified growth factor specific for epithelial cells. *Proc. Natl. Acad. Sci. USA* 1989;86:802–806.
67. Sasada R, Kurokawa T, Iwane M, Igarashi K. Transformation of mouse BALB/c 3T3 cells with human basic fibroblast growth factor cDNA. *Mol. Cell. Biol.* 1988;8:588–594.
68. Sherr C, Rettenmier CW, Sacca R, Roussel MF, Look AT, Stanley ER. The *c-fms* proto-oncogene product is related to the receptor for the mononuclear phagocyte growth factor, CSF-1. *Cell* 1985;41:665–676.
69. Shing Y, Folkman J, Sullivan R, Butterfield C, Murray J, Klagsbrun M. Heparin affinity: purification of a tumor-derived capillary endothelial cell growth factor. *Science* 1984;223:1296–1299.
70. Slack J, Darlington B, Heath JK, Godsave SF. Mesoderm induction in early *Xenopus* embryos by heparin-binding growth factors. *Nature* 1987;326:197–200.
71. Sommer A, Brewer MT, Thompson RC, Moscatelli D, Presta M, Rifkin DB. A form of human basic fibroblast growth factor with an extended amino terminus. *Biochem. Biophys. Res. Commun.* 1987;144:543–550.
72. Thomas KA, Gimenez-Gallego G. Fibroblast growth factors: broad spectrum mitogens with potent angiogenic activity. *Trends Biochem. Sci.* 1986;11:81–84.
73. Thomas KA, Rios-Candelore M, Gimenez-Gallego G, DiSalvo J, Bennett C, Rodkey J, Fitzpatrick S. Pure brain-derived acidic fibroblast growth factor is a potent angiogenic vascular endothelial cell mitogen with sequence homology to interleukin 1. *Proc. Natl. Acad. Sci. USA* 1985;82:6409–6413.
74. Togari A, Dickens G, Kuzuya H, Guroff G. The effect of fibroblast growth factor on PC12 cells. *J. Neurosci.* 1985;5:307–316.
75. Trowell OA, Chir B, Willmer EN. Studies on the growth of tissues *in vitro*: VI. The effects of some tissue extracts on the growth of periosteal fibroblasts. *J. Exp. Biol.* 1939;16:60–70.
76. Wagner JA, D'Amore PA. Neurite outgrowth induced by an endothelial cell mitogen isolated from retina. *J. Cell. Biol.* 1986;103:1363–1367.
77. Walicke P, Cowan WM, Ueno N, Baird A, Guillemin R. Fibroblast growth factor promotes survival of dissociated hippocampal neurons and enhances neurite extension. *Proc. Natl. Acad. Sci. USA* 1986;83:3012–3016.
78. Wang W-P, Lehtoma K, Varban ML, Krishnan I, Chiu I-M. Cloning of the gene coding for human class 1 heparin-binding growth factor (HBGF-1) and its expression in fetal tissues. *Mol. Cell. Biol.* 1989;9:2387–2395.
79. Wilkinson DG, Peters G, Dickson C, McMahon AP. Expression of the FGF-related protooncogene *int-2* during gastrulation and neurulation in the mouse. *EMBO J.* 1988;7:691–695.
80. Winkles JA, Friesel R, Burgess WH, Howk R, Mehlman T, Weinstein R, Maciag T. Human vascular smooth muscle cells both express and respond to heparin-binding growth factor I (endothelial cell growth factor). *Proc. Natl. Acad. Sci. USA* 1987;84:7124–7128.
81. Yoshida T, Miyagawa K, Odagiri H, Sakamoto H, Little PFR, Terada M, Sugimura T. Genomic sequence *hst*, a transforming gene encoding a protein homologous to

- fibroblast growth factors and the *int-2*-encoded protein. *Proc. Natl. Acad. Sci. USA* 1987;84:7305–7309.
- 82. Yoshida MC, Wada M, Satoh H, Yoshida T, Sakamoto H, Miyagawa K, Yokota J, Koda T, Kakinuma M, Sugimura T, Terada M. Human HST1(HSTF1) gene maps to chromosome band 11q13 and coamplifies with the *int-2* gene in human cancer. *Proc. Natl. Acad. Sci. USA* 1988;85:4861–4864.
  - 83. Zhan X, Bates B, Hu X, Goldfarb M. The human FGF-5 oncogene encodes a novel protein related to fibroblast growth factors. *Mol. Cell. Biol.* 1988;8:3487–3495.
  - 84. Zhan X, Culpepper A, Reddy M, Loveless J, Goldfarb M. Human oncogenes detected by a defined medium culture assay. *Oncogene* 1987;1:369–376.

# Biological and Immunochemical Properties of Recombinant Human NGF

L. Callegaro, E. Bigon, G. Vantini, A. Di Martino, S.D. Skaper,  
A. Leon and G. Toffano

*FIDIA Research Laboratories  
Via Ponte della Fabbrica 3/A  
35031 Abano Terme (PD) Italy*

**B**iotechnology provides powerful new tools by which to confront a wide range of human disorders—such as neurodegenerative diseases—for which few means of treatment exist. One such group of therapeutic agents may be neuronotrophic factors, proteins which play a fundamental role in the development and maintenance of neurons. Nerve Growth Factor (NGF) represents the first, and best characterized example of a neuronotrophic factor. Originally identified in mouse sarcoma (12), NGF has been purified from many sources including submandibular salivary glands of adult male mice (2,18), snake venom (1), guinea pig prostate (6), and human placenta (4,19).

The biological activity of NGF is associated exclusively with the  $\beta$  subunit ( $\beta$ NGF), also known as 2.5S NGF, which stimulates the differentiation and survival of both sensory and sympathetic neurons (5,13,16) and appears to affect certain populations of cholinergic neurons in the central nervous system. In several animal models, intraventricular infusion of NGF has been shown to prevent the loss of cholinergic neurons after septo-hippocampal lesions (7,9,20) and to restore cognitive function in aged rats when brain levels of NGF were reduced (3,11). Some investigators have proposed that Alzheimer's disease, which affects similar neuronal populations, results from deficient supplies of NGF (8). The investigation and treatment of a possible NGF deficit in Alzheimer's disease and in brain aging requires the availability of cDNA probes and biotechnology applicable to large scale production.

The human  $\beta$ NGF gene has been isolated and cloned (17). The synthesis of a gene and its insertion in an expression vector should, in principle, allow for the production of proteins as desired for biological and chemical analysis or for pharmacological use. However, the production of a protein which is identical to its naturally occurring prototype requires the same amino acid sequence, as well as correct intra- and inter-chain bonds to achieve the proper 3-D conformation. While recombinant human  $\beta$ NGF can be produced in large quantities, studies of its struc-

ture and function may reveal problems of molecular design inherent in the process of synthesis. As a first step in evaluating the potential for pharmacological application of recombinant human  $\beta$ NGF, we have partially purified and characterized  $\beta$ NGF from human placenta, and compared its immunoreactive characteristics with those of recombinant human  $\beta$ NGF (rh $\beta$ NGF) and mouse  $\beta$ NGF (m $\beta$ NGF).

## EXPERIMENTAL PROCEDURE

### **Human Placental NGF Purification**

The preliminary isolation steps are based on methods previously developed for mouse  $\beta$ NGF (14). To obtain the dissociated  $\beta$  subunit, human placental tissue was applied to an acidified carboxymethyl-cellulose column, dialyzed against 2 liters of 0.05 M sodium acetate, pH 5.5, and applied to a cationic exchange Mono S column (HR 5/5, Pharmacia, Sweden) equilibrated in the same buffer. Elution was carried out using a gradient of 0-1.0 M NaCl in 50 mM sodium acetate, pH 6.6, at a flow rate of 1 ml/min. The protein fractions associated with the biological activity were pooled, dialyzed against 0.05 M sodium acetate containing 0.1 M NaCl, pH 5.0, and stored in aliquots at  $-80^{\circ}\text{C}$  until used. The purity of human placental  $\beta$ NGF (h $\beta$ NGF) was evaluated under nonreducing conditions by SDS slab gel electrophoresis (10) with minor modifications.

### **Immunoblotting**

Immunoblotting was carried out with a Phastsystem™ instrument (Pharmacia, Sweden). The nitrocellulose strip containing the protein blots was incubated for 1 hr in a Petri dish at room temperature in 20 mM Tris-HCl, 0.15 M NaCl, 5% bovine serum albumin, 0.1% sodium azide, pH 8.2. This strip was then washed three times for 5 min in 20 mM Tris-HCl, pH 8.2, 0.15 M NaCl, 0.1% sodium azide and incubated for 2 hr with 5.7  $\mu\text{g}$  rabbit IgG anti-mouse  $\beta$ NGF affinity-purified polyclonal antibodies diluted in the same buffer. The membrane was again washed extensively and incubated for 2 hr with 500  $\mu\text{l}$  of gelatin and 100  $\mu\text{l}$  of Auroprobe™ BL plus goat antirabbit immunogold reagent (Janssen, Belgium). The membrane was washed and color developed by means of an IntentSE™ silver enhancement kit (Janssen).

### **Biological Assay**

Six millimeter microwells in 96-well culture plates were first incubated 1 hr at room temperature with 50  $\mu\text{l}$  polyornithine (0.1 mg/ml in 15 mM borate buffer,

pH 8.4), followed by two 50  $\mu$ l washes with distilled water. Wells were then exposed to 50  $\mu$ l of a 1  $\mu$ g/ml laminin solution in phosphate buffered saline (PBS) overnight at 37°C, followed by one 50  $\mu$ l wash with PBS. Rows of microwells were filled with twofold dilutions of the IgG to be tested. Dilutions were carried out in culture medium consisting of Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal calf serum (FCS). Where indicated, human NGF was also present and was incubated with IgG for 2 hr at 37°C prior to addition of cells. Control wells were incubated with human NGF and IgG preparations obtained from preimmune rabbit serum.

Neurons were obtained from dorsal root ganglia (DRG) of day 8 (E8) chick embryo, as described (15). In brief, ganglia were removed from white leghorn chick embryos, trypsinized, dissociated in DMEM + 10% FCS and purified by a pre-plating step. Enriched neurons were dispersed in culture medium, and 50  $\mu$ l of cell suspension (2,000 neurons) added to microwells containing 50  $\mu$ l of test agent in medium, as described above. After 24 hr in a humidified 5% CO<sub>2</sub>-95% air incubator at 37°C, the cultures were fixed with 2% glutaraldehyde and the number of surviving neurons evaluated by phase-contrast microscopy.

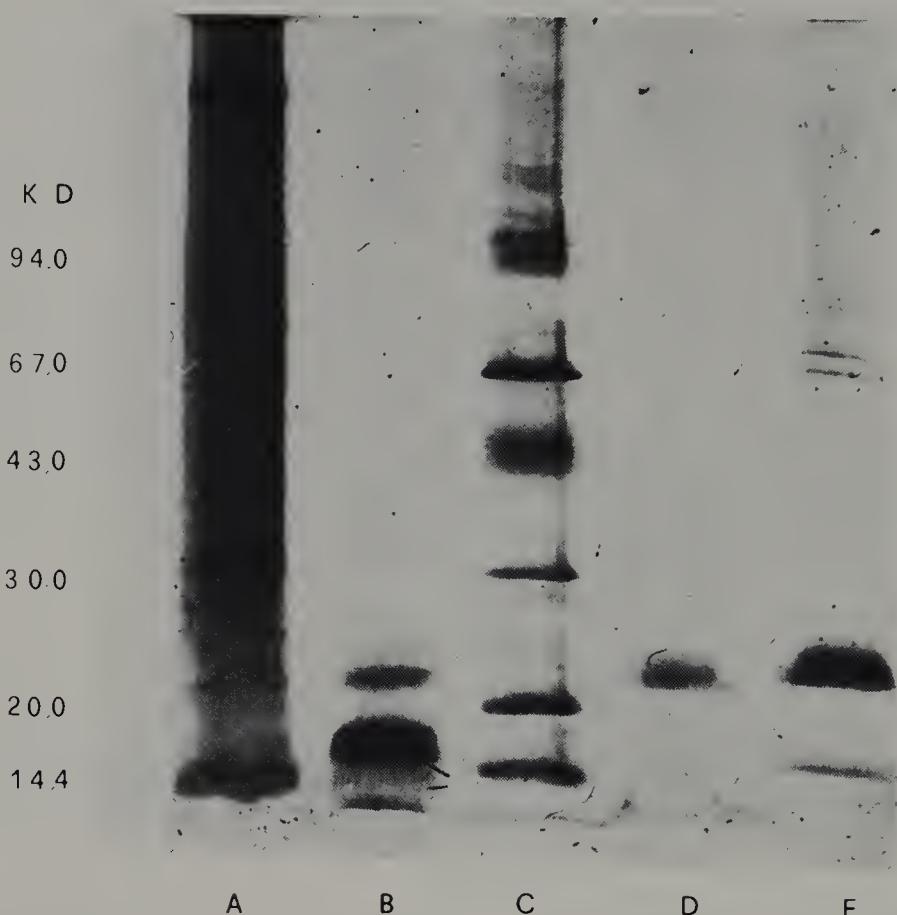
### NGF from Recombinant DNA

The human  $\beta$ NGF gene was isolated using mouse  $\beta$ NGF complementary DNA; *E. coli* was used as the expression vector as described (17).

## RESULTS AND DISCUSSION

The chemical, immunochemical, and biological characteristics of human placental NGF were compared with m $\beta$ NGF and rh $\beta$ NGF. SDS gel electrophoresis of highly purified rhNGF showed a band with a molecular weight of approximately 25.0 kDa similar to m $\beta$ NGF. The partially purified h $\beta$ NGF showed a diffuse band in the range of 24.0–25.0 kDa (Fig. 1). The band at 25 kDa corresponds to the dimeric subunit of  $\beta$ NGF. For further characterization, the rh $\beta$ NGF and m $\beta$ NGF were reduced using beta-mercaptoethanol and evaluated by SDS gel electrophoresis. Under these conditions, both the mouse and recombinant NGF species showed bands of approximately 13 kDa (Fig. 2). In addition, the immunochemical characteristics of the three different  $\beta$ NGF preparations were evaluated by immunoblot techniques. The h $\beta$ NGF and rh $\beta$ NGFs displayed antigenic similarities to m $\beta$ NGF, partially crossreacting with antimouse  $\beta$ NGF affinity purified polyclonal antibodies (Fig. 3).

Biologically, recombinant and placental human  $\beta$ NGFs stimulated neurite outgrowth from DRG neurons when added to their culture medium. The concentration of NGF necessary for half-maximal (ED50) and maximal (ED100) effects are reported in Table 1. The rh $\beta$ NGF displayed biological activity similar to that of



**FIG. 1.** Chemical characterization of nerve growth factor ( $\beta$  subunit) by SDS-slab gel electrophoresis. (A) Partially purified h $\beta$ NGF from placental tissue. (B) Recombinant h $\beta$ NGF. (C) Molecular weight protein standards (phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor,  $\alpha$ -lactalbumin). (D) Highly purified rh $\beta$ NGF. (E) Murine  $\beta$ NGF.

m $\beta$ NGF. Human placental NGF had a specific activity approximately 1% that of mouse NGF, a reflection of the inherent impurity of the human protein preparation.

In addition, the immunoreactivity of the three NGF species was compared by the addition of affinity-purified antimouse NGF IgG to the NGF-enriched culture media. The results are summarized in Table 2. Antimouse NGF IgG blocked the biological activities of m $\beta$ NGF, rh $\beta$ NGF, and h $\beta$ NGF, the latter with an ID<sub>50</sub> at least 10 times that of the other NGF proteins. These data demonstrate the limited biological immunoreactivity of the h $\beta$ NGF, a characteristic that appears to be intrinsic to this NGF species.

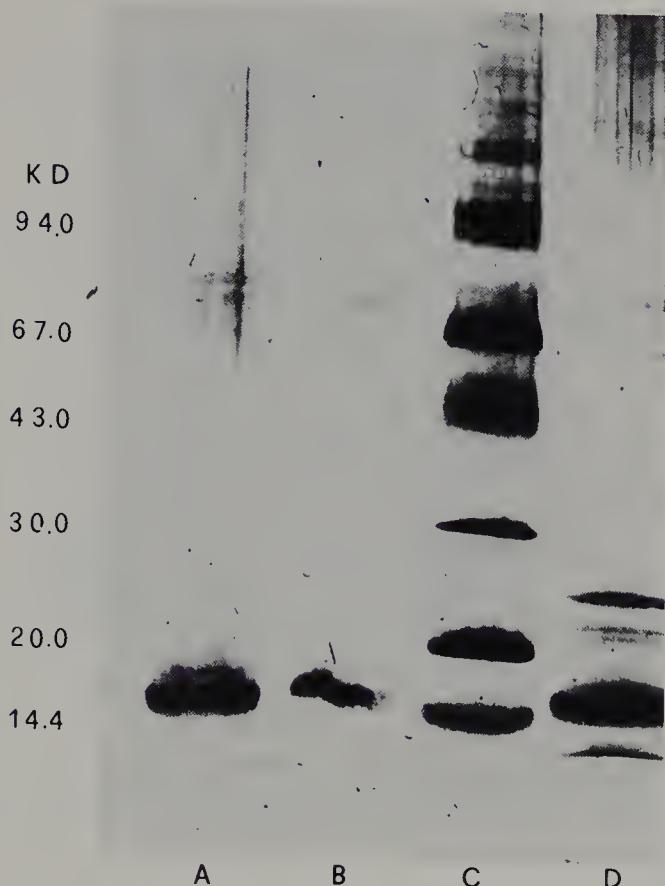
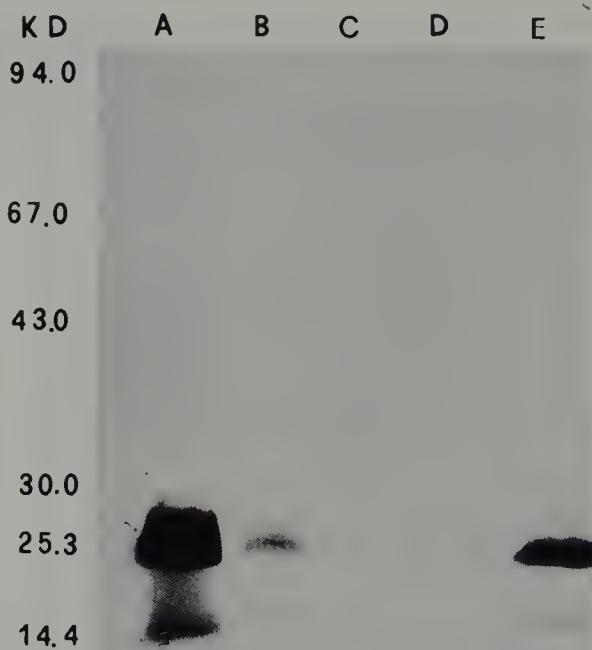


FIG. 2. Chemical characterization of nerve growth factors ( $\beta$  subunit) reduced by  $\beta$ -mercaptoethanol. (A) Murine  $\beta$ NGF. (B) Recombinant h $\beta$ NGF. (C) Molecular weight protein standard. (D) Recombinant h $\beta$ NGF before reduction with  $\beta$ -mercaptoethanol.

## CONCLUSION

The highly purified rh $\beta$ NGF preparation contained almost exclusively a 25.0 kDa species and exhibited trophic influence on dissociated sensory ganglion neurons *in vitro*. Though both rh $\beta$ NGF and h $\beta$ NGF molecules crossreacted to anti-m $\beta$ NGF IgG, h $\beta$ NGF appeared to be intrinsically less immunoreactive. This divergence of immuno-crossreactivity between the two human NGF molecules suggests differences in polypeptide conformation. For  $\beta$ NGF, a dimer with three interchain disulfide bonds, the altered folding of a recombinant DNA-derived product may compromise biological activity, stability, and immunoreactivity. Rigorous char-



**FIG. 3.** Immunoblot with affinity purified IgG anti-m $\beta$ NGF. (A) Murine  $\beta$ NGF. (B) Human  $\beta$ NGF. (C) Cytochrome C. (D) Human albumin. (E) Highly purified rh $\beta$ NGF.

**TABLE 1.** Biological activity of the diverse NGF species in fetal chick dissociated DRG neurons *in vitro*

Addition	ng/ml	
	ED50 ng/ml	ED100 ng/ml
Murine NGF	0.035	0.40
Recombinant NGF	0.05	0.6
Placental NGF	3.0	30.0

See text for materials and methods utilized. Biological activity was determined by assessing the number of surviving neurons possessing neurites after 24 hr *in vitro*.

**TABLE 2.** Titration of the biological immunoreactivity of the diverse NGF species following addition of high affinity purified anti-murine NGF antibodies to fetal chick dissociated DRG neurons *in vitro*

Addition	ID50 ng/ml
Murine NGF	10.0
Recombinant NGF	9.5
Placental NGF	>100.0

See Fig. 1 and note that the three NGF species were added to the cultures at concentrations yielding near-maximal (half-maximal for rhNGF) responses in the absence of antibody.

acterization of the active forms of native human NGF and the recombinant DNA derived product is essential to define the potential pharmacological applicability of the latter.

## ACKNOWLEDGMENTS

The authors wish to thank Genentech Inc. for the supply of recombinant human NGF used throughout the experiments.

## REFERENCES

1. Angeletti RH. Nerve growth factor from cobra venom. *Proc. Natl. Acad. Sci. USA* 1970;65:668-674.
2. Bocchini V, Angeletti PU. The nerve growth factor: purification as a 30,000-molecular-weight protein. *Proc. Natl. Acad. Sci. USA* 1969;64:787-794.
3. Fischer W, Wictorin K, Björklund A, Williams LR, Varon S, Gage FH. Intracerebral infusion of nerve growth factor ameliorates cholinergic neuron atrophy and spatial memory impairments in aged rats. *Nature* 1987;339:65-68.
4. Goldstein LD, Reynolds CP, Perez-Polo JR. Isolation of human nerve growth factor from parenteral tissue. *Neurochem. Res.* 1978;3:175-183.
5. Greene LA, Shooter EM. Nerve growth factor: biochemistry, synthesis and mechanism of action. *Annual Revue Neurosci.* 1980;3:353-402.
6. Harper GP, Barde YA, Burnstock G, Carstairs JE, Dennison ME, Suda K, Vernon C. Guinea pig prostate is a rich source of nerve growth factor. *Nature* 1979;279:160-162.

7. Hefti F. Nerve growth factor promotes survival of septal cholinergic neurons after fimbrial transections. *J. Neurosci.* 1986;6:2155–2162.
8. Hefti F, Weiner WJ. Nerve growth factor and Alzheimer's disease. *Ann. Neurol.* 1986; 20:275–281.
9. Kromer LF. Nerve growth factor treatment after brain injury prevents neuronal death. *Science* 1987;235:214–216.
10. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T<sub>4</sub>. *Nature* 1970;227:680–685.
11. Lärkfors L, Ebendal T, Whittemore SR, Persson H, Hoffler B, Olson L. Decreased levels of nerve growth factor (NGF) and its messenger RNA in the aged rat brain. *Mol. Brain Res.* 1987;3:55–60.
12. Levi-Montalcini R. The nerve growth factor. *Proc. NATO Adv. Study Invest.* 1966; 1:385–388.
13. Levi-Montalcini R, Hamburger U. Selective growth-stimulating effects of mouse sarcoma on the sensory and sympathetic nervous system of the chick embryo. *J. Exp. Zool.* 1951;116:321–361.
14. Mobley WC, Schenker A, Shooter EM. Characterization and isolation of proteolytically modified nerve growth factor. *Biochemistry* 1976;15:5543–5552.
15. Skaper SD, Varon S. Age-dependent control of dorsal root ganglion neuron survival by macromolecular and low molecular weight trophic agents and substratum-bound laminins. *Dev. Brain Res.* 1985;24:39–46.
16. Thoenen H, Barde YA. Physiology of nerve growth factor. *Physiol. Rev.* 1980;60:1284–1335.
17. Ullrich A, Gray A, Berman C, Dull TJ. Human β-nerve growth factor gene sequence highly homologous to that of mouse. *Nature* 1983;303:821–825.
18. Varon S, Nomura J, Shooter EM. The isolation of the mouse growth factor protein in a high molecular weight form. *Biochemistry* 1967;6:2202–2209.
19. Walker P, Weichsel ME Jr, Fischer DA. Human nerve growth factor: lack of immunoreactivity with mouse nerve growth factor. *Life Sci.* 1980;26:195–200.
20. Williams LR, Varon S, Peterson GM, Wictorin K, Fischer W, Bjöklund A, Gage FH. Continuous infusion of nerve growth factor prevents forebrain neuronal death after fimbrial-fornix transection. *Proc. Natl. Acad. Sci. USA* 1986;83:9231–9235.

## II. Trophic Factor Receptors



# Regulation of Nerve Growth Factor Gene Expression: *In vitro* and *In vivo* Studies

Italo Mocchetti,<sup>\*</sup> Roberto Dal Toso,<sup>\*\*</sup> Alexander G. Yakovlev,<sup>\*</sup>  
Maria A. De Bernardi<sup>\*</sup>,<sup>\*\*\*</sup> and Michele Fabrazzo<sup>\*</sup>

<sup>\*</sup>Department of Anatomy and Cell Biology, <sup>\*\*</sup>FIDIA-Georgetown Institute for the  
Neurosciences, and <sup>\*\*\*</sup>Department of Biochemistry and Molecular Biology,  
Georgetown University School of Medicine, Washington, D.C. 20007

**S**everal studies have indicated that the development and maintenance of neurons are controlled by a variety of trophic factors interacting with each other. These factors, probably released by target innervated cells, play a significant role in directing functional activities of neuronal cells, including intrinsic mechanisms for cell differentiation and contact-mediated cell interactions. The most well-characterized neuronal trophic factor is the beta-nerve growth factor (NGF). NGF has been shown to be a target-derived polypeptide which is taken up by nerve terminals and retrogradely transported to the nucleus where it enhances the growth and development of sympathetic and sensory neurons (15,23,33). Furthermore, the recent discovery in brain of NGF and NGF receptor mRNA molecules provided supporting evidence for a role of NGF in the central nervous system (CNS) (2,22,32,34), as previously suggested on the basis of *in vitro* studies on glial cells (27,28). In particular, *in vitro* and *in vivo* studies have demonstrated that NGF exerts trophic activity on cholinergic neurons of the basal forebrain (14,17,21,24,35). These and other evidences support the hypothesis that the response of the CNS to injury could be mediated by the release of trophic factors including NGF. Thus, alterations of NGF biosynthesis might be involved in the pathology of neurodegenerative disorders. To this end, it is important to identify in the adult brain those cells responsible for NGF production and to characterize transsynaptic mechanisms operative in the regulation of NGF gene expression.

## REGULATION OF NGF GENE EXPRESSION

Attempts to determine how the NGF gene expression is regulated in neuronal systems have been made by measuring NGF mRNA content. Ontogenetic studies

have indicated that the NGF gene is developmentally regulated in both the peripheral and central nervous system. These studies have shown a correlation between the onset of the expression of the NGF gene and the development of NGF-responsive neurons (18,22,31). However, NGF gene expression declines in the adult animal when the neuronal network is fully developed. These data suggest that NGF gene expression might be inhibited by a transsynaptic regulatory mechanism operative only during the adult life. The characterization of such a mechanism could aid in developing compounds able to increase NGF production. The clinical significance of these studies will be of utmost importance for the potential applications in the therapy of neurodegenerative disorders. These considerations brought us to investigate whether stimulation of specific neurotransmitter receptors could affect NGF gene expression.

### *In vitro Studies*

Neuronal-glial cell interactions are becoming the focus of attention for the role they play in the mechanisms of neuronal survival during development and differentiation, and in certain aspects of neuronal function of the adult nervous system. Glial cells may synthesize a variety of neurotrophic agents that subsequently interact with neuronal cells promoting maintenance and fiber outgrowth.

#### *Regulation of NGF Gene Expression in C6-2B Rat Glioma Cells*

The pioneering work of Schwartz and Costa has demonstrated that the stimulation of beta-adrenergic receptor (BAR) in C6 rat glioma cells increases the content and release of NGF (27,28). We used Northern blot hybridization analysis of poly(A)<sup>+</sup> RNA from C6 glioma (2B clone) cells (9) to assess whether the increase of NGF content elicited by BAR activation was due to the stimulation of NGF gene transcription. We found that the BAR-agonist isoproterenol (10 µM) increases NGF mRNA content within three hours (5,6). This increase is partially blocked by the BAR-antagonist propranolol (10 µM), but not by the alpha-adrenergic receptor antagonist phentolamine (10 µM) (Table 1). Pulses of isoproterenol (10 µM) lasting 10 and 20 min, followed by a washout period, produced a 1.6- to 2.4-fold increase in the NGF mRNA content measured after 3 hr (6). Hence, a brief activation of BAR is sufficient to trigger those mechanisms responsible for the increased production of NGF.

In glioma cells, BAR-agonists produce a dose and time dependent increase of the intracellular cAMP level by activating the catalytic subunit of adenylate cyclase (10,12). Therefore, we hypothesized that the increase of NGF gene expression could be regulated by the intracellular cAMP content. To test this hypothesis, a cell permeable analogue of cAMP, dibutyryl cAMP (Bt<sub>2</sub>-cAMP), was used. The

**TABLE 1.** Increase of NGF mRNA content by isoproterenol and PGE<sub>1</sub> in C6-2B cells

	Dose ( $\mu$ M)	NGF mRNA (arbitrary units)
No treatment		1
ISO	10	4.1 $\pm$ 0.8**
ISO + Propranolol	10 + 10	2.5 $\pm$ 0.4*
ISO + Phentolamine	10 + 10	4.0 $\pm$ 0.6**
Bt <sub>2</sub> -cAMP	1000	3.9 $\pm$ 0.9**
PGE <sub>1</sub>	10	1.7 $\pm$ 0.2
PGE <sub>1</sub> + IBMX	10 + 100	3.0 $\pm$ 0.2**
ISO + IBMX	10 + 100	4.8 $\pm$ 0.6**

C6-2B cells were incubated for 3 hr with the indicated drugs. RNA blot preparation was done as described previously (6). Hybridization of poly(A)<sup>+</sup> RNA was carried out using a high specific activity ( $10^9$  cpm/ $\mu$ g RNA) complementary NGF RNA probe (6). Quantization of the relative amount of NGF mRNA detected in the blots is estimated in arbitrary units that are calculated by dividing the peak densitometry area of NGF mRNA hybridization by the peak densitometry area of cyclophilin (7) mRNA hybridization. Cyclophilin is a non-regulated structural protein. The value of NGF mRNA from control has been arbitrarily set equal to 1 and other samples calculated relative to it. Data are expressed as mean  $\pm$  S.D. of three separate experiments. (\* p < 0.05 vs control, \*\* p < 0.01 vs control, Anova with Dunnett's test).

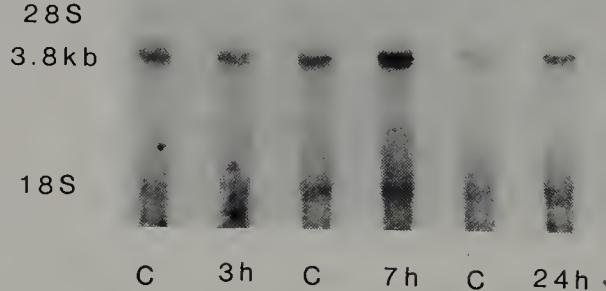
addition of Bt<sub>2</sub>-cAMP (1 mM) to C6-2B cells for 3 hr increased NGF mRNA content by an extent similar to that obtained with isoproterenol (Table 1).

In C6-2B cells, the stimulation of prostaglandin E receptors also leads to cAMP accumulation (6,12). PGE<sub>1</sub> (10  $\mu$ M) was used to further determine the role of cAMP in the regulation of NGF gene expression. The addition of PGE<sub>1</sub> to C6-2B cultures increased NGF mRNA, although to an extent smaller than that elicited by isoproterenol (Table 1). These data are consistent with the smaller increase in cellular cAMP content induced by PGE<sub>1</sub> when compared with the response elicited by isoproterenol (6). The intensity of the cAMP response may be related either to the relatively low density of PGE receptors present in these cells, as compared with the BAR, or to a difference in the coupling efficiency of PGE and BAR to adenylate cyclase. However, a greater increase of cAMP level is obtained by coincubating the cells with PGE<sub>1</sub> and 3-isobutyl,1-methylxanthine (IBMX) (100  $\mu$ M) (6), a phosphodiesterase inhibitor. Under these conditions, the effect of PGE<sub>1</sub> on NGF mRNA content was potentiated (Table 1). These results further indicate that intracellular cAMP is an important regulatory second messenger affecting NGF mRNA content. However, the five-fold increase of NGF mRNA level elicited by isoproterenol appears to be the maximum effect obtained. When cells were coincubated with a maximal dose of isoproterenol (10  $\mu$ M) and IBMX (100  $\mu$ M), no significant increase in NGF mRNA content, over that elicited by isoproterenol alone, was observed (Table 1).

### Regulation of NGF Receptor mRNA in C6-2B Rat Glioma Cells

A very close relationship exists between NGF and NGF receptor (NGFR). NGF exerts its trophic effect after being internalized and retrogradely transported to the neuronal cell body as NGF-receptor complex (19,30,33). Thus, to enhance NGF biological activity, it is crucial to increase the rate of both NGF and NGFR production. This hypothesis was tested by measuring NGFR mRNA content in C6-2B cells after isoproterenol incubation.

A single strand DNA complementary to NGFR mRNA was used to hybridize poly(A)<sup>+</sup> RNA from C6-2B cells. This probe detected an mRNA species of approximately 3.8 kb in accordance with the size of the NGFR mRNA previously reported (3,26). Thus, glioma cells are capable of synthesizing NGFR, supporting the suggestion that also non-neuronal cells can express NGFR (20). Incubation of C6-2B cells with isoproterenol (10  $\mu$ M) for 3 hr failed to change NGFR mRNA content, while a longer exposure to the agonist (7 and 24 hr) induced a two-fold increase of NGFR mRNA content (Fig. 1). The cAMP induced increase of NGFR mRNA was not due to a general increase of the total mRNA content since the mRNA for structural proteins, such as cyclophilin and calmodulin, failed to change. Thus, it appears that in the regulation of NGFR gene expression a cAMP-dependent mechanism might be involved. However, it is difficult to infer from these data whether cAMP is the only mechanism underlying the increase of NGFR mRNA



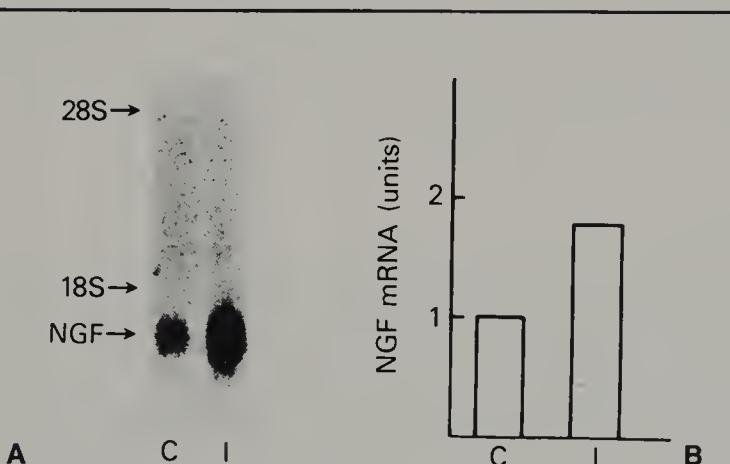
**FIG. 1.** Northern blot analysis of the isoproterenol-mediated increase of NGFR mRNA content, in C6-2B cells. C6-2B rat glioma cells were cultured as previously described (6). Isoproterenol incubation was performed at 3, 7 and 24 hr, at 37°C, in serum-free medium. Cells were then harvested and RNA was extracted (4). NGFR mRNA content was determined by Northern blot analysis on poly(A)<sup>+</sup> RNA using a single strand DNA probe constructed by subcloning a 700 bp NGFR cDNA (26) in M13mp18 vector.

since the accumulation of NGFR mRNA is preceded by the increase of NGF content. Therefore, the induction of NGFR gene could be triggered by the elevated NGF content in line with the evidence that NGF can stimulate transcription of a number of genes. This hypothesis must be further proven with more direct experiments (e.g., NGF treatment).

#### Regulation of NGF mRNA in Primary Culture of Rat Astrocytes

Primary cultures of astrocytes, prepared from cerebral cortex of 1-day-old rats, were used to study whether NGF gene expression could be regulated by BAR stimulation also in normal astrocytes.

Northern blot analysis of poly(A)<sup>+</sup> RNA from these cells revealed the presence of a NGF mRNA species with an apparent molecular weight of about 1.3 kb. The size of this NGF mRNA is similar to that detected in C6-2B cells and in brain (a band of approximately 1.7 kb can be also identified after a longer time exposure) further proving that, in the CNS, NGF can be synthesized by astroglial cells (1,11). Incubation with isoproterenol (10  $\mu$ M) for 3 hr elicited a 1.8-fold increase of NGF mRNA content (Fig. 2). As in C6 glioma cells, isoproterenol increased the intracellular cAMP level in primary cultures of astrocytes (*control* =  $20 \pm 2.5$



**FIG. 2.** Increase of NGF mRNA content by isoproterenol in primary culture of astrocytes. (A) Astrocyte cell cultures were made from cerebral cortex of 1-day-old Sprague Dawley rats. Confluent cells were incubated in serum-free conditioned medium with isoproterenol for 3 hr at 37°C. Medium was removed, cells were harvested, homogenized in guanidine isothiocyanate (4), and then processed for the determination of NGF and cyclophilin mRNA content as previously described (6). (B) Data are expressed in arbitrary units using p1B15 (7) mRNA densitometric area to correct for the relative amount of NGF mRNA detected in the blot. Control (C), isoproterenol (I).

$\text{pmol/mg prot., isoproterenol} = 200 \pm 30.6 \text{ pmol/mg prot.}$ ). Although preliminary, these data suggest that also in normal astrocytes, the NGF gene might be regulated by a cAMP-dependent mechanism similar to that observed in glioma cells.

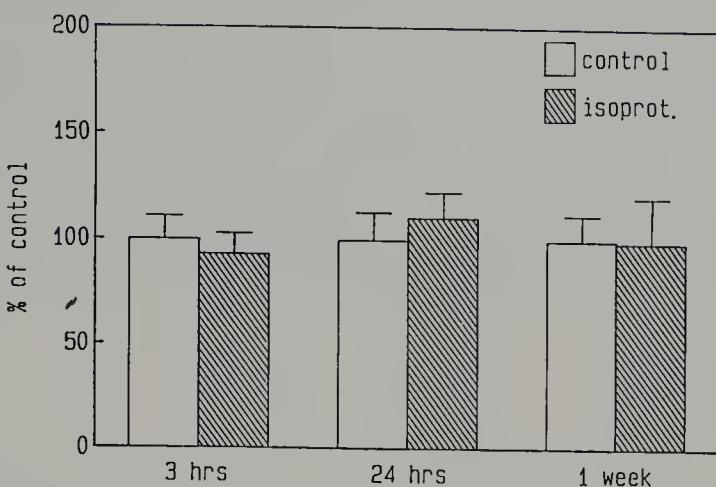
### Summary

In C6-2B rat glioma cells, synthesis and release of NGF are modulated by stimulation of specific neurotransmitter receptors coupled to adenylate cyclase. Therefore, NGF gene expression in cells derived from the neural plaque can be regulated by a cAMP-dependent mechanism. In primary cultures of astrocytes, a similar mechanism appears to be operative in the regulation of NGF gene expression. Two possibilities can be considered: After the increase of cAMP, the activated catalytic subunit of protein kinase A either translocates to the nucleus and stimulates gene expression (16) (through the phosphorylation of a nuclear regulatory protein) or phosphorylates a cytosolic transcription activating protein which, in turn, translocates to the nucleus. Previous studies, however, have reported that the increase of NGF does not require translocation of protein kinase (29). We are therefore considering the alternative possibility that the phosphorylation of a protein, which binds to a specific DNA sequence within the promoter or enhancer region of NGF gene, e.g., *c-fos*, (see Costa E., in this volume), increases the transcription rate for NGF gene. Future challenging research projects will certainly include the attempts to identify those specific DNA sequences within the NGF gene controlling the transcription rate of the gene itself.

### *In vivo* Studies

Although the data obtained from the *in vitro* studies suggest that a cAMP-dependent mechanism, coupled to a specific neurotransmitter receptor, is operative in the regulation of NGF gene expression, some basic questions still await answers: *Can a specific neuronal population control the expression and release of NGF from target cells? Can a primary neurotransmitter regulate NGF gene expression?* To answer these questions, we should use the *in vivo* model where the wide variety of neurotransmitter receptors and the heterogeneity of neuronal cells play critical roles in the modulation of NGF gene expression.

BAR stimulation was performed in adult and 21-day-old rats by an acute i.c.v. injection of isoproterenol ( $20 \mu\text{g}/5 \mu\text{l}$ ). This dose of isoproterenol has been chosen because it increases heart rate and blood pressure when injected acutely (8) and down-regulates the brain beta-2 adrenergic receptor subtype when given chronically (25). An acute dose of isoproterenol failed to change NGF mRNA content in cerebral cortex (Fig. 3), hippocampus, hypothalamus, cerebellum, brain stem, mid-brain, and striatum (data not shown) when rats were killed either 3 hr or 1 day



**FIG. 3.** NGF mRNA content in cerebral cortex of rats receiving isoproterenol. P-10 cannula was implanted i.c.v. in 21-day-old male Sprague Dawley rats. 24 hr after the surgery, isoproterenol (20 µg/5 µl) was infused through the cannula. Rats were sacrificed 3 or 24 hr after the injection. Another group of animals received isoproterenol once a day for one week and were sacrificed 24 hr after the last injection. Brain regions were dissected according to Glowinski and Iversen (13). Northern blot and hybridization analysis were carried out as previously described (6). Data are expressed as percentage of control and are the mean  $\pm$  S.D. of three separate experiments.

after the injection. From these data, it appears that a short stimulation of BAR cannot trigger those mechanisms responsible for the increase of NGF gene expression. Thus, we reasoned that to enhance NGF transcription, promoter and enhancer elements of the NGF gene must be activated repeatedly. Therefore, a daily injection of isoproterenol for 1 week was performed. This prolonged stimulation also failed to change NGF mRNA in any of the brain regions examined (Fig. 3) suggesting that, perhaps, *in vivo* BAR stimulation does not trigger NGF gene expression.

Several possibilities can explain the lack of effect of BAR stimulation in the *in vivo* studies. Among others, the most likely seems to be the fact that *in vivo* BAR activation is tonically modulated by noradrenaline whereas, *in vitro*, it is "artificially" modulated by isoproterenol since astrocytoma cells do not synthesize noradrenaline. Therefore, *in vitro*, the transduction signal at BAR and the subsequent chain of events leading to NGF regulation could be "up-regulated" by the presence of isoproterenol. Perhaps, to induce NGF gene expression *in vivo*, one should stimulate BAR localized only in NGF-containing cells or stimulate those receptors localized

in cells deprived of neurotransmitter because of nerve injury. This hypothesis is corroborated by the finding that a massive depletion of monoamine-containing terminals, induced by a subchronic treatment with reserpine, elicits a two-fold increase of NGF mRNA in specific brain areas (Fabrazzo et al., in preparation).

Another explanation should be considered. *In vivo*, connection and interaction of astroglia cells with neurons containing different neurotransmitters might play a key role in the induction of NGF gene. Therefore, the accumulation of intracellular cAMP, triggered by BAR stimulation, could activate a cascade of events not necessarily similar to those observed in cultures of a single cell population.

## CONCLUSIONS

In conclusion, NGF gene expression *in vitro* appears to be modulated by a cAMP-dependent mechanism. However, *in vivo*, the increase of intracellular cAMP by BAR stimulation failed to change NGF gene expression. Therefore, stimulation of BAR within a heterogeneous cell population, containing and releasing different neurotransmitters, does not necessarily result in the same biological effect as observed in a homogeneous population, such as astrocytes cultured in the absence of noradrenaline. These experiments further document the complexity of neuronal interactions. A better understanding of how these interactions can regulate NGF gene expression will help to gain insight into the pathology of neurodegenerative diseases.

## REFERENCES

1. Assouline JG, Bosch P, Lim R, Kim IS, Jensen R, Pantazis J. Rat astrocytes and Schwann cells in culture synthesize nerve growth factor-like neurite-promoting factors. *Dev. Brain Res.* 1987;31:103–118.
2. Buck CR, Martinez HJ, Black IB, Chao MV. Developmental regulated expression of the nerve growth factor receptor gene in the periphery and brain. *Proc. Natl. Acad. Sci. USA* 1987;84:3060–3063.
3. Chao MV, Bothewell MA, Ross H, Koprowski H, Lanahan AA, Buck R, Sehgal A. Gene transfer and molecular cloning of the human NGF receptor. *Science* 1986;232:518–521.
4. Chirgwin JM, Przybyla AE, MacDonald RJ, Rutter WJ. Isolation of biologically active ribonucleic acid from source enriched in ribonuclease. *Biochemistry* 1978;18:5294–5299.
5. Dal Toso R, De Bernardi MA, Costa E, Mocchetti I. Beta-adrenergic receptor regulation of NGF mRNA in rat C6-2B glioma cells. *Neuropharmacology* 1987;26:1783–1785.
6. Dal Toso R, De Bernardi MA, Brooker G, Costa E, Mocchetti I. Beta-adrenergic and prostaglandin receptor activation increases nerve growth factor mRNA content in C6-2B rat astrocytoma cells. *J. Pharmacol. Exp. Ther.* 1988;246:1190–1193.

7. Danielson PE, Forss-Petter S, Brown MA, Calavetta L, Douglass J, Milner RJ, Sutcliffe GJ. p1B15: A cDNA clone of the rat mRNA encoding cyclophilin. *DNA* 1988;4:261–267.
8. Day MD, Roach AG. Central alpha and beta-adrenoceptors modifying arterial blood pressure and heart rate in conscious cats. *Brit. J. Pharmacol.* 1974;51:315–333.
9. De Vellis J, Brooker G. Induction of enzymes of glucocorticoids and catecholamines in a rat glial cell line. In: Sato G, ed. *Tissue Cultures of the Nervous System*. New York: Plenum Press, 1973;231–245.
10. De Vellis J, Brooker G. Reversal of catecholamine refractoriness by inhibitors of RNA and protein synthesis. *Science* 1974;186:1221–1223.
11. Furukawa S, Furukawa Y, Satoyoshi E, Hayashi K. Synthesis and secretion of nerve growth factor is associated with cell growth in cultured mouse astroglial cells. *Biochem. Biophys. Commun. Res.* 1987;142:395–402.
12. Gilman A, Nirenberg M. Effect of catecholamines on the adenosine 3'5'-cyclic monophosphate concentration of clonal satellite cells of neurons. *Proc. Natl. Acad. Sci. USA* 1971;68:2165–2168.
13. Glowinski J, Iversen LL. Regional studies of catecholamines in rat brain. The disposition of <sup>3</sup>H-noradrenaline, <sup>3</sup>H-dopamine and <sup>3</sup>H-DOPA in various regions of the brain. *J. Neurochem.* 1966;13:655–669.
14. Gnahn H, Hefti F, Heumann R, Schwab ME, Thoenen H. NGF-mediated increase of choline acetyltransferase (ChAT) in the neonatal rat forebrain: evidence for a physiological role of NGF in the brain? *Dev. Brain Res.* 1983;9:45–52.
15. Greene LA, Shooter EM. The nerve growth factor: biochemistry, synthesis, and mechanism of action. *Ann. Rev. Neurosci.* 1980;3:353–402.
16. Guidotti A, Costa E. Trans-synaptic regulation of tyrosine 3-monophosphate biosynthesis in rat adrenal medulla. *Biochem. Pharmacol.* 1977;26:817–823.
17. Hefti F. Nerve growth factor promotes survival of septal cholinergic neurons after fimbrial transections. *J. Neurosci.* 1986;6:2155–2162.
18. Heumann R, Korschning S, Scott J, Thoenen H. Relationship between levels of nerve growth factor and its messenger RNA in sympathetic ganglia and peripheral target tissues. *EMBO J* 1984;3:3183–3189.
19. Johnson EM Jr, Andres RY, Bradshaw RA. Characterization of the retrograde transport of nerve growth factor using high specific activity (<sup>125</sup>I) nerve growth factor. *Brain Res.* 1978;150:319–331.
20. Johnson EM, Taniuchi M, Di Stefano P. Expression and possible function of nerve growth factor receptor on Schwann cells. *Trend. Neurosci.* 1988;11:299–304.
21. Kromer LF. Nerve growth factor treatment after brain injury prevents neuronal death. *Science* 1987;235:214–216.
22. Large TH, Bodary SC, Clegg DO, Weskamp G, Otten U, Reichardt LF. Nerve growth factor gene expression in the developing rat brain. *Science* 1986;234:352–355.
23. Levi-Montalcini R, Angeletti PV. Nerve growth factor. *Physiol. Rev.* 1968;48:534–569.
24. Mobley WC, Rutkowski LJ, Tennekoon GI, Buchanan K, Johnston MV. Choline acetyltransferase activity in striatum of neonatal rats increased by nerve growth factor. *Science* 1985;239:284–287.
25. Ordway GA, Gambara C, Frazer A. Quantitative autoradiography of central beta-adrenoceptor subtypes: comparison of the effects of chronic treatment with desimipramine or centrally administered 1-isoproterenol. *J. Pharmacol. Exp. Ther.* 1988;247:379–389.

26. Radeke MJ, Misko TP, Hsu C, Herzenberg LA, Shooter EM. Gene transfer and molecular cloning of the rat nerve growth factor receptor. *Nature* 1987;325:593–597.
27. Schwartz JP, Chuang D-M, Costa E. Increase in nerve growth factor content of C6 glioma cells by the activation of a beta-adrenergic receptor. *Brain Res.* 1977;137:369–375.
28. Schwartz JP, Costa E. Regulation of nerve growth factor content in C6 glioma cells by beta-adrenergic receptor stimulation. *Naunyn-Schmiedeberg's Arch. Pharmacol.* 1977; 300:123–129.
29. Schwartz JP, Costa E. The role of cyclic AMP-dependent protein kinase in the catecholamine mediated regulation of nerve growth factor contained in C6 glioma cells. In: Usdin E, Kopin IJ, Barchas JD, eds. *Catecholamines: Basic and Clinical Frontiers*. New York: Pergamon Press, 1980;773–775.
30. Seiler M, Schwab ME. Specific retrograde transport of nerve growth factor (NGF) from neocortex to nucleus basalis in the rat. *Brain Res.* 1984;300:33–39.
31. Shelton DL, Reichardt LF. Expression of the beta-nerve growth factor gene correlates with the density of sympathetic innervation in effector organs. *Proc. Natl. Acad. Sci. USA* 1984;81:7951–7955.
32. Taniuchi M, Schweitzer JB, Johnson EM. Nerve growth factor receptor molecules in rat brain. *Proc. Natl. Acad. Sci. USA* 1986;83:1950–1954.
33. Thoenen H, Barde YA. Physiology of nerve growth factor. *Physiol. Rev.* 1980;60:1284–1335.
34. Whittemore SR, Ebendal T, Larkfors L, Olson L, Seiger A, Stromberg I, Persson H. Developmental and regional expression of beta-nerve growth factor mRNA and protein in the rat central nervous system. *Proc. Natl. Acad. Sci. USA* 1986;83:817–821.
35. Williams LR, Varon S, Peterson GM, Wictorin K, Fisher W, Bjorklund A, Gage FH. Continuous infusion of nerve growth factor prevents basal forebrain neuronal death after fimbria fornix transection. *Proc. Natl. Acad. Sci. USA* 1986;83:9231–9235.

# Cell-specific Expression of NGF Receptors in the Basal Forebrain

C.F. Dreyfus\*, P. Bernd\*\*, H.J. Martínez\*, \*\*\*, S.J. Rubin\* and I.B. Black\*

\*Division of Developmental Neurology, Cornell University Medical College, New York, NY 10021

\*\*Department of Anatomy and Cell Biology, SUNY Health Science Center, Brooklyn, NY 11203

\*\*\*Instituto de Investigaciones Clínicas, Universidad del Zulia Maracaibo, Venezuela

**E**xtenstive work now suggests that nerve growth factor (NGF), well known for its actions on peripheral neurons (9,37), may also play a critical role in brain function and development. In particular, abundant evidence suggests that NGF regulates ontogeny and mature function of cholinergic basal forebrain (bf) projection neurons. For example, NGF specifically elevates choline acetyltransferase (CAT), the acetylcholine synthesizing enzyme, in bf neurons developing in culture (11,13,15,22) and *in vivo* (8,23). In addition, NGF appears to promote survival of lesioned bf neurons (12,17,38) in the adult.

These actions of NGF may be mediated through specific receptors for the trophic factor. Recent reports indicate that brain NGF receptors are present and resemble those of the periphery with respect to molecular weight (36) and dissociation constants (29,41). Moreover, recent mapping studies suggest that the site of action of NGF may be on cholinergic neurons (1,10,14,28,30). However, direct evidence that high-affinity receptor sites are associated with these neurons has been lacking. It is possible that actions of NGF may be mediated directly through receptors on cholinergic neurons, or, alternatively, they may be mediated indirectly through other cell types.

To approach this problem and determine the site of action of NGF on basal forebrain neurons, we have used a combined immunochemical and autoradiographic approach to visualize high-affinity NGF binding sites in dissociated basal forebrain tissue cultures and identify the transmitter phenotype associated with these potentially NGF receptive cells. Our data suggest that neuronal and non-neuronal cell groups exhibit NGF binding. However, high-affinity binding sites appear to be restricted to the neuronal population. Moreover, at least two, apparently distinct, subtypes of neurons express high-affinity receptors. Consequently, NGF may act on entirely different populations of neurons to effect its actions on the rat basal forebrain.

## METHODS

### Dissociated Cultures

Basal forebrain dissociated cultures were established from embryonic day 17 (E17) rat fetuses as described previously (3,27) and maintained for one week.

### Preparation of [ $^{125}\text{I}$ ]Nerve Growth Factor

$\beta$ -NGF was prepared from adult mouse salivary glands (24) and iodinated as described previously (2).

### Exposure to [ $^{125}\text{I}$ ]Nerve Growth Factor

Binding studies were conducted as previously (3,6). In brief, dissociates were thoroughly rinsed (2 hr) to eliminate nonradioactive NGF. They then were exposed to [ $^{125}\text{I}$ ]NGF (0.02 nM or 0.2 nM, 1 hr, 37°C) in a medium containing Eagle's minimum essential medium with Earle's salts, L-glutamine (2 mM), glucose (600 mg %), and bovine serum albumin (5 mg/ml). Control cultures were incubated as above but with an excess of nonradioactive NGF (0.2  $\mu\text{M}$ ) in addition to the radioactive ligand. After incubation, cultures were rinsed rapidly with ice-cold phosphate buffered saline (PBS) and fixed (4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4, 1 hr, room temperature). To distinguish high- and low-affinity sites some dissociated cultures were incubated with [ $^{125}\text{I}$ ]NGF (0.2 nM, 1 hr, 37°C) and then exposed to ice-cold PBS containing nonradioactive NGF (0.2  $\mu\text{M}$ ) in HEPES buffer (25 mM) for 30 min at 4°C. Cultures were rinsed and prepared for radioautography using procedures previously described (3,6).

### Immunocytochemistry

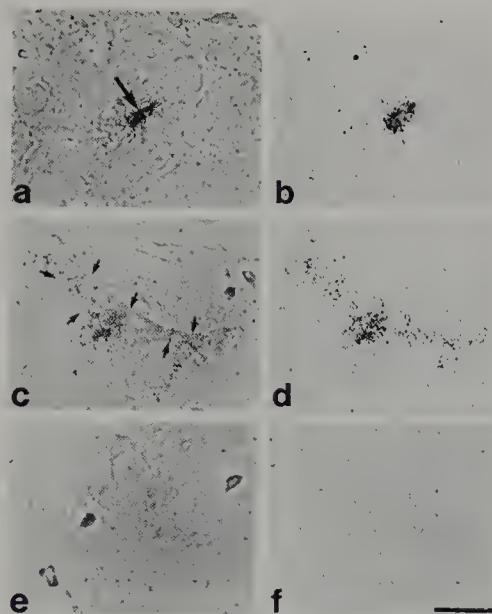
NGF receptors were identified immunocytochemically using a monoclonal antibody to the rat NGF receptor (192 IgG). Cultures were rinsed, fixed, and processed for immunocytochemistry using the avidin/biotin technique (3). A monoclonal antibody directed against the rat NGF receptor (192 IgG) was used to visualize receptor sites. Antibody was diluted 1:100. In some cases, after binding studies with [ $^{125}\text{I}$ ]NGF, as described above, cultured cells were assessed for CAT using a modification (6) of the method of Houser et al. (16) and processed for autoradiography. Gamma-aminobutyric acid (GABA) was detected using the avidin/biotin technique (6). Two polyclonal antisera were employed. One, raised

against a bovine serum albumin conjugate of GABA (Incstar Corp.) was used at a 1:2000 dilution. This antiserum has been characterized (20). The second, raised against a glutaraldehyde conjugate of GABA (Chemicon Corp.), was used at a dilution of 1:100. The specificities of both antisera were assessed by preincubating antisera with GABA itself (25).

## RESULTS

### Visualization of [ $^{125}\text{I}$ ]Nerve Growth Factor Binding Sites

In initial studies we identified bf cell populations that express NGF binding sites. Cultures were incubated with [ $^{125}\text{I}$ ]NGF (0.2 nM) and processed for radioautography. Light microscopic analysis (Fig. 1) revealed two subpopulations of basal

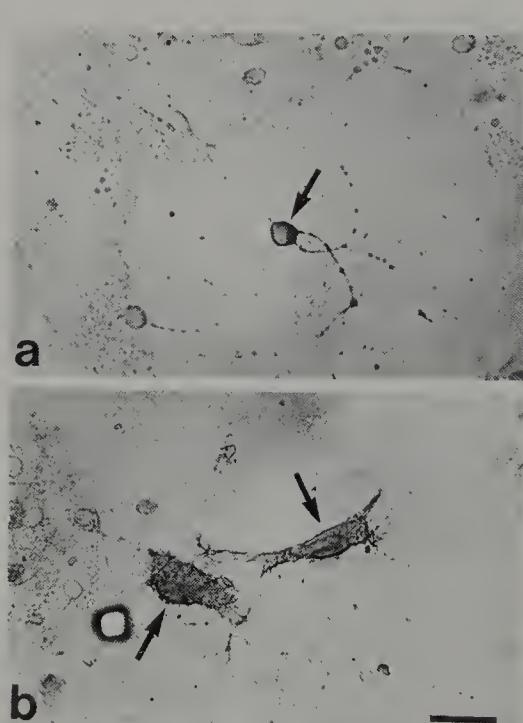


**FIG. 1.** Light microscopic radioautographic visualization of specific [ $^{125}\text{I}$ ]NGF binding sites in unstained bf cultures viewed by phase optics (a,c,e), or transmitted light (b,d,f). Two populations of cells exhibit specific binding with [ $^{125}\text{I}$ ]NGF, small cells (a,b), and large, flat cells (c,d). The arrows indicate labeled cells. Only background levels of silver grains are seen when cultures are incubated in the presence of non-radioactive NGF (0.2  $\mu\text{M}$ ) in addition to the radiolabeled ligand (e,f). Bar = 25  $\mu\text{m}$ . (From ref. 3.)

forebrain cells that accumulated silver grains. One consisted of a subset of small, round neuron-like cells. The second consisted of large flat cells that resembled support cells. Binding was specific, since only background levels of silver grains were observed when incubation was performed in the presence of nonradioactive NGF (0.2  $\mu$ M) in addition to the iodinated compound.

#### Localization of NGF Receptive Cells using a Monoclonal Antibody Directed against the Receptor

To confirm that NGF receptors were indeed being visualized, we used an immunocytochemical approach to identify receptive cells using the specific NGF receptor monoclonal antibody 192 IgG. Two subpopulations of NGF receptive bf cells were revealed. One group was composed of small, round neuron-like cells and the second was composed of large flat support-like cells (Fig. 2).

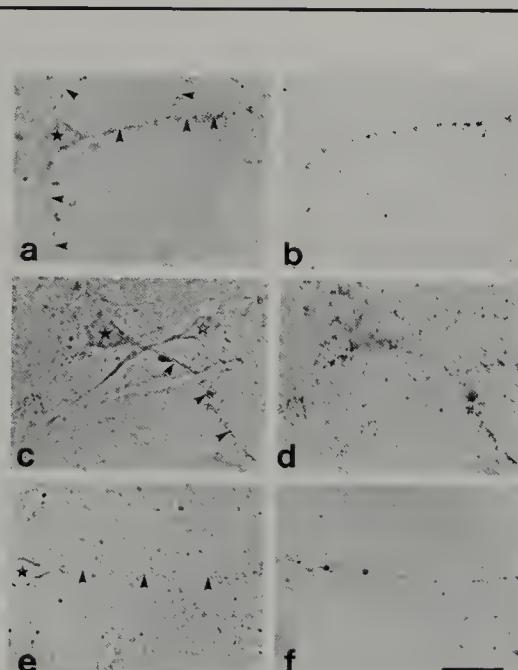


**FIG. 2.** Immunocytochemical detection of NGF receptor-positive cells in dissociated cultures using monoclonal antibody 192 IgG. Neuron-like (a) and large, flat cells (b) are labeled. Positive cells are indicated by arrows. Bar = 30  $\mu$ m. (From ref. 3.)

## Distribution of High-affinity Receptor Sites on Bf Populations

NGF receptors have been reported to exist as two species consisting of high- and low-affinity subtypes. While the high-affinity subtype has been linked with the biological actions of NGF in the periphery (9,37), the role of the low-affinity receptor is less clear. It was important, therefore, to determine the receptor subtype associated with the two cell populations. To approach this problem, we used a pulse-chase paradigm to take advantage of the different rates of dissociation exhibited by low- and high-affinity NGF receptors. Bf cultures were incubated with [<sup>125</sup>I]NGF (0.2 nM), and then washed in the cold in PBS containing excess non-radioactive NGF. This procedure largely eliminates NGF bound to low-affinity receptor which exhibits a half time of dissociation of approximately 5 minutes (18,34).

In fact, light microscopic radioautographic analysis revealed that the neuronal cells continued to bind NGF after the wash procedure (Fig. 3). Consequently, the neuron-like populations exhibit high-affinity receptors in addition to low-affinity receptors. Conversely, no high-affinity sites were detected on the support-like cells, suggesting that low-affinity sites are associated with this cell type.



**FIG. 3.** Light microscopic localization of [<sup>125</sup>I]NGF binding in bf dissociates after a wash in nonradioactive NGF. Cultures are viewed by phase optics (a,c,e) or transmitted light (b,d,f). A sub-group of neuron-like cells exhibited silver grains (arrowheads). Note that other neuron-like cells (c,d) did not exhibit silver grains after the wash procedure (open star). Bar = 25  $\mu$ m. (From ref. 3.)

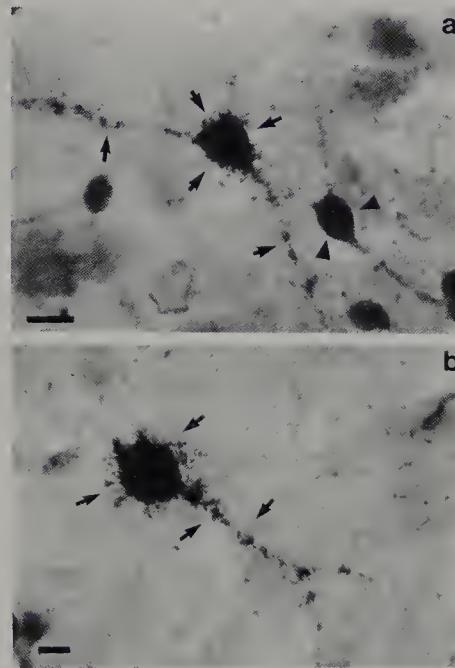
## Cholinergic Populations Express High-affinity NGF Binding

To further identify the neuron-like population, we performed simultaneous radioautography, to visualize [ $^{125}\text{I}$ ]NGF binding sites, and immunocytochemistry, to detect choline acetyltransferase. Analysis of the dissociates revealed a cell population exhibiting accumulations of silver grains and brown reaction product, indicating the presence of binding sites on CAT-positive neurons. Cholinergic neuron-like cells, therefore, appear to exhibit NGF binding. However, only a subset of CAT-positive cells exhibited NGF receptor binding. Consequently, CAT-positive cells may comprise several populations, those exhibiting receptor sites and those either devoid of receptors or expressing levels of receptor below the threshold of detectability. In summary, our observations support the hypothesis that NGF acts directly on specific subpopulations of cholinergic cells to elicit effects noted previously, consistent with the work of others on bf *in vivo* (1,28) and in culture (10).

To define the receptor subtype associated with these cells we used two experimental designs. Initially, to preferentially label high-affinity sites, we incubated cultures with an order of magnitude less NGF (0.02 nM) than used in previous studies. Alternatively, we performed pulse-chase experiments to preferentially dissociate low-affinity binding sites (Fig. 4a). Simultaneous immunocytochemical techniques were employed to visualize CAT-positive populations. In both experimental procedures CAT-positive cells exhibited specific accumulations of silver grains, suggesting that cholinergic cells express high-affinity receptors.

## Identification of a Non-cholinergic Population that Binds NGF

Unexpectedly, following these procedures, we also observed a population of non-cholinergic, NGF receptor-positive cells, indicating that non-cholinergic neurons may respond to NGF as well. A prominent CAT-negative cell population in the bf contains gamma aminobutyric acid (GABA) (4), a neurotransmitter used by neurons throughout the central nervous system (5). To determine whether GABA neurons exhibit high-affinity NGF receptors, cells were identified immunocytochemically. Two different polyclonal antisera directed against GABA were used. Dissociates were incubated with [ $^{125}\text{I}$ ]NGF and subjected to the pulse-chase procedure. In both cases, GABA-positive cells exhibited silver grains after the wash procedure, suggesting that high-affinity NGF receptors were associated with GABAergic cells (Fig. 4b). However, only a subset of GABA-positive cells exhibited NGF receptors, consistent with results noted with cholinergic populations. Moreover, a subgroup of NGF receptor-positive cells were GABA-negative (data not shown). Our observations suggest that a previously unidentified GABA-positive population expresses high-affinity NGF receptors in the basal forebrain. The transmitter associated with the non-GABAergic group remains to be identified,



**FIG. 4.** Combined light microscopic radioautographic visualization of specific [ $^{125}\text{I}$ ]NGF binding and immunocytochemical localization of CAT-positive (**a**) or GABA-positive (**b**) cells in dissociated bf cultures after the wash procedure (arrows). Note the presence of neurons that bind NGF (arrows) as well as the presence of neurons that do not (arrowheads). Bar = 10  $\mu\text{m}$ .

but it is probable that at least some of the cells represent the cholinergic populations described above.

Finally, we sought to define the neuron-like populations exhibiting high-affinity binding. Cultures were incubated with [ $^{125}\text{I}$ ]NGF, washed in nonradioactive NGF, and stained for the glial marker, glial fibrillary acidic protein (GFAP) or the neuronal marker, neuron specific enolase (NSE). Although NSE-positive cells exhibited silver grains, GFAP populations were not labeled after the chase procedure, indicating that CAT and GABA populations were indeed neuronal.

## DISCUSSION

A variety of studies indicate that NGF influences function of maturing and adult cholinergic neurons of the basal forebrain. In the present experiments, dissociated cultures were employed to identify NGF-responsive cells and define receptor subtypes.

Both neurons and flat, support-like cells exhibited specific NGF binding sites. However, high-affinity sites appeared to be predominantly associated with the neuron-like population; low-affinity sites were associated with both the neuron-like and support cell-like populations.

The localization of low-affinity binding sites to an apparent support cell population is consistent with recent studies of the peripheral (31,35,40) and central nervous systems (21): low-affinity sites are associated with Schwann-like cells and astrocytes. In the brain, NGF receptors have also been identified on tanyocytes (1) and meningeal cells (39). Our current work is directed toward identification of the large support-like cells in the basal forebrain cultures.

Expression of high-affinity sites by the neuronal subgroup in the bf is of particular functional significance. In the peripheral nervous system, high-affinity sites mediate biological responsiveness (9,37). The association of high-affinity receptors with the neuronal population, and in particular with cholinergic neurons, suggests that effects of NGF observed on cholinergic neurons may be mediated directly through receptors on this cell population.

Unexpectedly, in defining potentially receptive cell populations, high-affinity sites also were identified that were associated with GABA-positive neurons. Thus, the actions of NGF may not be restricted to cholinergic neurons. Different brain populations may potentially respond to NGF, reproducing the heterogeneity of responsive populations described in the periphery (9,19,37). These data complement recent work *in vivo*, indicating that NGF may bind to unidentified non-cholinergic neurons (1, 28). The studies suggest that novel and diverse populations of brain neurons may be receptive to the NGF protein.

More generally, the observation that GABA-positive neurons exhibit high-affinity NGF binding sites is of special significance. GABA-containing neurons are a prominent brain population that plays critical inhibitory roles throughout the central nervous system. In the basal forebrain, GABA may modulate cholinergic function in systems that subserve spatial memory (7,26) and that degenerate in Alzheimer's disease (32). The present studies, then, raise the possibility that the action of NGF may extend beyond the cholinergic bf neuron system. Consequently, NGF may influence brain function, and, in particular, basal forebrain-hippocampal function, through as yet unexplored mechanisms.

## SUMMARY

Previous studies have indicated that nerve growth factor (NGF) regulates the development and physiology of basal forebrain cholinergic neurons. To define underlying mechanisms, we have used dissociated, rat basal forebrain cultures to identify specific cells that may be responsive to NGF. Initially, NGF binding sites were detected using two morphological techniques, radioautography after incubation in [ $^{125}\text{I}$ ]NGF and immunocytochemistry using a monoclonal antibody to the NGF receptor. Two populations of cells expressed putative receptors. One

consisted of a population of neurons, and the second contained large, flat, support-like cells. Since biologic actions of NGF have been associated almost exclusively with high-affinity binding sites, experiments using a pulse-chase paradigm were performed to estimate binding affinities associated with the two cell groups. Only the neuron group exhibited high-affinity binding sites.

To identify the transmitter expressed by this neuron population, we used radioautography to visualize high-affinity sites, and immunocytochemistry to visualize CAT. A subgroup of CAT-positive cells was labeled with silver grains, suggesting that NGF directly regulates cholinergic neurons by binding to high-affinity receptor sites. Unexpectedly, a population of GABA-positive cells also exhibited high-affinity NGF receptors. Our observations indicate that GABA-positive as well as CAT-positive cells exhibit high-affinity NGF receptors. NGF, therefore, may influence multiple neuron systems in the brain.

## ACKNOWLEDGMENTS

The authors gratefully acknowledge the generous gift of anti-NGF receptor monoclonal antibody, 192 IgG, from Dr. Eugene M. Johnson, Jr. and the kind gift of anti-choline acetyltransferase monoclonal antibody from Drs. G.D. Crawford and P. Salvaterra.

This work was supported by NIH grants NS20788, HD23315, DA05132, NSF grant BNS8896101, the Alzheimer's Disease and Related Disorders Assoc., Inc., March of Dimes Birth Defects Fdn. and the Bristol Myers Co. Unrestricted Grant Award. The authors thank D. Medford for excellent technical assistance.

## REFERENCES

1. Batchelor PE, Armstrong DM, Blaker SN, Gage FH. Nerve growth factor receptor and choline acetyltransferase. Co-localization in neurons within the rat forebrain: response to fimbria-fornix transection. *J. Comp. Neurol.* 1989;284:187-204.
2. Bernd P. Characterization of nerve growth factor binding to cultured neural crest cells: evidence of an early developmental form of the NGF receptor. *Devel. Biol.* 1986; 115:415-424.
3. Bernd P, Martínez HJ, Dreyfus CF, Black IB. Localization of high-affinity and low-affinity nerve growth factor receptors in cultured rat basal forebrain. *Neuroscience* 1988; 26:121-129.
4. Brashears HR, Zaborszky L, Heimer L. Distribution of GABA and cholinergic neurons in the rat diagonal band. *Neuroscience* 1986;17:439-451.
5. Cooper JR, Bloom FE, Roth RH. *The Biochemical Basis of Neuropharmacology*, 5th ed. New York: Oxford Univ. Press, 1986;124-155.
6. Dreyfus CF, Bernd P, Martínez HJ, Rubin SJ, Black IB. GABAergic and cholinergic neurons exhibit high-affinity nerve growth factor binding in rat basal forebrain. *Exp. Neurol.* 1989;104:181-185.

7. Fischer W, Wictorin K, Bjorklund A, Williams LR, Varon S, Gage FH. Amelioration of cholinergic neuron atrophy and spatial memory impairment in aged rats by nerve growth factor. *Nature* 1987;329:65–68.
8. Gnahn H, Hefti F, Heumann R, Schwab ME, Thoenen H. NGF-mediated increase of choline acetyltransferase (ChAT) in the neonatal rat forebrain: evidence for a physiological role of NGF in the brain? *Brain Res.* 1983;9:45–52.
9. Greene LA, Shooter EM. The nerve growth factor: biochemistry, synthesis and mechanism of action. *Ann. Rev. Neurosci.* 1980;3:353–402.
10. Hartikka J, Hefti F. Development of septal cholinergic neurons in culture: plating density and glial cells modulate effects of NGF on survival, fiber growth and expression of transmitter-specific enzymes. *J. Neurosci.* 1988;8:2967–2985.
11. Hatanaka H, Tsukui H, Nihonmatsu I. Developmental change in nerve growth factor action from induction of choline acetyltransferase to promotion of cell survival in cultured basal forebrain cholinergic neurons from postnatal rats. *Devel. Brain Res.* 1988;39:85–95.
12. Hefti F. Nerve growth factor promotes survival of septal cholinergic neurons after fimbrial transection. *J. Neurosci.* 1986;6:2155–2162.
13. Hefti F, Hartikka J, Eckenstein F, Gnahn H, Heumann R, Schwab M. Nerve growth factor increases choline acetyltransferase but not survival or fiber outgrowth of cultured fetal septal cholinergic neurons. *Neuroscience* 1985;14:55–68.
14. Hefti F, Hartikka J, Salvatierra A, Weiner WJ, Mash DC. Localization of nerve growth factor receptors in cholinergic neurons of the human basal forebrain. *Neurosci. Lett.* 1986;69:37–41.
15. Honegger P, Lenoir D. Nerve growth factor (NGF) stimulation of cholinergic telencephalic neurons in aggregating cell cultures. *Devel. Brain Res.* 1982;3:229–238.
16. Houser CR, Crawford GD, Barber RP, Salvaterra PM, Vaughn JE. Organization and morphological characteristics of cholinergic neurons: an immunocytochemical study with a monoclonal antibody to choline acetyltransferase. *Brain Res.* 1983;266:97–119.
17. Kromer LF. Nerve growth factor treatment after brain injury prevents neuronal death. *Science* 1987;235:214–216.
18. Landreth GE, Shooter EM. Nerve growth factor receptors on PC12 cells: ligand-induced conversion from low- to high-affinity states. *Proc. Natl. Acad. Sci. USA* 1980;77:4751–4755.
19. Levi-Montalcini R. The nerve growth factor: thirty-five years later. *Embo J.* 1987;6:1145–1154.
20. Maley B, Newton BW. Immunohistochemistry of  $\lambda$ -aminobutyric acid in the cat nucleus tractus solitarius. *Brain Res.* 1985;330:364–368.
21. Marchetti D, Stach RW, deVellis J, Perez Polo JR. Binding constants of soluble NGF receptors in rat oligodendrocytes and astrocytes in culture. *Biochem. Biophys. Res. Commun.* 1987;147:422–427.
22. Martínez HJ, Dreyfus CF, Jonakait GM, Black IB. Nerve growth factor selectively increases cholinergic markers but not neuropeptides in rat basal forebrain in culture. *Brain Res.* 1987;412:295–301.
23. Mobley WC, Rutkowski JL, Tennekoon GI, Buchanan K, Johnston MV. Nerve growth factor increases choline acetyltransferase activity in developing basal forebrain neurons. *Molec. Brain Res.* 1986;1:53–62.
24. Mobley WC, Schenker A, Shooter EM. Characterization and isolation of proteolytically modified nerve growth factor. *Biochemistry* 1976;15:5543–5552.

25. Naegele JR, Arimatsu Y, Schwartz P, Barnstable CJ. Selective staining of a subset of GABAergic neurons in cat visual cortex by monoclonal antibody VC 1.1. *J. Neurosci.* 1988;8:79–89.
26. Olton DS, Wenk GL. Dementia: animal models of the cognitive impairments produced by degeneration of the basal forebrain cholinergic system. In: Meltzer HY, ed. *Psychopharmacology: The Third Generation of Progress*. New York: Raven Press, 1987; 941–953.
27. Prochiantz A, DiPorzio U, Kato A, Berger B, Glowinski J. *In vitro* maturation of mesencephalic dopaminergic neurons from mouse embryos is enhanced in the presence of their striatal target cells. *Proc. Natl. Acad. Sci. USA* 1979;76:5387–5391.
28. Raivich G, Kreutzberg GW. The localization and distribution of high-affinity  $\beta$ -nerve growth factor binding in the central nervous system of the adult rat. A light microscopic autoradiographic study using [ $^{125}$ I]-nerve growth factor. *Neuroscience* 1987; 30:23–36.
29. Richardson PM, Verge Issa VMK, Riopelle RJ. Distribution of neuronal receptors for nerve growth factor in the rat. *J. Neurosci.* 1986;6:2312–2321.
30. Riopelle RJ, Richardson PM, Verge VMK. Distribution and characteristics of nerve growth factor binding on cholinergic neurons of rat and monkey forebrain. *Neurochem. Res.* 1987;12:923–928.
31. Rohrer H. Nonneuronal cells from chick sympathetic and dorsal root sensory ganglia express catecholamine uptake and receptors for nerve growth factor during development. *Devel. Biol.* 1985;111:95–107.
32. Rossor MN, Iversen LL, Reynolds GP, Mountjoy CQ, Roth R. Neurochemical characteristics of early and late onset types of Alzheimer's disease. *Br. Med. J.* 1984; 288:961–968.
33. Schatteman GC, Gibbs L, Lanahan AA, Claude P, Bothwell M. Expression of NGF receptor in the developing and adult primate central nervous system. *J. Neurosci.* 1988; 8:860–873.
34. Schechter AL, Bothwell MA. Nerve growth factor receptors on PC12 cells: evidence for two receptor classes with differing cytoskeletal association. *Cell* 1981;24:867–874.
35. Sutter A, Riopelle RJ, Harris-Warrick RM, Shooter EM. The heterogeneity of nerve growth factor. In: Bitensky M, ed. *Transmembrane Signalling*. New York: Alan R. Liss, 1979;659–667.
36. Taniuchi M, Schweitzer JB, Johnson Jr EM. Nerve growth factor receptor molecules in rat brain. *Proc. Natl. Acad. Sci. USA* 1986;83:1950–1954.
37. Thoenen H, Barde Y-A. Physiology of nerve growth factor. *Physiol. Rev.* 1980; 60:1284–1335.
38. Williams LR, Varon S, Peterson GM, Wictorin K, Fischer W, Bjorklund A, Gage FH. Continuous infusion of nerve growth factor prevents basal forebrain neuronal death after fimbria fornix transection. *Proc. Natl. Acad. Sci. USA* 1986;83:9231–9235.
39. Yan Q, Johnson, Jr. EM. An immunohistochemical study of the nerve growth factor receptor in developing rats. *J. Neurosci.* 1988;8:3481–3498.
40. Zimmerman A, Sutter A.  $\beta$ -Nerve Growth Factor (NGF) receptors on glial cells. Cell-cell interactions between neurons and Schwann cells in cultures of chick sensory ganglia. *EMBO J.* 1983;2:879–885.
41. Zimmerman A, Sutter A, Samuelson J, Shooter EM. A serological assay for the detection of cell surface receptors of nerve growth factor. *J. Supramolec. Struct.* 1978;9:351–361.



# The Nerve Growth Factor Receptor

J. Regino Perez-Polo

University of Texas Medical Branch  
Galveston, TX 77550

**T**he nerve growth factor protein, NGF, has been shown to act as a trophic factor in the peripheral nervous system (PNS). In the central nervous system (CNS) it has been shown to elicit neuronal sprouting and induction of the cholinergic enzyme, choline acetyl transferase (ChAT) in those brain structures that are involved in learning and the appearance of aging-related cognitive deficits. This is of interest because aging-associated reductions in NGF and its receptor, NGFR, in rodent and human cholinergic CNS areas have been reported (12). NGF has been shown to be essential to the proper development, maintenance and regeneration of some peripheral and central neurons (29,36–42,58–60,64–66). Both the NGF and NGFR proteins and mRNA have been demonstrated to be present in brain and spinal cord (2,12,24,48,54,55,64,65), where NGF promotes neuronal survival after deafferenting lesions that increase NGF protein and mRNA synthesis (9,12). Moreover, NGF receptors are also present in adrenal and lymphoid cells where they act as a mitogen (30,62). The NGFR in PNS, CNS, some cell lines, and lymphocytes have been partially characterized (1,3,13–15,19,28,31–33,37,71).

## ROLE OF NGF IN AGING

The hypothesis that inadequate functioning of the NGF trophic mechanism may underlie neurological deficits associated with aging has been advanced although not confirmed (12,26). There are increased risks due to free radical damage in the aging process that might be due to shifts in oxidant-antioxidant metabolic balance. There is some evidence that NGF can protect neuronal cells *in vitro* against hydrogen peroxide insults through the induction of catalase and glutathione transferase activities (41–46). Since NGF may induce antioxidant enzymes *in vitro*, the NGF regulation of neuronal oxidant-antioxidant balance in neuronal cells is of interest to an understanding of the mechanism of action of NGF. Our overall goal here is to discuss the mechanism of action of NGF in terms of its initial binding

to NGFR and its intracellular effects on oxidant-antioxidant balance. Two hypotheses to be discussed are:

- 1) Are there deficits in the action of NGF in the CNS associated with the aging process?
- 2) Does NGF have a regulatory role on neuronal oxidant-antioxidant metabolic balance that is relevant to the aging of CNS neurons?

## CELL DEATH

Cell death during the ontogeny of the nervous system allows for competitive innervation of targets (18,23,27,34,35,38,58,59). At critical stages during development, neurons that successfully compete for target-derived trophic factors, such as NGF, are spared cell death. Thus, exogenous NGF, or its withdrawal by anti-NGF, has significant and permanent effects on axonal sprouting and survival of sympathetic, sensory, and striatal neurons (4,21,22,29). The relationship among neuronal cell death during development and its counterparts in injury and aging is not known. It has been shown that cycloheximide, a protein synthesis inhibitor, does protect sensory neurons from neuronal death during development where it may suppress expression of "suicide genes" (34). However, there are other alternatives to this finding. Since neurons have low endogenous levels of antioxidants, the regulation of oxidant-antioxidant balance by neurotrophic factors can control neuronal death in development, injury, and aging. It is a corollary of this hypothesis that aging associated deficits result partly from reductions in trophic factors and their receptors resulting in damaging shifts in the oxidant-antioxidant balance (38,40–42,46). A consequence of such a shift is likely to be release of calcium from mitochondrial stores or increased calcium influx into cells due to membrane peroxidation-induced damage and, in turn, activation of calcium dependent proteases resulting in crosslinking of proteins and nucleic acids (8,49–52). A variant of this explanation for aging-induced damage is, that since mitochondrial oxidative DNA damage is high and DNA repair enzymes are absent, or not particularly effective inside mitochondria as compared with nuclear DNA repair mechanisms (49–52), oxidatively generated DNA fragments escape from mitochondria and become randomly integrated into nuclear genes, disrupting the regulation of transcription in a cumulative fashion over time (50). These alternative explanations for cell death in the nervous system are not mutually exclusive but rather may be coupled. For example, expression of a "suicide gene" could result in increases in superoxide dismutase while catalase is repressed, or increased levels of proteases, or increased levels of calcium transport into cytoplasm from extracellular spaces or the mitochondrial domain. It is known that a consequence of aging is oxidative stress partially manifested by an increase in peroxidative events that disturb energy metabolism and damage proteins and membranes. The issue is further complicated because, whereas at low concentrations hydrogen peroxide has inductive effects

on neuronal metabolism, at high concentrations it has toxic effects (10,16,17,40, 41,42,46). Thus, oxidative stress can induce xenobiotic responses much like heavy metals. The regulation of free radical scavenging mechanisms is important because free radicals can accumulate and adversely affect the functions of neurons which are highly specialized, have limited regenerative capacity, and a relatively high requirement for oxygen.

## NGF

The structural features of NGF have been amply chronicled (5,13,29,37,39,69). Although the subunit composition of NGF varies for different sources, only  $\beta$ -NGF, henceforth called NGF, has neuronotrophic activity. NGF mRNA levels have been determined for brain, superior cervical ganglia, and spinal cord and correlated with NGF protein levels as a function of development and response to injury (64,65). Levels of NGF mRNA and protein in PNS and CNS correlate with the density of innervation (58–60). The highest levels of NGF mRNA and protein are found in cortex and hippocampus, terminal axonal regions for basal forebrain cholinergic neurons, which is where NGF effects on ChAT induction and cell sparing have been best documented (64). There is NGF mRNA present in the thalamus/hypothalamus, brainstem, striatum, cerebellum, and spinal cord, in that order of decreasing prevalence. In other CNS regions, such as cerebellum, where there is also NGFR, the low levels of NGF that are present are not associated with cholinergic neurons (64,65).

## NGF EFFECTS

NGF can directly influence lymphoid cells (61). There are NGF receptors on B-cells where NGF acts as a mitogen (62). Treatment of sensory and sympathetic ganglia with NGF, at appropriate stages in development, results in a decrease in neuronal cell death, neuronal hypertrophy, increased anabolic activity, neurotransmitter synthesis, and exaggerated neurite outgrowth (13,29). Depression of NGF levels in early development due to injections of anti-NGF, results in irreversible sympathectomy and neuronal cell loss in sensory ganglia (29). The NGF responses for PNS have been amply reviewed (13,23,29). In CNS, NGF stimulates ChAT in the hippocampus, septum, and cortex of neonatal and adult rats after fimbrial lesions (12,64). NGF increases ChAT activity and neurite outgrowth in cultures of dissociated fetal basal forebrain and septal cells (4,11,64). The retrograde transport of NGF from innervated tissues to cell bodies has been demonstrated for PNS and CNS neurons (24,25,48,54). NGF in spinal cord is also retrogradely transported by central processes to sensory neurons (24,25).

## NGF RECEPTORS

Like other protein hormones, NGF binds to a cell surface receptor (NGFR) and is internalized (13,56). Studies on NGF receptors, NGFR, are of three different types: binding studies, detection of NGFR protein, and detection of NGFR mRNA. Binding studies use  $^{125}\text{I}$ -NGF as ligand for *in vitro* kinetic and equilibrium studies, or as a probe for autoradiography of neural tissues (48,53,56,57). NGFR protein has been characterized by affinity crosslinking of  $^{125}\text{I}$ -NGF to NGFR followed by immunoprecipitation (14,15,20,26,28,70,71); direct iodination of receptor followed by immunoprecipitation, or using indirect techniques or monoclonal antibodies to rodent and human NGFR that do not crossreact (15,20). Immunoprecipitates have been analyzed exclusively by SDS-PAGE. NGFR mRNA has been detected by Northern analysis using two independently derived cDNA probes (6,7,47). PNS neurons display two NGF-binding activities consisting of a high Bmax, low-affinity ( $K_d: 10^{-9}\text{M}$ ) binding site called type II or fast dissociating NGFR, and a low Bmax, high-affinity ( $K_d: 10^{-11}\text{M}$ ) binding site called type I or slowly dissociating NGFR (56). Evidence exists as to the interconvertability of the two types of binding (32). There are preliminary reports as to the NGF binding properties of different CNS structures (1,53). Immunoprecipitation studies with rat PNS and CNS tissues reveal a major 75–80 KDa protein and a minor 200 KDa protein with an additional 123 KDa protein being expressed early in development (3). Although the reported size of the immunoprecipitated or crosslinked NGFR varies, in general there are two species detected. Based on comparisons of the deduced amino acid sequence of NGFR with SDS-PAGE data and the interaction between NGFR and lectins, the minimal NGFR is a 42–49 KDa protein with a 10 KDa sugar moiety.

## MODEL SYSTEMS

There are difficulties in studying the mechanism of action of NGF *in vivo*. Cell lines have distinctive advantages (37); although caution must be exercised before extrapolating from studies with transformed cell lines. Three neuronal cell lines that have proved useful are the PC12 rat pheochromocytoma line, and the SK-N-SH-SY5Y, SY5Y, and the LAN-1 human neuroblastoma lines. PC12 display type I and type II NGFR and respond to NGF with extended neurites and increased neurotransmitter synthesis, both cholinergic and adrenergic (37). SY5Y and LAN-1 are nearly diploid lines. The SY5Y display only NGFR I, whereas LAN-1 display both type I and II NGFR (56). Exposure of SY5Y and LAN-1 to NGF induces neurites, electrically excitable membranes, inhibits cell division and stimulates the anabolic machinery of the cell (36–38,41–44). For PC12 and SY5Y, NGF provides protection from peroxidative events by inducing catalase and glutathione transferase activity (36–44). A particular advantage of rodent sensory ganglia is that

neuronal cell number for different developmental stages is well documented and there are established paradigms that perturb neuronal cell death. Also, tissue culture techniques for sensory neurons and viability assays have been available for some time (37). This is also true for some NGF-responsive neurons in CNS (37).

## OXIDATIVE STRESS IN AGING

Many neuronal deficits resulting from neuronal aging are secondary consequences of free radical mediated events. Aging is associated with deficits in metabolic expression and altered energy and diminished immunogenic response that in turn will result in unimpeded generation of free radicals. There are subsequent events that involve permanent changes at the blood-brain barrier and astrocytic activation that will not be addressed here, although they are important and free radicals may be involved. To a large extent, studies on NGF effects on neuronal development have focused on axonal growth and little is known as to the effects of NGF on the oxidant-antioxidant balance of aging neurons. In addition, perturbation of the level of intracellular hydrogen peroxide can have toxic or activating effects on glucose metabolism since energy metabolism in neurons is particularly dependent on glucose utilization via the pentose phosphate pathway and lipogenesis. Thus, exposure of neurons to free radicals results in an imbalance in energy metabolism in addition to the effect of radicals on membrane lipids and proteins.

## CHARACTERIZATION OF NGFR

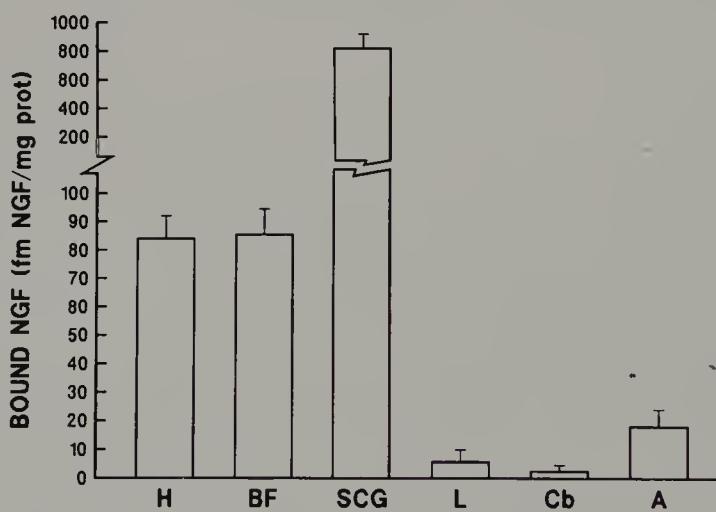
Results obtained with a soluble NGFR-binding assay using LAN-1 and PC12 cells would indicate that the properties of NGFR binding and molecular species present in these cells is very similar to that observed for dissociated chick embryo sensory ganglia. Using a partial purification of NGFR by lectin chromatography and preparative electrofocusing on granulated gel (PEGG) that separates high-affinity from low-affinity binding activities on LAN-1, it is possible to separate the two molecular species responsible for NGF binding (32).

One outcome of the comparative SDS-PAGE analysis of NGFR from dissociated sensory ganglia is the continued presence of a third molecular species. This could be due to the known presence of NGFR on Schwann cells (25). When  $^{125}\text{I}$ -NGF is infused into spinal cord, there is uptake and accumulation of label in large sensory neurons as well as satellite cells (25). There are also receptors to NGF in astrocyte but not oligodendroglial primary cell cultures (33). This is further complicated by the fact that there are effects of steroids on NGF binding and NGF levels in male and female rodent sympathetic and sensory ganglia during development *in vivo* (67,68). Taken together, these results would suggest that the strategy of using cell line models to better understand NGF-NGFR interactions prior to tackling *in vivo*

situations is a prudent approach and that careful attention to culture conditions is necessary because steroids affect NGF and NGFR expression *in vivo*.

## NGF RECEPTORS IN THE CNS

It is established that NGFR mRNA and protein are distributed throughout the CNS, but less is known about the binding properties of NGFR in CNS (Fig. 1). There is evidence that NGF levels in aged rodent and human cerebral cortical structures are reduced as a consequence of aging. Equilibrium binding studies on different CNS tissues from aged rats would suggest that the levels of NGF binding to solubilized brain tissues are significantly reduced for the aged rodent basal forebrain, cerebral cortex, and hippocampus (1, Fig. 2). These results are in agreement with reports of reduced NGF, NGF-binding activity, and NGFR antigen in aged rats (12,26). Although a cursory Scatchard analysis would suggest that there is only one binding site with an equilibrium dissociation constant in the nanomolar range, for the cerebral cortex and basal forebrain, where  $B_{max}$  levels were higher, there is good evidence for NGF binding displaying  $K_d$  values in the picomolar range (Fig. 3). It is known that aged rats have lower levels of NGFR in the superior cervical ganglion, in agreement with the findings of Angelucci et al. (1,63). There



**FIG. 1.**  $B_{max}$  obtained by Scatchard analysis of  $^{125}\text{I}-\beta\text{-NGF}$  binding to NP-40-solubilized tissues of rat brain, as described (1), and expressed as femtomoles of  $^{125}\text{I}-\beta\text{-NGF}$  bound per mg of protein. H, hippocampus; BF, basal forebrain; SCG, superior cervical ganglion; L, liver; Cb, cerebellum; A, adrenal.

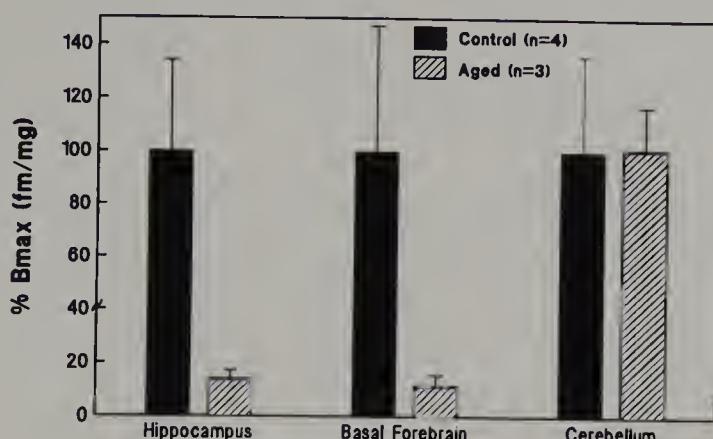


FIG. 2. Relative Bmax obtained by Scatchard analysis, as described (1), of  $^{125}\text{I}$ - $\beta$ -NGF binding to NP-40-solubilized brain tissues of 4-month-old and aged (26-month-old) rats expressed as % of values obtained for 4-month-old rats.

is also a report of a reduction in binding of NGF to basal forebrain neurons of 30-month old rats when compared with 10-month old rats as judged by immunocytochemistry relying on the monoclonal antibody 192 IgG to rodent NGFR (26). Because there are no significant changes in the Kd values reported for CNS, it is likely that the differences observed reflect a decrease in NGF receptor molecules. Because rodent astrocytes can display NGFR (56), the finding of reduced NGF binding sites in aged basal forebrain and hippocampus does not necessarily speak to a neuronal deficit alone. There may be fewer NGFR/cell or fewer cells containing the same number of NGFR/cell. Alternative explanations would be that there is more endogenous NGF in the aged rat samples competing for the iodinated NGF, an unlikely explanation because NGF levels are reduced. Monitoring of NGFR levels may provide a way of assessing dysfunction of CNS cholinergic systems relevant to some neurological disorders or aging and evaluating the outcome of a number of therapeutic approaches. It is clear from the findings to date that manipulation of NGF and NGFR, reported to be possible, may be in itself a promising therapeutic strategy (1,19,21).

## SUMMARY

The ability of neurons in the mammalian nervous system to establish and maintain functional connections with target tissues and respond satisfactorily to the metabolic demands typical for neurons is dependent in many instances on an adequate supply of trophic factors and an active repertoire of receptors to these factors

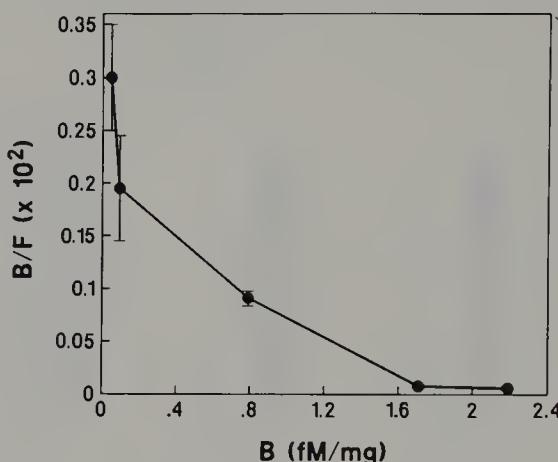


FIG. 3. Scatchard analysis of  $^{125}\text{I}$ - $\beta$ -NGF binding to NP-40-solubilized basal forebrain tissue from 4-month-old male rats expressed as the bound-to-free ratio of  $^{125}\text{I}$ - $\beta$ -NGF times 100 as a function of fentomoles (fM) of  $^{125}\text{I}$ - $\beta$ -NGF bound per mg of protein as described in (1).

being displayed at the plasma membrane. The best understood example of growth factor action in the nervous system is the nerve growth factor protein (NGF) and its receptor (NGFR). NGF is critical to the regulation of neuronal cell death in development, adulthood, and perhaps also in aging. The NGFR is a glycoprotein that is encoded by a single gene that can exist in two binding states: a high-affinity, low NGF binding capacity and a low-affinity, high NGF binding capacity state. Interconversion between the two states appears to involve a receptor-associated molecule that is present in the high-affinity state. In rodent CNS, degenerative events associated with cholinergic neurons of the basal forebrain mirror a drastic loss of NGF binding there and also in hippocampus and frontal cortex. These findings are consistent with the hypothesis that age-associated deficits may be due to deficits in trophic factor activity.

## REFERENCES

- Angelucci L, Ramacci MT, Taglialatela G, Hulsebosch C, Morgan B, Werrbach-Perez K, Perez-Polo R. Nerve growth factor binding in aged rat central nervous system: effects of acetyl-L-carnitine. *J. Neurosci. Res.* 1988;20:491–496.
- Auburger G, Heumann R, Korschning S, Thoenen H. Developmental changes of nerve growth factor and its mRNA in rat hippocampus: comparison with choline acetyltransferase. *Dev. Biol.* 1987;120:322–8.

3. Bernd P. Characterization of nerve growth factor binding to cultured neural crest cells: evidence of an early developmental form of the nerve growth factor receptor. *Dev. Biol.* 1986;115:415–24.
4. Bostwick JR, Appel SH, Perez-Polo JR. Distinct influences of nerve growth factor and central nervous system cholinergic factor on medial septal explants. *Brain Res.* 1987;422:92–98.
5. Bradshaw RA. Nerve growth factor. *Annu. Rev. Biochem.* 1978;47:191–216.
6. Buck CR, Martinez HF, Black IB, Chao MV. Developmentally regulated expression of the nerve growth factor receptor gene in peripheral nervous system and brain. *Proc. Natl. Acad. Sci. U.S.A.* 1987;84:3060–3.
7. Chao MV, Bothwell M, Ross A, Koprowski H, Lanahan A, Buck C, Sehgal A. Gene transfer and molecular cloning of the human nerve growth factor receptor. *Science* 1986;232:518–21.
8. Davies K. Protein damage and degradation by oxygen radicals. *J. Biol. Chem.* 1987;262:9895–9920.
9. Ebendal T, Olson L, Seiger A, Hedlund O. Nerve growth factor in rat iris. *Nature* 1980;286:25–8.
10. Estralita M, Martin E, Skaper SD, Varon S. Catalase protection of neuronal survival *in vitro* is not directed to the accumulation of peroxides in the culture medium. *Int. J. Devl. Neurosci.* 1987;5:1–10.
11. Gahwiler BH, Enz A, Hefti F. Nerve growth factor promotes development of the rat septo-hippocampal cholinergic projection *in vitro*. *Neurosci. Lett.* 1987;75:6–10.
12. Goedert M, Fine A, Hunt S, Ullrich A. Nerve growth factor mRNA in peripheral nervous system and central nervous system and in human central nervous system: lesion effects in rat brain and Alzheimers disease. *Mol. Brain Res.* 1986;1:85–92.
13. Greene LA, Shooter EM. The nerve growth factor: biochemistry, synthesis, and mechanism of action. *Annu. Rev. Neurosci.* 1980;3:353–402.
14. Greene SH, Greene LA. A single 103KDa nerve growth factor receptor activity species represents low- and high-affinity forms of the nerve growth factor receptor. *J. Biol. Chem.* 1986;261:15316–15327.
15. Grob PM, Ross AH, Koprowski H, Bothwell M. Characterization of the human melanoma nerve growth factor receptor. *J. Biol. Chem.* 1985;260:8044–8049.
16. Hall E, Braughler J. Effect of intravenous methylprednisolone on spinal cord lipid peroxidation and Na<sub>+</sub>, K<sub>+</sub>-ATPase activity: dose-response analysis during the first hour after contusion injury. *J. Neurosurg.* 1982;57:247–253.
17. Hall EH. Intensive anti-oxidant pretreatment retards motor nerve degeneration. *Brain Res.* 1987;413:175–178.
18. Hamburger V, Oppenheim RW. Cell death. *Neuroscience Commun.* 1982;1:39–55.
19. Haskell BE, Stach RW, Werrbach-Perez K, Perez-Polo JR. Effect of retinoic acid on nerve growth factor receptor. *Cell Tissue Res.* 1987;247:67–73.
20. Hosang M, Shooter EM. The internalization of nerve growth factor by high-affinity receptors on pheochromocytoma PC12 cells. *EMBO J.* 1987;6:1197–1202.
21. Hulsebosch C, Coggeshall R, Perez-Polo JR. Persistence of anti-nerve growth factor induced sensory axons: possible penetration in mammalian spinal cord. *Brain Res.* 1987;411:267–74.
22. Hulsebosch C, Perez-Polo JR, Coggeshall R. *In vivo* anti-nerve growth factor induces sprouting in dorsal roots. *J Comp. Neurol.* 1987;259(3):445–51.
23. Johnson EM, Rich K, Kip H. The role of nerve growth factor in sensory neurons *in vivo*. *Trends Neurosci.* 1986;9:33–7.

24. Johnson EM, Taniuchi M, Clark HB, Springer JE, Koh SY, Tayrien MW, Loy R. Demonstration of retrograde transport of nerve growth factor receptor in peripheral nervous system and central nervous system. *J Neurosci.* 1987;7:923–9.
25. Khan R, Green B, Perez-Polo JR. Effect of injury on nerve growth factor uptake by sensory ganglia. *J. Neurosci. Res.* 1987;18:562–567.
26. Koh S, Loy R. Age-related loss of nerve growth factor sensitivity in rat basal forebrain neurons. *Brain Res.* 1988;440:396–401.
27. Korschning S. The role of nerve growth factor in the central nervous system. *Trends Neurosci.* 1986;9:570–573.
28. Kouchalakos RN, Bradshaw RA. Nerve growth factor receptor from rabbit sympathetic ganglia membranes. *J. Biol. Chem.* 1986;261:16054–16059.
29. Levi-Montalcini R. The nerve growth factor 35 years later. *Science* 1987;237:1154–1162.
30. Lillien L, Claude P. Nerve growth factor mitogen for chromaffin cells. *Nature* 1985; 317:632–4.
31. Lyons CR, Stach RW, Perez-Polo JR. Binding constants of isolated nerve growth factor receptors from different species. *Biochem. Biophys. Res. Commun.* 1983;115:368–74.
32. Marchetti D, Perez-Polo JR. Nerve growth factor receptor in human neuroblastoma cells. *J. Neurochem.* 1987;49:475–486.
33. Marchetti D, Stach RW, Saneto RP, deVellis J, Perez-Polo JR. Binding constants of soluble nerve growth factor receptors in rat oligodendrocytes and astrocytes in culture. *Biochem. Biophys. Res. Comm.* 1987;147:422–427.
34. Martin DP, Schmidt RE, DiStefano PS, Lowry OH, Carter JG, Johnson Jr. EM. Inhibitors of protein synthesis and RNA synthesis prevent neuronal death caused by nerve growth factor deprivation. *J. Cell Biol.* 1988;106:829–844.
35. Oppenheim RW. Naturally occurring cell death during neural development. *Trends Neurosci.* 1985;8:487–493.
36. Perez-Polo JR. Neuronotrophic factors. In: Bottenstein J, Sato G, eds. *Cell Cultures in the Neurosciences*, vol. 3. New York: Plenum Press, 1985;95–123.
37. Perez-Polo JR. *Neuronal Factors*. CRC: Boca Raton, FL, 1–202.
38. Perez-Polo JR. Role of trophic factors in neuronal aging. In: *Developmental Neuroscience & Aging*. (eds). Lauder, Jr., In Press.
39. Perez-Polo JR, Haber B. Neuronotrophic interactions. In: *The Cün Neurosciences*, vol. 5. Rosenberg RN, ed. New York: Churchill Livingstone'1984;37–51.
40. Perez-Polo JR, Werrbach-Perez K. Effects of nerve growth factor on the response of neurons to injury. In: *Recent Achievements in Restorative Neurology: Upper Motor Neuron Functions and Dysfunctions*, Eccles J, Dimitrijevic JR, eds. pp. 30:321–377. Karger, Basel, Switzerland.
41. Perez-Polo R, Werrbach-Perez K. In vitro model of neuronal aging and development in the nervous system. In: Vernadakis A, ed. *Model Systems of Development and Aging of the Nervous System*. Boston: Martinus Nijhoff, 1987;442–443.
42. Perez-Polo JR, Werrbach-Perez K. Role of nerve growth factor in neuronal injury and survival. In: Gorio A, Perez-Polo JR, deVellis J, Haber B, eds. *Neural Development and Regeneration Cellular and Molecular Aspects*. Heidelberg: Springer, 1988;339–410.
43. Perez-Polo JR, Werrbach-Perez K, Tiffany-Castiglioni E. A human clonal line model of differentiating neurons. *Develop. Biol.* 1979;71:341–355.
44. Perez-Polo JR, Reynolds CP, Tiffany-Castiglioni E, Ziegler M, Schulze I, Werrbach-

- Perez K. Nerve growth factor effects on human neuroblastoma lines: a model system. In: Haber B, Perez-Polo JR, Coulter, JD, eds. *Proteins in the Nervous System: Structure and Functions*. New York: Alan R. Liss, 1982;285–299.
45. Perez-Polo JR, Tiffany-Castiglioni E, Werrbach-Perez K. Model clonal system for study of neuronal cell injury. In: Giuffrida AM, Haber B, Hashim G, Perez-Polo JR, eds. *Nervous system regeneration*. New York: Alan R. Liss, 1983;201–220.
46. Perez-Polo JR, Apffel L, Werrbach-Perez K. Role of central nervous system and peripheral nervous system trophic factors on free radical mediated aging events. *Clin. Neuropharmac.* 1986;9:98–100.
47. Radeke MJ, Misko TP, Hasu C, Herzenberg LA, Shooter EM. Gene transfer and molecular cloning of rat nerve growth factor receptor. *Nature* 1987;325:593–597.
48. Richardson PM, Riopelle RJ. Uptake of nerve growth factor along peripheral and spinal axons of primary sensory neurons. *J. Neurosci.* 1984;4:1683–1689.
49. Richter C. Biophysical consequences of lipid peroxidation in membranes. *Chem. Phys. Lipids* 1987;44:175–189.
50. Richter C. Do mitochondrial DNA fragments promote cancer and aging? *FEBS Lett.* 1988;241:1–5.
51. Richter C, Frei B. CA<sup>2+</sup> release from mitochondria induced by peroxidants. *Free Radical Biol. Med.* 1988;4:365–375.
52. Richter C, Park JW, Ames BN. Normal oxidative damage to mitochondrial and nuclear DNA is extensive. *Proc. Natl. Acad. Sci. USA* 1988;85:6465–7.
53. Riopelle RJ, Verge VMK, Richardson PM. Properties of receptors for nerve growth factor in the mature rat nervous system. *Mol. Brain Res.* 1987;3:45–53.
54. Seiler M, Schwab ME. Specific retrograde transport of nerve growth factor from neocortex to nucleus basalis in the rat. *Brain Res.* 1984;300:33–39.
55. Shine HD, Perez-Polo JR. 7S nerve growth factor protein in the golden hamster. *J. Neurochem.* 1976;27:1315–1318.
56. Stach W, Perez-Polo R. Binding of nerve growth factor to nerve growth factor receptor. *J. Neurosci. Res.* 1987;17:1–10.
57. Stach RW, Lyons CR, Perez-Polo JR. Characteristics of partially purified nerve growth factor receptor. *J. Neurochem.* 1987;49:1280–1285.
58. Thoenen H, Barde YA, Davies AM, Johnson JE. Neuronotrophic factors and neuronal death. *Ciba Found. Symp.* 1981;126:838–840.
59. Thoenen H, Barde YA, Edgar D. Factors involved in the regulation of the survival and differentiation of neurons. In: Nicholls JG, ed. *Repair and Regeneration of the Nervous System*. Berlin: Springer, 1982;173–185.
60. Thoenen H, Edgar D. Neurotrophic factors. *Science* 1985;229:238–242.
61. Thorpe LW, Stach RW, Hashim GA, Marchetti D, Perez-Polo JR. Receptors for nerve growth factor on rat spleen monocytes. *J. Neurosci. Res.* 1987;17:128–134.
62. Thorpe LW, Morgan B, Beck C, Werrbach-Perez K, Perez-Polo JR. Nerve growth factor and the immune system. In: Gorio A, Perez-Polo R, deVellis J, Haber B, eds. *Neural Development and Regeneration Cellular and Molecular Aspects*. Heidelberg: Springer, 1988;583–594.
63. Uchida Y, Tonionaga M. Loss of nerve growth factor receptors in sympathetic ganglia from aged mice. *Biochem. Biophys. Res. Commun.* 1987;146:797–801.
64. Whittemore SR, Seiger A. The expression, localization and functional significance of beta-nerve growth factor in the central nervous system. *Br. Res. Rev.* 1987;12:439–464.

65. Windebank A, Poduslo JF. Neuronal growth factors produced by adult peripheral nerve after injury. *Brain Res.* 1986;385:197–200.
66. Wright LL, Beck C, Perez-Polo JR. Sex differences in nerve growth factor levels in superior cervical ganglia and pineals. *Int. J. Develop. Neurosci.* 1987;5:383–390.
67. Wright LL, Marchetti D, Perez-Polo JR. Effects of gonadal steroids on nerve growth factor receptors in sympathetic and sensory ganglia of neonatal rats. *Int. J. Develop. Neurosci.* 1988;6:217–222.
68. Yankner B, Shooter E. Biology and mechanism of action of nerve growth factor. *Annu. Rev. Biochem.* 1982;51:845–68.
69. Yau Q, Johnson, Jr. EM. A quantitative study of the developmental expression of nerve growth factor receptor in rats. *Develop. Biol.* 1987;121:139–148.
70. Yip H, Johnson R. Developing dorsal root ganglia require trophic support from central processes: evidence for a role for retrogradely transported nerve growth factor from central nervous system to peripheral nervous system. *Proc. Natl. Acad. Sci. USA* 1984; 81:6245–9.
71. Yip HK, Johnson, Jr. EM. Nerve growth factor receptors in rat spinal cord: an autoradiographic and immunohistochemical study. *Neuroscience* 1987;22:267–279.

# Receptors for Gangliosides on Rat Brain Membranes: Specificity, Regional and Subcellular Distribution

Michael Tiemeyer and Ronald L. Schnaar

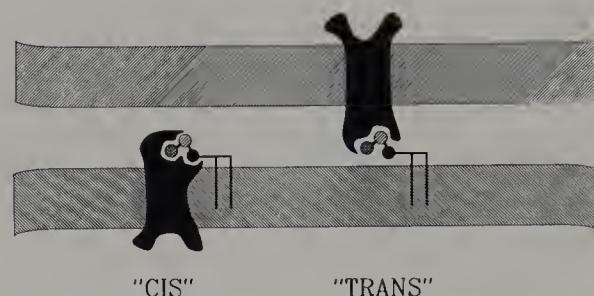
*Departments of Pharmacology and Neuroscience  
The Johns Hopkins University School of Medicine  
Baltimore, MD 21205*

Gangliosides are a varied class of sialic acid-bearing glycosphingolipids found predominantly on the outer membrane leaflet of vertebrate cell membranes (9). Their lipid portion (ceramide) is anchored in the plasma membrane and their oligosaccharide extends into the extracellular space. Although distributed broadly in vertebrate tissues, the highest concentration of gangliosides is in the brain, where they carry over half of the cell surface sialic acid (21). Although the physiological functions of gangliosides are not known, *in vitro* studies have demonstrated regulation of cell biochemistry (4,8,24), morphology (3,25), growth (2), and differentiation (14) by exogenously added gangliosides. Among other effects, gangliosides modulate brain protein kinases (4,8,24), enhance neurite outgrowth from certain neuroblastoma cell lines (3,25), and support recognition and adhesion of embryonic neural cells (1).

As trophic or recognition factors, gangliosides may bind to specific protein receptors on the cells they regulate. This may occur in two ways (Fig. 1): (i) Gangliosides may associate laterally with receptors in the *same* membrane bilayer (*cis*); or (ii) receptors on one cell may bind to gangliosides on an *apposing* cell surface (*trans*). These mechanisms are not mutually exclusive. *Cis*-effects may be responsible for the ability of gangliosides to inhibit growth factor receptor responses (2), while *trans*-effects are apparent in the ability of intact embryonic neural cells to adhere to purified gangliosides adsorbed on an apposing surface (1). Identification of protein receptors for gangliosides would greatly enhance our understanding of their actions. We developed new probes for ganglioside receptors and identified a novel ganglioside-specific receptor on rat brain membranes (22).

## NEOGANGLIOPROTEINS: NEW PROBES FOR GANGLIOSIDE RECEPTORS

Prior studies using radiolabeled gangliosides (23) reported multiple binding equilibria to brain membranes due to membrane insertion and nonspecific hydrophobic

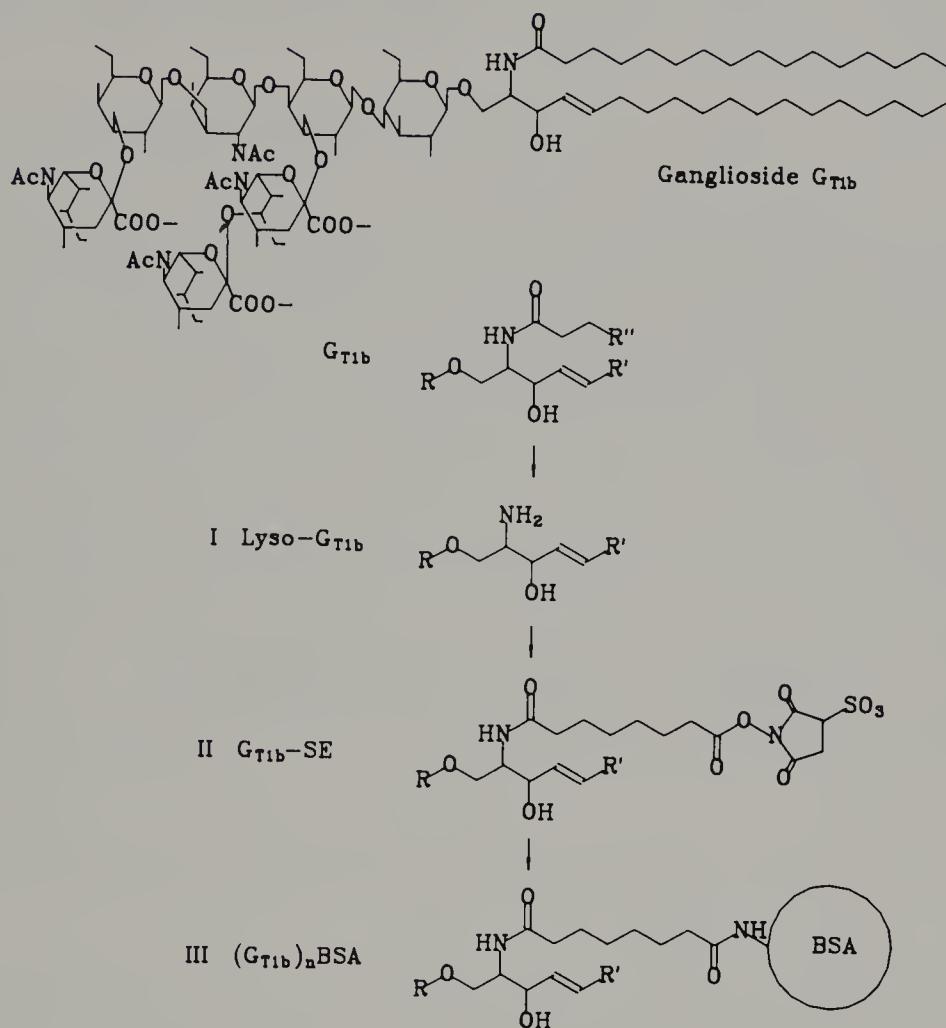


**FIG. 1.** Potential interactions between membrane gangliosides and complementary protein receptors. Apposing cell membranes are depicted.

adsorption. To focus on receptors that interact with their oligosaccharides, we covalently linked gangliosides, via their lipid moiety, to a protein carrier, bovine serum albumin (BSA). We refer to such conjugates as "neoganglioproteins" in analogy to the less complex neoglycoproteins introduced by Lee and co-workers (13) to probe vertebrate lectins. Neoganglioproteins are better probes for ganglioside receptors than are unconjugated gangliosides because: (i) the ganglioside's hydrophobic lipid portion is sequestered, minimizing nonspecific membrane adsorption or insertion; (ii) they can be readily radioiodinated to high specific activity; and (iii) attaching multiple glycoconjugates per BSA carrier results in a high-affinity probe (12 and see below).  $G_{T1b}$  (ganglioside nomenclature is that of Svennerholm, 21) was chosen for our initial studies because it was effective at supporting the adhesion of embryonic neural cells (1), and can be purified in abundance from bovine brain.

### Neoganglioprotein Synthesis

The ganglioside fatty acid amide was replaced with an active ester suitable for coupling to protein (22 and Fig. 2). Lysoganglioside (I, Fig. 2) was prepared by removal of ganglioside *N*-acyl groups (both from the ceramide and neuraminic acids) with strong base, then the neuraminic acids were selectively re-*N*-acetylated. The lysoganglioside was converted to a sulfosuccinimidyl ester (II, Fig. 2) by treating Compound I with an excess of the crosslinking reagent bis(sulfosuccinimidyl)suberate (20). Compound II was purified by silicic acid chromatography (to remove unreacted crosslinker) and linked to BSA in aqueous buffer (III, Fig. 2). The resulting neoganglioprotein was purified by gel filtration and anion exchange HPLC, which revealed one major peak that contained >90% of the protein and migrated differently from either the starting BSA or unconjugated



**FIG. 2.** Preparation of  $(G_{T1b})_n$ BSA. Lyso- $G_{T1b}$  (I) was prepared by selective alkaline deacylation of the sphingosine amine of purified bovine brain  $G_{T1b}$ . Reaction of the primary amine of lyso- $G_{T1b}$  with excess bis(sulfosuccinimidyl)suberate generated the activated ganglioside-sulfosuccinimidyl ester ( $G_{T1b}$ -SE, II) which was separated from unreacted bis(sulfosuccinimidyl)suberate before addition to BSA. Attack of the sulfosuccinimidyl ester of  $G_{T1b}$ -SE by primary amines on BSA resulted in the formation of stable amide bonds linking the derivatized ganglioside to the protein  $((G_{T1b})_n$ BSA, III). The same preparative steps, utilizing lyso- $G_{M1}$  as starting material, generated the neoganglioprotein  $(G_{M1})_n$ BSA. Adapted from Tiemeyer et al. (22), where detailed descriptions of the syntheses are reported.

ganglioside ester. Fractions containing neoganglioprotein were pooled, lyophilized, resuspended in water, desalted by gel filtration, and stored at 20°C.

### Neoganglioprotein Characterization

Carbohydrate to protein ratios of neoganglioproteins were characterized by hydrolysis and HPLC saccharide analysis (Table 1). NeuAc:Gal:GalNAc:Glc ratios were characteristic of the ganglioside attached, and allowed calculation of the average number of ganglioside molecules covalently bound per BSA molecule which was denoted  $(G_{T1b})_n$ BSA, with the type of ganglioside in parenthesis and the average number of ganglioside molecules attached per BSA indicated by the subscript "n." While  $(G_{T1b})_4$ BSA was used in most of our studies, we also synthesized  $(G_{T1b})_8$ BSA,  $(G_{T1b})_2$ BSA,  $(G_{T1b})_1$ BSA, and  $(G_{M1})_2$ BSA. Covalent attachment of gangliosides to BSA was demonstrated by SDS polyacrylamide gel electrophoresis followed by cholera toxin binding. Purified neoganglioproteins migrated as broad bands of Coomassie Blue-stained material with apparent molecular weights greater than underivatized BSA (22).  $^{125}I$ -Cholera toxin B-subunit (a specific probe for the monosialoganglioside  $G_{M1}$ , 11) bound to  $(G_{T1b})_4$ BSA (coincident with Coom-

TABLE 1. Carbohydrate analysis of neoganglioproteins

Neoganglio-protein	Sugar	mol Sugar per mol BSA	Sugar Ratio <sup>a</sup>	
			Observed	Expected
$(G_{T1b})_4$ BSA	NeuAc	12.8	3.0	3
	GalNAc <sup>b</sup>	4.2	1.0	1
	Gal	8.7	2.0	2
	Glc	4.4	1.0	1
$(G_{T1b})_1$ BSA	NeuAc	3.3	3.0	3
	GalNAc	1.1	1.0	1
	Gal	2.3	2.1	2
	Glc	1.5	1.4	1
$(G_{M1})_2$ BSA	NeuAc	2.0	1.0	1
	GalNAc	2.2	1.1	1
	Gal	4.1	2.0	2
	Glc	3.3	1.6	1

Neutral sugars and hexosamines were determined after hydrolysis by ion exchange HPLC and pulsed amperometric detection using a Dionex carbohydrate system, and NeuAc was determined by a micro-thiobarbituric acid assay. Adapted from Tiemeyer et al. (22), which should be consulted for details of the analyses.

<sup>a</sup> The ratio of individual monosaccharides to each other was calculated by setting the determined value for NeuAc equal to 3 for  $G_{T1b}$  derivatives and equal to 1 for  $G_{M1}$  derivatives. Minor glucose contamination is a common problem associated with the sensitive detection method used.

<sup>b</sup> GalNAc was measured as galactosamine since the hydrolysis conditions quantitatively de-N-acetylate GalNAc.

asie Blue) after transferring the electrophoresed proteins to nitrocellulose and treating the blots with neuraminidase, which converts  $G_{T1b}$  to  $G_{M1}$  (19). No detectable toxin binding to  $(G_{T1b})_n$ BSA without neuraminidase treatment or to lanes containing underivatized BSA was detected.  $(G_{T1b})_4$ BSA, radioiodinated with  $Na^{125}I$  (22) was used in the binding studies described below.

### Ganglioside Receptor Assay

Attempts to detect specific binding of  $^{125}I\text{-}(G_{T1b})_4$ BSA directly to isolated rat brain membranes in suspension were unsuccessful. The possibility that ganglioside receptors might be masked by endogenous gangliosides in the brain membrane preparation seemed likely. Therefore, we removed endogenous gangliosides from membrane proteins by detergent extraction. Rat brain membranes (P2 fraction) were agitated gently in 0.4% deoxycholate at 37°C for 20 min, then passed through microporous filters resulting in retention of a small, but reproducible, portion ( $\approx 2.5\%$ ) of the membrane protein on each filter (22). Membrane-adsorbed filters were placed in vials containing 1 ml of binding buffer (50 mM Hepes buffer, pH 7.4, supplemented with 1 mg/ml BSA, 10 mM  $CaCl_2$  and 0.016%, w/v, Triton X-100) at 8°C.  $^{125}I\text{-}(G_{T1b})_n$ BSA and inhibitors (as indicated) were added in small volumes, the vials incubated for 90 min at 8°C, then each filter was transferred to a manifold, washed three times with 2 ml of ice-cold 50 mM Hepes (pH 7.4), placed in a test tube, and bound radioactivity measured using a gamma-radiation counter.

## GANGLIOSIDE BINDING PROTEIN ON RAT BRAIN MEMBRANES

Binding isotherms performed with increasing concentrations of  $^{125}I\text{-}(G_{T1b})_4$ BSA incubated with filters treated with 30  $\mu g$  rat brain membrane protein revealed high-affinity saturable binding (Fig. 3). Background binding was low ( $\approx 10\%$  of specific binding using 0.5 nM ligand) and characteristically nonsaturable. Since 10  $\mu M$  underivatized  $G_{T1b}$  reduced radioligand binding to background levels (see below), specific binding was defined as binding in the absence of  $G_{T1b}$  (total) less binding in the presence of 10  $\mu M$   $G_{T1b}$  (nonspecific). Scatchard transformation (18) of the binding data was linear with a  $K_D$  of 2.0 nM and a  $B_{max}$  of 20 pmol/mg membrane protein (Fig. 4A); analysis by the method of Hill (10) generated a coefficient of 1.0 (Fig. 4B), consistent with the presence of a single class of binding sites. This ganglioside receptor is a protein, since even limited trypsin pretreatment (1  $\mu g/ml$  trypsin, 20 min, 37°C) of membrane-adsorbed filters blocked neoganglioprotein binding by >80% (Fig. 5). In contrast, binding was reduced by less than 10% when filters were treated with the same amount of trypsin in the presence of soybean trypsin inhibitor.

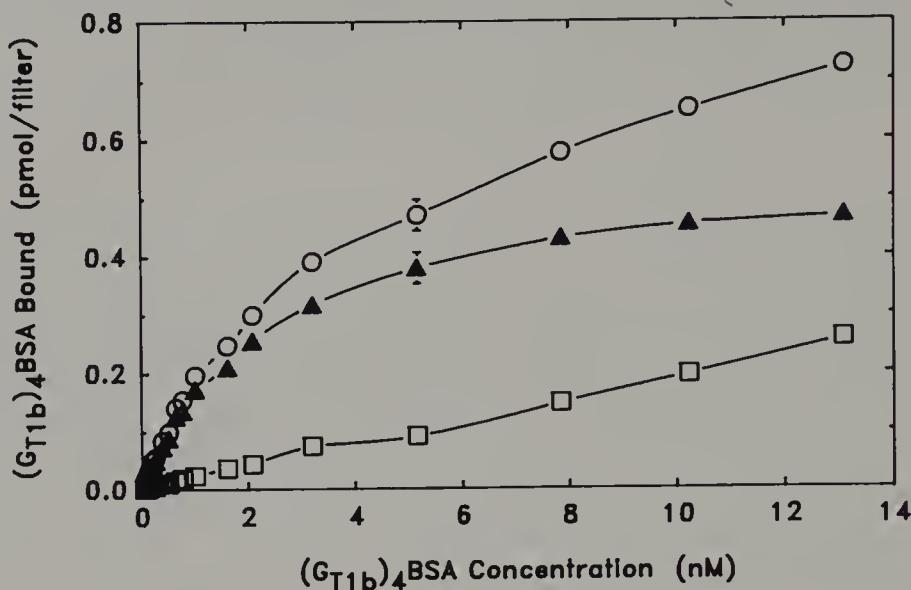
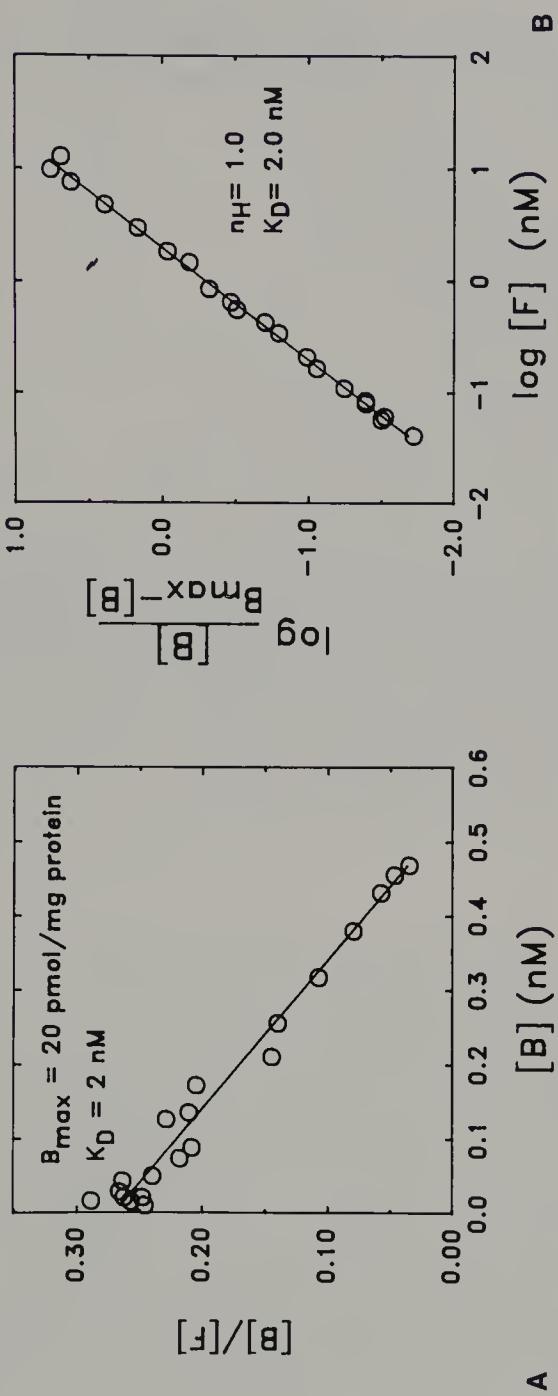


FIG. 3.  $^{125}\text{I}$ -(G<sub>T1b</sub>)<sub>4</sub>BSA binding to detergent-treated rat brain membranes. Brain P2 membranes were suspended at 60  $\mu\text{g}/\text{ml}$  protein in 0.4% deoxycholate in 50 mM Hepes buffer pH 7.4 and incubated at 37°C for 20 min. An aliquot (0.5 ml) was passed through a polyvinylidene difluoride filter (Millipore HV, 2.5 cm diam.) on a manifold, the filters washed and incubated with increasing concentrations of  $^{125}\text{I}$ -(G<sub>T1b</sub>)<sub>4</sub>BSA in 1 ml of binding buffer for 90 min at 8°C in the presence or absence of 10  $\mu\text{M}$  G<sub>T1b</sub>. Specific binding (filled triangles) is defined as total binding (open circles, binding in the absence of G<sub>T1b</sub>) minus nonspecific binding (open squares, binding in the presence of G<sub>T1b</sub>). Values are the mean  $\pm$  S.D. for triplicate determinations (S.D. values that fall within the symbols are not shown). Adapted from Tiemeyer et al. (22).

## CARBOHYDRATE SPECIFICITY OF BRAIN GANGLIOSIDE RECEPTORS

Inhibition of neoganglioprotein binding by various compounds in solution revealed carbohydrate specificity in  $^{125}\text{I}$ -(G<sub>T1b</sub>)<sub>4</sub>BSA binding to rat brain membranes. The three most potent ganglioside inhibitors (G<sub>D1b</sub>, G<sub>T1b</sub>, and G<sub>Q1b</sub>, Fig. 6) have similar inhibitory potencies ( $\text{IC}_{50} \leq 100$  nM), and similar oligosaccharides (Fig. 7). By comparison, disialogangliosides lacking either the internal NeuAc $\alpha$ 2-8NeuAc $\alpha$ 2-3 group or the gangliotetraose backbone (G<sub>D1a</sub> and G<sub>D3</sub>, respectively) are one sixth as potent inhibitors compared with G<sub>D1b</sub>, although of equal anionic charge. G<sub>M1</sub>, which varies from G<sub>D1b</sub> only by the absence of the NeuAc $\alpha$ 2-8 group was the least potent ganglioside inhibitor with an inhibitory potency <4% that of



**FIG. 4.** Linear transformations of  $^{125}\text{I}-(\text{GTr1b})_4\text{BSA}$  binding to detergent-treated rat brain membranes. Data from saturation isotherms performed as in Fig. 3 were transformed by the method of Scatchard (18). **A:**  $B_{\text{max}}$ , total receptor concentration;  $K_D$ , apparent dissociation constant or Hill (10). **B:**  $n_H$ , Hill coefficient.  $[B]$ , concentration of membrane-bound ligand;  $[F]$ , concentration of unbound ligand. Adapted from Tiemeyer et al. (22).

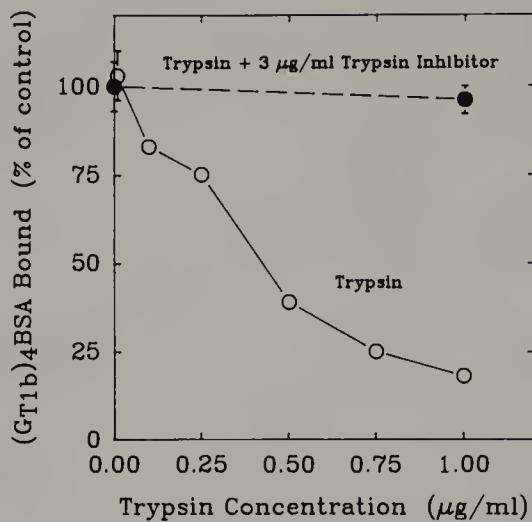
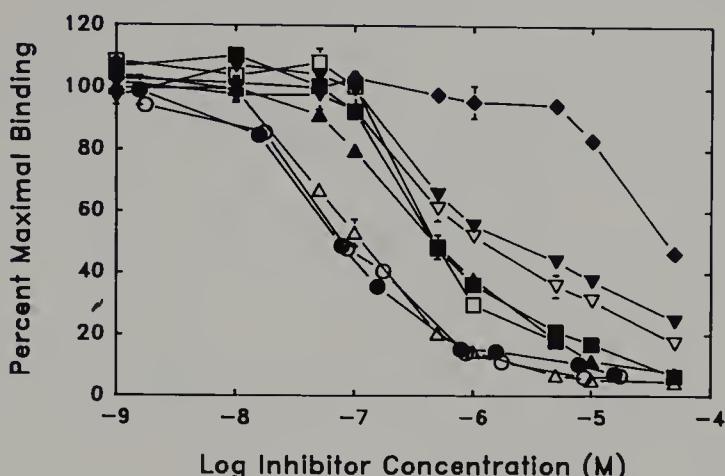
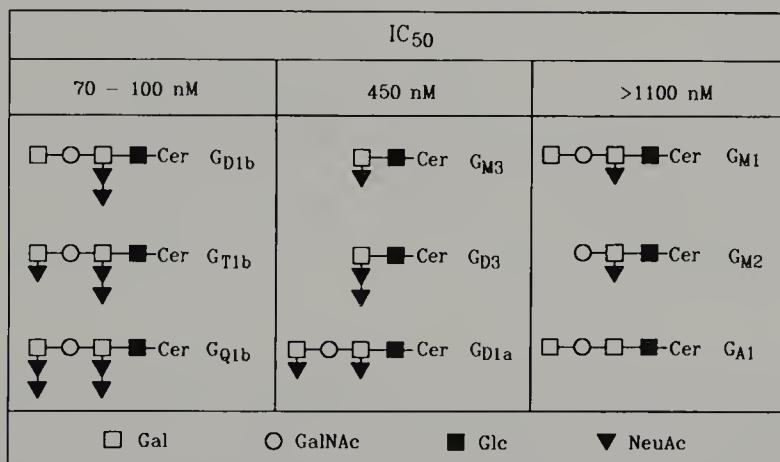


FIG. 5. Detergent-treated brain membranes were filter-adsorbed as described in the legend to Fig. 3. Before incubation with radioligand, filters were incubated with the indicated concentrations of trypsin in phosphate buffered saline for 20 min at 37°C. Proteolysis was stopped by the addition of a five-fold weight excess of soybean trypsin inhibitor after which the filters were washed and placed in scintillation vials containing binding buffer with 0.5 nM  $^{125}\text{I}$ - $(\text{G}_{\text{T}1\text{b}}\text{-}_4\text{BSA})$   $\pm$  10  $\mu\text{M}$   $\text{G}_{\text{T}1\text{b}}$ . In control experiments, membrane-adsorbed filters were pre-incubated in the presence of trypsin plus trypsin inhibitor as indicated. Adapted from Tiemeyer et al. (22).

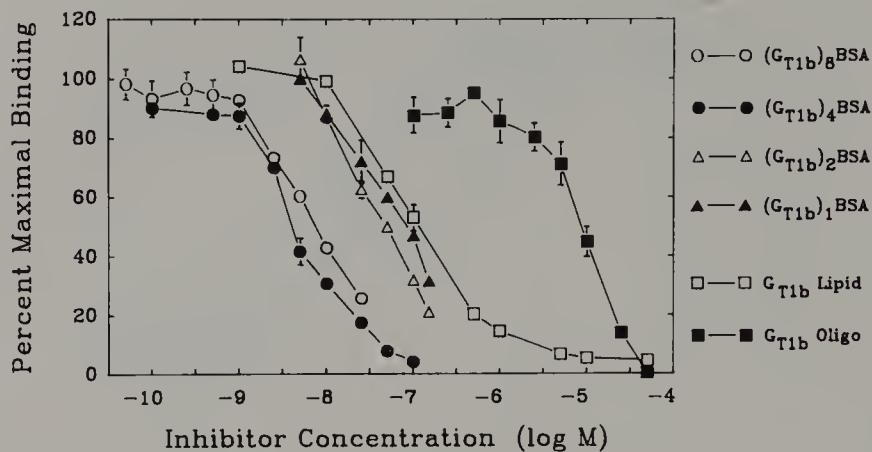
$\text{G}_{\text{D}1\text{b}}$ . Other anionic or neutral sphingolipids including asialo-GM<sub>1</sub>, globotetraosylceramide, sphingomyelin, sphingosine, and psychosine (galactosyl-sphingosine) were inefficient at blocking  $^{125}\text{I}$ - $(\text{G}_{\text{T}1\text{b}}\text{-}_4\text{BSA})$  binding. Phospholipids varied in their effects on neoganglioprotein binding. Addition of 10  $\mu\text{M}$  phosphatidylethanolamine, phosphatidylcholine, or the highly anionic phosphatidylinositol 4,5-bisphosphate did not reduce binding, while phosphatidylglycerol and phosphatidylinositol exhibited IC<sub>50</sub> values of  $\approx$ 500 nM. However, kinetic analyses of the inhibition of  $^{125}\text{I}$ - $(\text{G}_{\text{T}1\text{b}}\text{-}_4\text{BSA})$  binding by gangliosides and phospholipids demonstrated that they act through different inhibitory mechanisms (22): Ganglioside inhibition is competitive and reversible while phospholipid inhibition is noncompetitive and irreversible. These data demonstrate that the  $^{125}\text{I}$ - $(\text{G}_{\text{T}1\text{b}}\text{-}_4\text{BSA})$  binding protein on brain membranes is selective for gangliosides carrying a defined oligosaccharide structure (Fig. 7). This conclusion was supported by inhibition with the oligosaccharide released from  $\text{G}_{\text{T}1\text{b}}$  ( $K_1 = 8 \mu\text{M}$ , Fig. 8). In contrast, the oligosaccharide from GM<sub>1</sub> inhibited only  $\approx$ 20% at 10  $\mu\text{M}$  (the highest concentration tested), and 5 mM Gal, Glc, GalNAc, GlcNAc, NeuAc, lactose, N-acetylglucosamine, and N-acetylgalactosamine were noninhibitory.



**FIG. 6.** Inhibition of  $^{125}\text{I}$ -(GT<sub>1b</sub>)<sub>4</sub>BSA binding to detergent-treated rat brain membranes by gangliosides. Filters adsorbed with 30  $\mu\text{g}$  of rat brain membrane protein (see Fig. 3 legend) were incubated for 90 min at 8°C with 0.5 nM  $^{125}\text{I}$ -(GT<sub>1b</sub>)<sub>4</sub>BSA in 1 ml of binding buffer containing gangliosides at the indicated concentrations. Binding in the presence of added ganglioside is expressed as a percent of the binding measured in the absence of added inhibitor (maximal binding was always comparable to that shown in Fig. 3) and are the mean  $\pm$  S.D. for triplicate determinations at each concentration (S.D. values which fall within the symbols are not shown). The gangliosides tested were: ○, G<sub>Q1b</sub>; △, GT<sub>1b</sub>; ●, GD<sub>1b</sub>; ▲, GD<sub>1a</sub>; □, GD<sub>3</sub>; ■, GM<sub>3</sub>; ▽, GM<sub>2</sub>; ▾, GM<sub>1</sub>; ◆, GA<sub>1</sub>. Adapted from Tiemeyer et al. (22).



**FIG. 7.** Comparison of ganglioside oligosaccharide structures with their potency in inhibiting  $^{125}\text{I}$ -(GT<sub>1b</sub>)<sub>4</sub>BSA binding to detergent-treated rat brain membranes (IC<sub>50</sub> values derived from data in Fig. 6).



**FIG. 8.** Inhibition of  $^{125}\text{I}-(G_{T1b})_4\text{BSA}$  binding to detergent-treated rat brain membranes by neoganglioproteins with different valencies,  $G_{T1b}$ , and the soluble oligosaccharide prepared from  $G_{T1b}$ . Inhibition studies were performed as described in the legend to Fig. 6, using 0.5 nM  $^{125}\text{I}-(G_{T1b})_4\text{BSA}$  and the indicated concentrations of  $^{125}\text{I}-(G_{T1b})_n\text{BSA}$  where  $n = 8, 4, 2$ , or 1. For comparison, inhibition by  $G_{T1b}$  itself and the oligosaccharide removed from  $G_{T1b}$  are presented. Binding data in the presence of inhibitor is expressed as a percent of the binding measured in its absence (maximal binding was always comparable to that shown in Fig. 3) and are the mean  $\pm$  S.D. for triplicate determinations at each concentration (S.D. values which fall within the symbols are not shown). Adapted from Tiemeyer et al. (22).

The most potent protein inhibitor of  $^{125}\text{I}-(G_{T1b})_4\text{BSA}$  binding was unlabeled  $(G_{T1b})_4\text{BSA}$  ( $K_I = 2.6$  nM). Inhibition by other  $G_{T1b}$ -neoganglioproteins varied with the degree of derivatization (Fig. 8). While  $(G_{T1b})_8\text{BSA}$  was equipotent to the tetravalent ligand, the divalent ligand,  $(G_{T1b})_2\text{BSA}$ , was 15-fold less inhibitory and the monovalent ligand,  $(G_{T1b})_1\text{BSA}$ , nearly 30-fold less inhibitory ( $K_I = 46$  nM and 81 nM, respectively). Several other glycoproteins including fetuin, asialofetuin, orosomucoid, asialo-orosomucoid, and myelin basic protein were, at most, weakly inhibitory, with  $IC_{50}$  values 100- to 1000-fold greater than  $(G_{T1b})_4\text{BSA}$  (22).

The relative inhibitory potencies (Fig. 8) of  $G_{T1b}$  (100 nM),  $(G_{T1b})_1\text{BSA}$  (100 nM), and  $G_{T1b}$ -oligosaccharide (8  $\mu\text{M}$ ) support a role for the ceramide portion of the ganglioside in binding to this receptor. All three inhibitors are monovalent under the conditions of the binding assay; the "free" ganglioside exists in Triton micelles, which are approximately equimolar with the ganglioside at its  $IC_{50}$ , and the oligosaccharide does not self-associate in aqueous solution. However,  $G_{T1b}$  itself and  $(G_{T1b})_1\text{BSA}$ , which retains most of the ceramide structure, are 100-fold more potent inhibitors than  $G_{T1b}$  oligosaccharide.

## TISSUE, REGIONAL, AND SUBCELLULAR DISTRIBUTION OF BRAIN GANGLIOSIDE RECEPTORS

The above studies revealed the presence of a specific binding protein for gangliosides in brain but did not address the potential functions of such a receptor. To gather more information about its potential functions, we determined when and where the binding activity appeared in rat brain.

Tissue and regional distribution studies revealed marked specificity for central nervous system (CNS) tissue and differences among the different brain regions (Fig. 9). Rat liver membranes (17) supported <3% as much  $^{125}\text{I}$ -(G<sub>T1b</sub>)<sub>4</sub>BSA binding as brain P2 membranes, and binding of the ligand to peripheral nerve membranes was insignificant. Within the CNS, areas rich in white matter supported more binding than those rich in gray matter. These data suggested that the receptor we found might be associated with myelinated fibers rather than neuronal soma or synaptosomes. Subcellular fractionation supported this hypothesis. Sucrose density separation of myelin (5), followed by osmotic shock and separation of myelin membranes from axolemmal membranes (6) resulted in  $^{125}\text{I}$ -(G<sub>T1b</sub>)<sub>4</sub>BSA binding activity partitioning with the myelin membranes. Ontogenetic studies further support the association of this activity with myelin;  $^{125}\text{I}$ -(G<sub>T1b</sub>)<sub>4</sub>BSA binding appears first

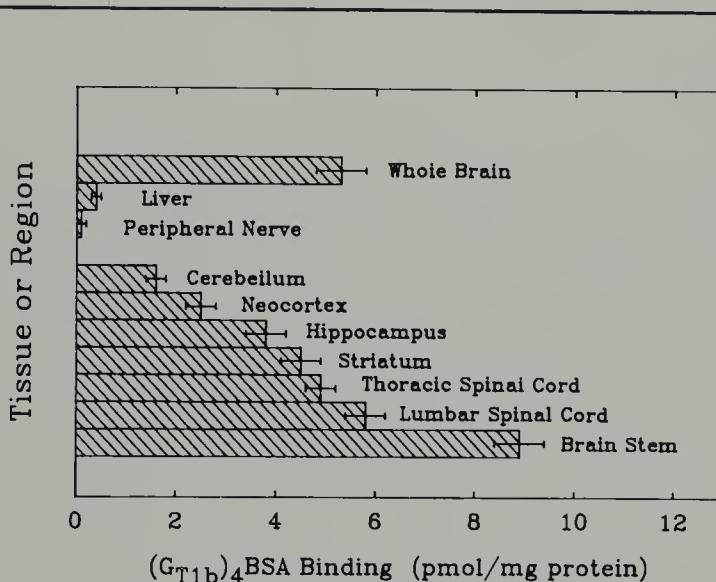


FIG. 9. Tissue and regional distribution of  $^{125}\text{I}$ -(G<sub>T1b</sub>)<sub>4</sub>BSA binding. The binding of 0.5 nM  $^{125}\text{I}$ -(G<sub>T1b</sub>)<sub>4</sub>BSA to detergent-treated rat brain P2 fraction ("Whole Brain", conditions as in Fig. 3) was compared with binding to similarly treated liver plasma membranes (17), membranes prepared from peripheral nerve, and membranes prepared from the indicated anatomic areas dissected from mature rat brain. Binding data are reported as the mean  $\pm$  S.D. for triplicate determinations.

at 15 days postnatal and is half-maximal at  $\approx$ 50 days paralleling appearance of myelination (15). Direct binding of biotinylated  $(G_{T1b})_4$ BSA to sections of adult rat brain revealed predominant ganglioside-specific binding to fiber tracks, notably corpus callosum and the corticospinal tract.

The above data led us to investigate further the nature of the detergent-treated brain membranes used in our studies. Pereyra et al. (16) had recently reported that detergent-treated CNS myelin retained a characteristic membrane lamellar structure while losing the major myelin proteins. Therefore, we subjected our standard membrane preparation (treated with deoxycholate 22) to ultracentrifugation ( $90,000 \times g$ , 30 min). All of the  $(G_{T1b})_4$ BSA binding activity pelleted under these conditions. Solubilization of the pellet in sodium dodecyl sulfate (SDS) followed by SDS polyacrylamide gel electrophoresis revealed that most of the myelin basic protein and proteolipid protein (the major proteins of myelin) were absent from our most active ganglioside receptor preparation. Further detergent treatment of this preparation with 1% Triton X-100 removed all apparent myelin basic protein and proteolipid protein from the membranes, yet half of the  $(G_{T1b})_4$ BSA binding activity was retained in an ultracentrifuged pellet. These data demonstrate that the ganglioside receptor revealed in our study is among the minor detergent-resistant residual proteins of the myelin membrane. At present its functional role in myelin is unknown.

## CONCLUSIONS

The hypothesis that gangliosides modulate cell behavior by interacting with specific cell-surface receptors is supported by studies using a new class of synthetic ganglioside probes, neoganglioproteins. In particular, BSA derivatized with ganglioside  $G_{T1b}$  has well-behaved, high-affinity, and saturable binding kinetics to detergent-treated brain membranes. Because the lipid portion of the ganglioside is sequestered by its covalent attachment to protein, lipid insertion and/or hydrophobic adsorption is reduced or absent when using neoganglioproteins. The resulting binding to a brain membrane protein shows specificity for the ganglioside oligosaccharide structure and is blocked by the soluble oligosaccharide released from  $G_{T1b}$ . One interpretation of the data is that neoganglioprotein binding is to a ganglioside receptor in a *trans* configuration (Fig. 1) which normally interacts with gangliosides on an apposing cell membrane rather than within the same cell membrane. The regional and subcellular distribution and oligosaccharide specificity of the receptor we revealed supports this notion. Because complex gangliosides such as  $G_{T1b}$ ,  $G_{D1b}$ , and  $G_{Q1b}$  predominate in neuronal rather than glial elements of the brain, it was surprising that a receptor with the specificity demonstrated in Fig. 7 was found to be associated regionally, subcellularly, and ontogenically with myelin. Prior studies that separate myelin from associated axolemmal membranes (7) revealed that  $G_{M1}$ , which binds poorly to the receptor we revealed, predom-

inates in myelin (up to 96% of total gangliosides). In contrast, axolemma has relatively high concentrations of G<sub>D1b</sub> and G<sub>T1b</sub>. This leads us to speculate that the myelin ganglioside-binding protein may be involved in *trans* interaction between oligodendroglial cell membranes and neuronal axolemma. More exacting subcellular localization via electron microscopy may resolve whether the ganglioside receptor we revealed is preferentially localized in areas of myelin/axolemma contact. Regardless of its distribution, the role of the ganglioside-binding protein remains unknown.

Because gangliosides have been shown to have direct effects on neuronal cells (3,24,25), we considered the implications of finding a single class of myelin-associated ganglioside receptors in rat brain. We speculate that our findings may result from the nature of our assay. Detergent treatment, used to remove the bulk of endogenous gangliosides and reveal ganglioside receptors, also solubilized >97% of the membrane proteins. Therefore, experimentally, we have probed only for detergent-resistant ganglioside receptors. The possibility that additional, perhaps neuronal, brain ganglioside receptors can be identified among the detergent-solubilized proteins is currently under investigation. To the extent that gangliosides exert their effects on neuronal and non-neuronal cells by interacting with specific ganglioside receptors, neoganglioproteins may prove to be valuable tools in dissecting the underlying molecular mechanisms.

## ACKNOWLEDGMENTS

The authors are grateful to Patti Swank-Hill for valuable technical assistance and to Yoshinobu Yasuda for insightful contributions early in this work. These studies were supported by NIH Grant HD14010 and Training Grant MH18030 (to MT). MT is supported by the Lucille P. Markey Charitable Trust and RLS by the American Cancer Society (FRA-280).

## REFERENCES

1. Blackburn CC, Swank-Hill P, Schnaar RL. Gangliosides support neural retinal cell adhesion. *J. Biol. Chem.* 1986;261:2873–2881.
2. Bremer EG, Schlessinger J, Hakomori S. Ganglioside-mediated modulation of cell growth: specific effects of GM3 on tyrosine phosphorylation of the epidermal growth factor receptor. *J. Biol. Chem.* 1986;261:2434–2440.
3. Byrne MC, Ledeen RW, Roisen FJ, Yorke G, Sclafani JR. Ganglioside-induced neurogenesis: verification that gangliosides are the active agents, and comparison of molecular species. *J. Neurochem.* 1983;41:1214–1222.
4. Chan KJ. Ganglioside-modulated protein phosphorylation. Partial purification and characterization of a ganglioside-stimulated protein kinase in brain. *J. Biol. Chem.* 1987;262:5248–5255.

5. Cotman CW, Matthews DA. Synaptic plasma membranes from rat brain synaptosomes: isolation and partial characterization. *Biochim. Biophys. Acta* 1971;249:380–394.
6. DeVries GH, Matthieu J, Beny M, Chicheportiche R, Lazdunski M, Dolivo M. Isolation and partial characterization of rat CNS axolemma enriched fractions. *Brain Res.* 1978;147:339–352.
7. DeVries GH, Zmachinski CJ. The lipid composition of rat CNS axolemma-enriched fractions. *J. Neurochem.* 1980;34:424–430.
8. Goldenring JR, Otis LC, Yu RK, Delorenzo RJ. Calcium/ganglioside-dependent protein kinase activity in rat brain membranes. *J. Neurochem.* 1985;44:1229–1234.
9. Hakomori S. Glycosphingolipids in cellular interaction, differentiation, and oncogenesis. In: Kanfer JN, Hakomori S, eds. *Sphingolipid Biochemistry*, New York: Plenum Press, 1983; 327–379.
10. Hill AV. The possible effects of the aggregation of the molecules of haemoglobin on its dissociation curves. *J. Physiol. (London)* 1910;40:4–7.
11. Holmgren J, Lonnroth I. Structure and function of enterotoxins and their receptors. In: Ouchterlony O, Holmgren J, eds. *43rd Nobel Symposium: Cholera and Related Diarrheas. Molecular Aspects of a Global Health Problem*, Basel Karger, 1980; 88–103.
12. Kuhlenschmidt TB, Lee YC. Specificity of chicken liver carbohydrate binding protein. *Biochemistry* 1984;23:3569–3575.
13. Lee YC, Stowell CP, Krantz MJ. 2-Imino-2-methoxyethyl 1-thioglycosides: new reagents for attaching sugars to proteins. *Biochemistry* 1976;15:3956–3963.
14. Nojiri H, Kitagawa S, Nakamura M, Kirito K, Enomoto Y, Saito M. Neolacto-series gangliosides induce granulocytic differentiation of human promyelocytic leukemia cell line HL-60. *J. Biol. Chem.* 1988;263:7443–7446.
15. Norton WT, Poduslo SE. Myelination in rat brain: changes in myelin composition during brain maturation. *J. Neurochem.* 1973;21:759–773.
16. Pereyra PM, Horvath E, Braun PE. Triton X-100 extractions of central nervous system myelin indicate a possible role for the minor myelin proteins in the stability of lamellae. *Neurochem. Res.* 1988;13:583–595.
17. Ray TK. A modified method for the isolation of the plasma membrane from rat liver. *Biochim. Biophys. Acta*. 1970;196:1–9.
18. Scatchard G. The attractions of proteins for small molecules and ions. *Ann. N. Y. Acad. Sci.* 1949;51:660–672.
19. Schauer R, Rudiger WV, Sander M, Corfield AP, Wiegandt H. “Neuraminidase-resistant” sialic acid residues of gangliosides. *Adv. Exp. Med. Biol.* 1980;125:283–294.
20. Staros J. N-Hydroxysulfosuccinimide active esters: Bis(N-hydroxysulfosuccinimide) esters of two dicarboxylic acids are hydrophilic, membrane-impermeant, protein cross-linkers. *Biochemistry* 1982;21:3950–3955.
21. Svennerholm L. Gangliosides and synaptic transmission. *Adv. Exp. Med. Biol.* 1980; 125:533–544.
22. Tiemeyer M, Yasuda Y, Schnaar RL. Ganglioside-specific binding protein on rat brain membranes. *J. Biol. Chem.* 1989;264:1671–1681.
23. Toffano G, Benvegnu D, Bonetti AC, Facci L, Leon A, Orlando P, Ghidoni R, Tettamanti G. Interactions of GM1 ganglioside with crude rat brain neuronal membranes. *J. Neurochem.* 1980;35:861–866.
24. Tsuji S, Yamashita T, Nagai Y. A novel, carbohydrate signal-mediated cell surface

- protein phosphorylation: ganglioside GQ1b stimulates ecto-protein kinase activity on the cell surface of a human neuroblastoma cell line, GOTO. *J. Biochem. (Tokyo)* 1988; 104:498-503.
25. Tsuji S, Yamashita T, Tanaka M, Nagai Y. Synthetic sialyl compounds as well as natural gangliosides induce neuritogenesis in a mouse neuroblastoma cell line (Neuro2a). *J. Neurochem.* 1988;50:414-423.



# Bioactive Gangliosides Modulating Transmembrane Signaling

Sen-itiroh Hakomori<sup>1</sup>, Yasuyuki Igarashi<sup>1</sup>, Hisao Nojiri<sup>1</sup>, Eric Bremer<sup>2</sup>, Nobuo Hanai<sup>1,3</sup> and Gustavo A. Nores<sup>1,4</sup>

<sup>1</sup> The Biomembrane Institute and University of Washington, Seattle, WA 98119;

<sup>2</sup> Dept. of Immunology/Microbiology, Rush University, Chicago, IL 60612; <sup>3</sup> Kyowa Hakko Kogyo Co., Tokyo 194, Japan; <sup>4</sup> Institut für Phisiologische Chemie I, Philipps-Universitat-Marburg, D-3550 Marburg/Lahn, West Germany.

**G**lycosphingolipids (GSLs) are functionally bimodal, i.e., a single GSL may play two basically different functions: (1) as a receptor to interact with the outer environment of cells (i.e., with other cells, extracellular matrices, microbes, and toxins); and (2) as a modulator of functional membrane proteins such as growth factor receptors, transducers, and transporters. The latter situation can be regarded as a key mechanism regulating cell proliferation through alteration of transmembrane signaling. In this article, we shall present evidence for the latter view, i.e., effect of gangliosides and their breakdown products on cell growth and the associated modulatory effects of these compounds on receptor-associated tyrosine kinase, PK-C, and other kinases.

## EVIDENCE THAT GANGLIOSIDES MAY REGULATE CELL PROLIFERATION THROUGH TRANSMEMBRANE SIGNALING, AND INDUCE DIFFERENTIATION: A BRIEF OVERVIEW

Several lines of evidence that gangliosides may regulate cell proliferation have accumulated in the past decade and are listed in Table 1. Results from these experimental systems have been interpreted to suggest that gangliosides by themselves may transduce signals stimulating or inhibiting cell growth, or may modulate cell growth through influencing the function of receptors, transducers, or transporters that are essential for cell growth regulation. The effect of endogenous gangliosides stimulated by ligand has been demonstrated through utilization of cholera toxin subunit B, which binds to GM<sub>1</sub> and stimulates 3T3 cell growth in the quiescent state through an unknown mechanism associated with Ca<sup>2+</sup> influx (52,61). The same ligands inhibited growth of transformed cells (52). Cell growth is also mod-

**TABLE 1.** Evidence that glycolipids may regulate cell proliferation

1. Contact inhibition of cell growth accompanies change of glycolipid synthesis ("cell-contact response" of glycolipids) (13, 21, 32, 38, 48, 63).
2. Cell cycle-dependent change of glycolipid organization: Exposure at G1 or G0 phase (19, 40).
3. Butyrate induces cell growth inhibition and enhances GM<sub>3</sub> synthesis (18).
4. Retinoids induce contact inhibition of cell growth and enhance GM<sub>3</sub> synthesis and glycolipid response (45).
5. Antibodies to GM<sub>3</sub> but not to globoside inhibit 3T3 and NIL cell growth and enhance GM<sub>3</sub> synthesis (40); MoAb to GD<sub>3</sub> inhibits melanoma cell growth (14).
6. Cholera toxin subunit B, which binds GM<sub>1</sub>, induces DNA synthesis in quiescent 3T3 cells, while the same ligand induces stimulation of transformed 3T3 cells (52, 54).
7. Sialidase induces and sialidase inhibitor reduces cell proliferation (59–61).
8. Membrane-associated sialidase increases in various tumor cell lines (49), and decreases in association with contact inhibition (63); sialidase inhibitor for GM<sub>3</sub> induces cell growth behavior similar to that of normal cells (23).
9. Exogenous addition of glycolipids incorporated into cell membranes inhibits cell growth through extension of the G1 phase (31, 37).

ulated by anti-ganglioside antibodies (14,15,40) and sialidase (59,60) (Table 1), which may also affect organization, mobility, and quantity of membrane gangliosides, as a consequence triggering a cascade reaction leading to cell growth inhibition or stimulation. Ganglioside-dependent cell growth modulation may well operate by modified signal transduction through the receptor/transducer kinases, since gangliosides are modulators for receptor-associated tyrosine kinases (4,5,24,25), PK-C (26,35), and other kinases (7,8,20) (Table 2). On the other hand, ganglioside-dependent induction of differentiation has been observed, and the inductive phenomenon is increasingly apparent in hematopoietic cells (42,43) and in neuroblastoma (56), although the mechanism of the phenomenon is still unknown. The presence of GQ<sub>1b</sub>-sensitive cell surface protein kinase and its activation could be the cause of GQ<sub>1b</sub>-induced neurite formation in neuroblastoma (58). This research area is still in a stage of ferment and our knowledge is highly fragmented. Various aspects of phenomena involving bioactive gangliosides are summarized in Table 2.

We describe in this chapter a series of our own studies on the role of gangliosides and their breakdown products in modulation of transmembrane signaling.

### **Effect of GM<sub>3</sub> on FGF-dependent Growth of BHK Fibroblasts**

Baby hamster kidney (BHK) fibroblasts are highly dependent on FGF as a growth factor in chemically defined, serum-free medium. FGF-dependent cell growth was strongly inhibited by exogenous addition of GM<sub>3</sub> in chemically defined medium. No other ganglioside was as effective as GM<sub>3</sub> (5). BHK cells precultured in medium containing 20–30 μM GM<sub>3</sub> became refractory to FGF-dependent mitogenesis, and accumulated <sup>125</sup>I-labeled FGF at the cell surface, indicating that internalization of FGF was blocked (Fig. 1), and further evidence for this possibility was presented

TABLE 2. Bioactive gangliosides

Phenomenon	Involved ganglioside	References
Receptor for bacterial toxins	GM <sub>1</sub> (cholera toxin B subunit)	17
Receptor for other bioactive substances (toxins and hormones)	various gangliosides (evidence is ambiguous)	22 (review)
<u>Growth modulator</u>		
FGF-dependent BHK cell growth	GM <sub>3</sub> (NIL cells, growth ↓)	5
PDGF-dependent 3T3 cell growth	GM <sub>1</sub> (Swiss 3T3, growth ↓)	3
EGF-dependent A431 and EB cell growth	GM <sub>3</sub> (A431 human epidermoid carcinoma, growth ↓)	4, 24
B subunit-dependent DNA synthesis	GM <sub>1</sub> (quiescent 3T3, growth ↑, transformed 3T3 and rapidly dividing normal 3T3, growth ↓)	52
EGF-dependent A431 cells	de-N-Ac-GM <sub>3</sub> (A431, growth ↑)	24
Ion-transport modulator	lyso-GM <sub>3</sub> (A431, growth ↓)	25
β-adrenergic receptor	GM <sub>1</sub> (Na <sup>+</sup> transport)	53
	unknown ganglioside coupling with receptor molecule	10, 39
<u>Differentiation-inducer (receptors and involved mechanism unknown)</u>		
Neurite formation in human neuroblastoma cell lines	GQ <sub>1b</sub>	56
Monocytic differentiation in human leukemia HL60, U937	GM <sub>3</sub>	43
Granulocytic differentiation in HL60	neolactogangliosides (nLc-Gg)	42
<u>Modulator for protein kinases</u>		
PK-C	polysialogangliosides (HL60 PK-C ↓) “lysoganglioside” (brain PK-C ↓) GM <sub>3</sub> , lyso-GM <sub>3</sub> (A431 PK-C ↓)	35 26 Igarashi et al., unpubl.
Tyrosine kinase	GM <sub>3</sub> (EGR-R autophosphorylation ↓) lyso-GM <sub>3</sub> (EGF-R autophosphorylation ↓) de-N-Ac-GM <sub>3</sub> (EGF-R autophosphorylation ↑) 2,3-SPG (insulin-R autophosphorylation ↓)	4 25 24 Nojiri et al., unpubl.
Other kinases	GD <sub>1a</sub> (Ca <sup>2+</sup> /CaM-dependent kinase ↑) GQ <sub>1b</sub> (Ca <sup>2+</sup> /GQ <sub>1b</sub> activated kinase ↑) polysialoganglioside-dependent (brain-kinase PKJ ↑) polysialoganglioside-dependent (brain-kinase PKL ↓)	20 57 7 8

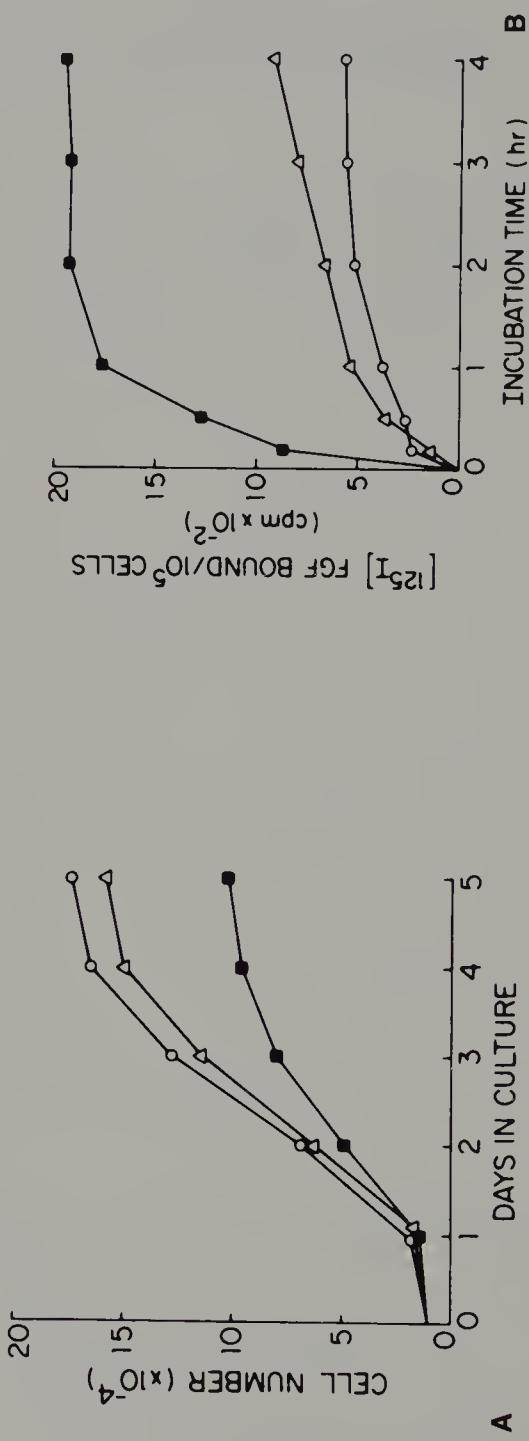


FIG. 1. A: BHK cells were grown in the presence or absence of exogenous ganglioside in chemically defined medium as described previously (5). B: Binding of  $[^{125}\text{I}]$ -labeled FGF to BHK cells cultured in the presence of GM<sub>3</sub> or GM<sub>1</sub> in chemically defined medium as described previously (5).  $[^{125}\text{I}]$ -FGF binding was assayed after four days of cell culture. Nonspecific binding was determined using 500 ng cold FGF, and was  $<10\%$  in all cases. Note the great accumulation of  $[^{125}\text{I}]$ -labeled FGF at the surface of cells whose growth was inhibited by GM<sub>3</sub>, although GM<sub>3</sub> does not bind to FGF directly (data not shown). ○, no ganglioside added. △, cells cultured in the presence of 140  $\mu\text{g}/\text{ml}$  GM<sub>1</sub>. ■, cells cultured in the presence of 100  $\mu\text{g}/\text{ml}$  GM<sub>3</sub>. Each point represents the average of four determinations by Coulter counter.

(5). However, FGF did not interact directly with GM<sub>3</sub>. Properties of the FGF receptor were not well-defined at that time, and further studies on inhibition of FGF-dependent mitogenesis by GM<sub>3</sub> were not conducted.

### Effect of GM<sub>1</sub> and GM<sub>3</sub> on PDGF-dependent Swiss 3T3 Cell Growth and on PDGF Receptor Tyrosine Kinase

A similar approach as applied for FGF-dependent cell growth modification by GM<sub>3</sub> (as described above) was applied to 3T3 cell growth, which is highly dependent on PDGF. In this system, the receptor was well-characterized, and PDGF-dependent 3T3 cell growth is known to be correlated with receptor-associated tyrosine kinase (46,47). PDGF-dependent 3T3 cell growth was inhibited strongly by 5–10 μM GM<sub>1</sub>, and slightly by GM<sub>3</sub>, but not by other gangliosides or neutral GSLs (Fig. 2). GM<sub>1</sub>-fed cells altered (increased) the affinity of the receptor for PDGF. GM<sub>1</sub> and GM<sub>3</sub>, but not other GSLs, inhibited receptor tyrosine kinase activity in a dose-dependent manner (Fig. 3). However, PDGF did not interact directly with GM<sub>1</sub>, GM<sub>3</sub>, or any other ganglioside. Experimental details of these studies are described in the following five paragraphs.

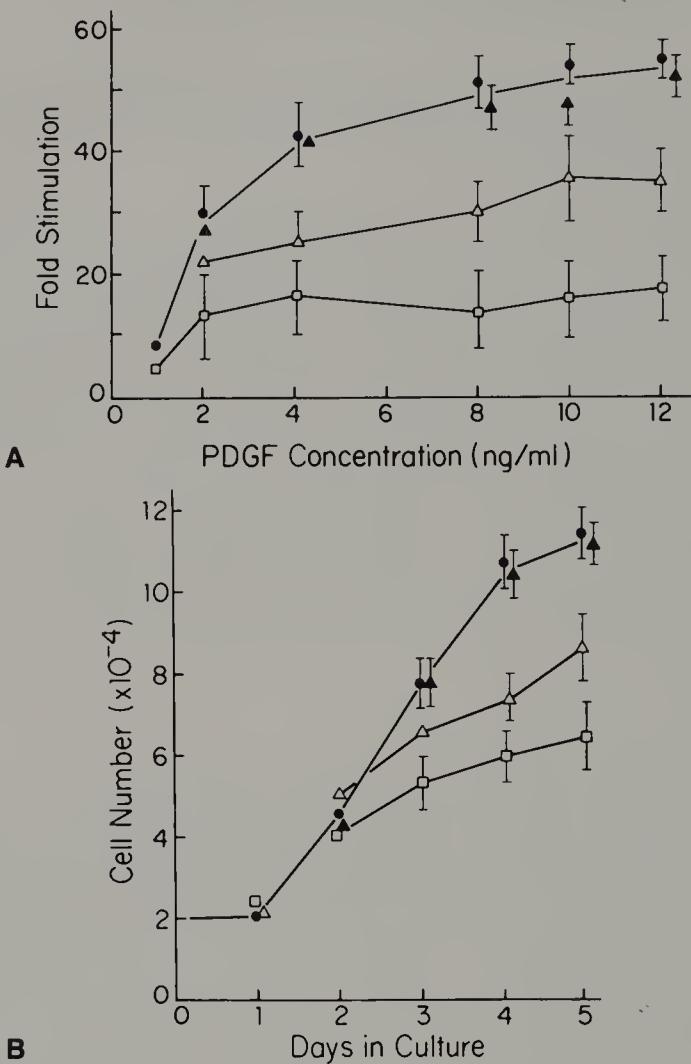
#### Cell Culture

The medium used for cell culture was a mixture of DME and Ham's F-12 medium, in the weight ratio of 3:1, and supplemented with 0.4 g/l of L-histidine and 15 mM HEPES (DME/F-12). Cells were grown in the above basal medium supplemented with 5% FCS and penicillin-streptomycin in a humidified atmosphere of 95% air/5% CO<sub>2</sub>. Serum-free cell growth was in the same basal medium, supplemented with 1 μg/ml of insulin, 1 μg/ml of transferrin, 1 μg/ml of hydrocortisone, 100 μg/ml of fatty acid-free BSA plus linoleic acid, 2 μg/cm<sup>2</sup> of fibronectin, 10 ng/ml of PDGF, and 10 ng/ml of EGF (1). For assays, cells were seeded into 24-well plates (Costar, Cambridge, MA) at a density of 1–2 × 10<sup>4</sup> cells/well.

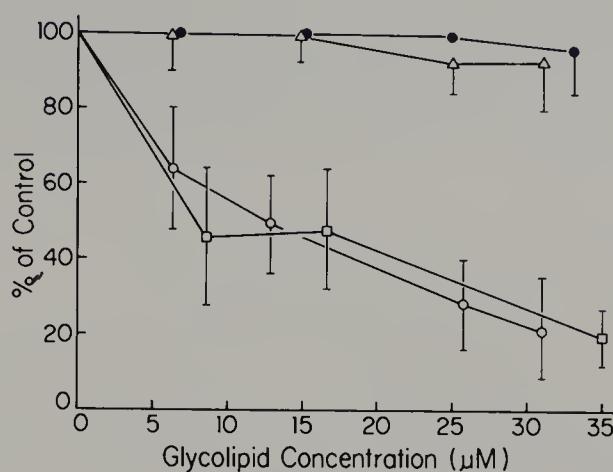
Purified gangliosides were added to cell culture medium as follows. Ganglioside in C/M solution was transferred to a glass screw-cap test tube, evaporated under N<sub>2</sub> stream, and dissolved in 0.5 ml of distilled water with the aid of sonication. This solution was then diluted with 5 ml of DME/F-12, passed through a 0.2-μm sterilizing filter (Gelman, Ann Arbor, MI), and diluted with sterile medium to a final ganglioside concentration of 50 μM.

#### Growth Factors

Highly purified PDGF was isolated from outdated human platelet-rich plasma as described by Raines and Ross (47). EGF and FGF were purchased from Collaborative Research, Waltham, MA.



**FIG. 2. A:** 3T3 cells were seeded into 24-ml plates at a density of  $2 \times 10^4$  cells/well, and grown in serum-free chemically defined DME/F-12 containing no ganglioside (●), 50 nmol/ml of GM<sub>1</sub> (□), 50 nmol/ml of GM<sub>3</sub> (△), or 50 nmol/ml of NeuAcnLc<sub>4</sub> (▲). The medium was changed on days 2 and 4. Each day, cells were harvested with trypsin and numbers were counted. Each data point represents the average of 6 determinations  $\pm$  SD. **B:** 3T3 cells were cultured for 3 days in DME/F-12 supplemented with 5% FCS and containing no ganglioside (●), 50 nmol/ml of GM<sub>1</sub> (□), 50 nmol/ml of GM<sub>3</sub> (△), or 50 nmol/ml of NeuAcnLc<sub>4</sub> (▲). The medium was then replaced with DME/F-12 (without serum) containing various concentrations of PDGF (as indicated in the abscissa) for 48 hr, and incorporation of [<sup>3</sup>H]-thymidine in TCA-insoluble material was measured 18 hr later. The results are plotted as fold stimulation (ratio of [<sup>3</sup>H]-thymidine incorporation in the presence vs. absence of added PDGF) as compared with initial PDGF concentrations. Each data point represents the average of four determinations  $\pm$  SD.



**FIG. 3.** Swiss 3T3 membranes were incubated with 10 nM [ $\gamma$ -<sup>32</sup>P]-ATP and 60 nmol of PDGF with glycolipid at the concentrations indicated in the abscissa for 30 min at 30°C, and proteins were separated by polyacrylamide slab gel electrophoresis (36). After visualization of <sup>32</sup>P-labeled protein by autoradiography, the  $M_r = 170,000$  protein was cut from the gel and counted in a liquid scintillation counter. Results are expressed as a percent of maximum response (glycolipid concentration 0) versus concentration of glycolipid added. Data points represent the mean  $\pm$  SE of at least three determinations. ○, GM<sub>1</sub>; □, GM<sub>3</sub>; ●, Gb<sub>4</sub>; Δ, NeuAcnLc<sub>4</sub>.

#### PDGF-dependent Mitogenesis and its Inhibition by Exogenous Addition of GM<sub>3</sub>

Swiss 3T3 cells were grown in DME/F-12 in the presence or absence of gangliosides, as described above. The confluent cell cultures were pre-incubated for 1–2 days in 1 ml of the medium plus 1% human plasma-derived serum, followed by incubation for 3 days in DME/F-12 plus 5% FCS, with or without addition of 50 nmol/ml of ganglioside. This medium was then replaced by DME/F-12 without serum, but with various concentrations of PDGF (2–12 ng/ml), with or without ganglioside, for 48 hr. Each well was supplemented with 1  $\mu$ Ci/ml of [<sup>3</sup>H]thymidine (60 Ci/mmol quantity; ICM, Irvine, CA). Cells were harvested 18 hr later, washed 3 times with ice-cold 5% TCA, and the TCA-insoluble material was solubilized with 0.5 ml of 1 N NaOH. The base was neutralized with 0.1 ml of glacial acetic acid, 0.5 ml of the solution was added to 5 ml of scintillation mixture (e.g., Formula 963, New England Nuclear), and activity was determined by scintillation counter.

#### Membrane Preparation

Membranes were prepared by scraping the cells, grown in 30–50 150-mm dishes, in the presence of PBS by rubber policeman. The scraped cells were pelleted and

resuspended in 6 ml of 5 mM HEPES (pH 7.4), 5 mM MgCl<sub>2</sub>, and 5 mM  $\beta$ -mercaptoethanol. The cells were then homogenized with 30–50 strokes in a Dounce homogenizer using a tight-fitting pestle, and 2.1 ml of 1 M sucrose were added to the homogenate, which was then transferred to polycarbonate tubes and centrifuged at 100,000 g for 1 hr. The pellet was resuspended for assay in 20 mM HEPES (pH 7.4) and 100 mM NaCl. Membrane protein was determined by fluorescamine protein assay.

### *Phosphorylation Assays and the Effect of Gangliosides*

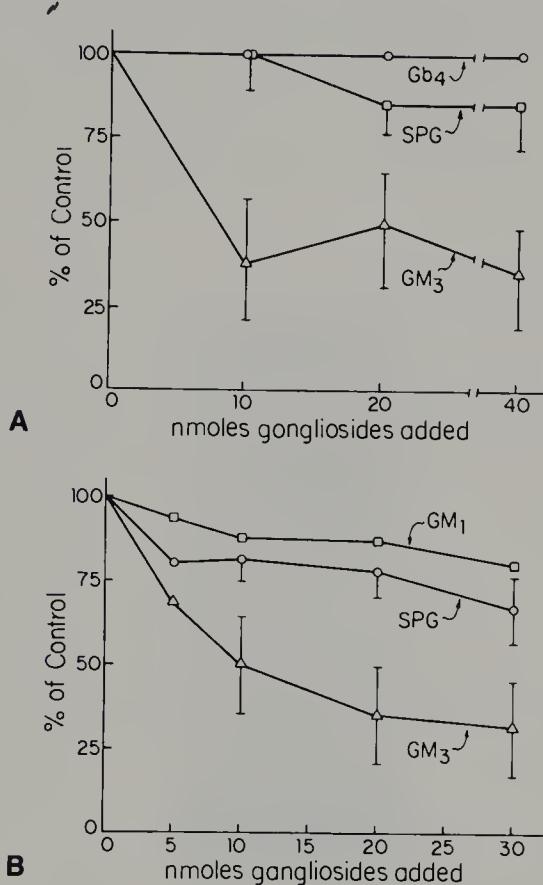
Phosphorylation of membrane proteins by membrane kinase was carried out in a total volume of 40  $\mu$ l containing (in final concentration): 20 mM HEPES (pH 7.4), 100 mM NaCl, 0.2% Triton X-100, 10 mM MnCl<sub>2</sub>, 40  $\mu$ M adenosine 5'-( $\beta,\lambda$ -imino)triphosphate, 5 mM *p*-nitrophenyl phosphate, 10 nM [ $\gamma$ -<sup>32</sup>P]ATP (1000–3000 Ci/mmol), and approximately 30–40  $\mu$ g of membrane protein. In addition, the assay system contained 70 nM PDGF plus 5  $\mu$ g of carrier BSA or 5  $\mu$ g of BSA for controls. These conditions were similar to those described by Pike et al. (46). To observe the effect of gangliosides, various quantities of gangliosides (GM<sub>1</sub>, GM<sub>3</sub>, NeuAcnLc<sub>4</sub>) and Gb<sub>4</sub> in C/M solution were placed in individual test tubes and evaporated to dryness. To each tube, the buffer solution containing Triton X-100 was added and sonicated. The concentration of glycolipid in each test tube varied from 5–30  $\mu$ M. The whole assay system, containing [ $\gamma$ -<sup>32</sup>P]ATP, PDGF, carrier BSA, and many other components as described above, was then added to the solution in each test tube. Assays were started by addition of the membrane suspension and incubated at 30°C for 30 min. The reactions were terminated by addition of 40  $\mu$ l Laemmli sample buffer and subsequently boiled for 3 min prior to application to an 8% polyacrylamide gel (36). To hydrolyze serine or threonine O-phosphate, the gels were fixed and then treated with 1 N NaOH at 50°C for 1 hr according to the method of Cheng and Chen (9), dried, and autoradiographed for 2–8 hr. After visualization of the gel by autoradiography, the region containing the  $M_r = 170,000$  component was cut out of the gel, and the <sup>32</sup>P activity was determined by a liquid scintillation counter.

### **Effect of GM<sub>3</sub> on EGF-dependent Mitogenesis and EGF Receptor Phosphorylation**

#### *Basic Studies*

Studies similar to those described above were extended to EGF-dependent cell growth in human epidermoid carcinoma KB and A431 cell lines, both of which are rich in EGF receptor and show EGF-dependent growth. The EGF receptor has

been well-characterized, and receptor tyrosine kinase activity is associated with the cytoplasmic domain. Growth of both KB and A431 cells was inhibited by GM<sub>3</sub> (15–30  $\mu$ M; 25–50 nmol/ml), and the receptor kinase activity induced by 100 ng EGF was inhibited by 5–40 nmol GM<sub>3</sub> per 40  $\mu$ mol total assay system in a dose-dependent manner (Fig. 4A, B). This phenomenon was reproduced in the immunoprecipitate from anti-EGF receptor antibody affixed on Sepharose beads. Re-

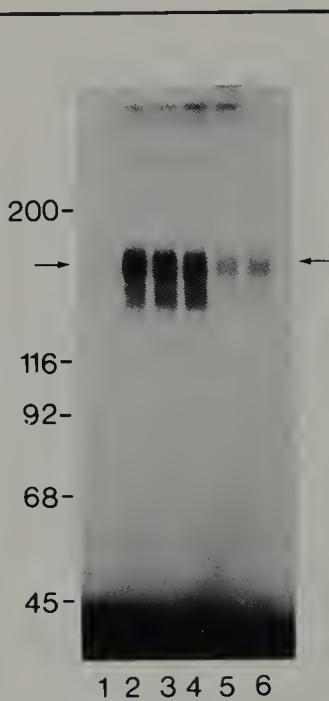


**FIG. 4.** A: A431 cells. A431 membranes were incubated with 100 ng of EGF for 20 min at room temperature and 10 nM [ $\gamma$ -<sup>32</sup>P]-ATP for 10 min at 0°C in the presence or absence of glycolipids as described in the text. After visualization of <sup>32</sup>P-labeled protein by autoradiography, the EGF receptor ( $M_r = 170,000$ ) was cut from the gel and radioactivity detected in a liquid scintillation counter. Results are expressed as a percent of maximum response (glycolipid concentration 0) versus concentration of glycolipid added. Data points represent the average  $\pm$  SE of at least three determinations. B: KB cells. KB cell membranes were incubated with EGF followed by [ $\gamma$ -<sup>32</sup>P]-ATP in the presence or absence of various amounts of glycolipids (GM<sub>3</sub>, GM<sub>1</sub>, and SPG). After visualization of [ $\gamma$ -<sup>32</sup>P]-labeled protein by autoradiography, the activity of the EGF receptor area was determined as described for A.

sults of a typical experiment are shown in Fig. 5. Phosphorylation of tyrosine, but not serine or threonine, was inhibited by GM<sub>3</sub>.

#### Studies with A431 Cell Mutant Clones

Two mutant clones of cell line A431 showing different responses to EGF were utilized in subsequent studies. Clones A1S and A5I, showing EGF-dependent growth stimulation and inhibition respectively, have been well-characterized (25). A1S cells contain GM<sub>3</sub> and the receptor-associated GM<sub>3</sub> can be detected by anti-GM<sub>3</sub> antibody DH2. In contrast, A5I cells do not contain GM<sub>3</sub>, and the isolated receptor did not associate with GM<sub>3</sub> as shown by absence of DH2 reactivity. EGF-dependent growth stimulation was inhibited by exogenous addition of GM<sub>3</sub> in A1S



**FIG. 5.** The EGF receptor was immunoprecipitated from solubilized A431 cell membranes and the immunoprecipitated material was then assayed for EGF-stimulated phosphorylation as described in the text, with the addition of 0.65 mM phosphatidylethanolamine. Glycolipids were included in the reaction mixture as indicated below. After phosphorylation, the samples were subjected to gel electrophoresis, dried, and exposed to x-ray film. The resulting autoradiogram is shown above. Arrow indicates the location of the EGF receptor. Molecular weight standards are shown  $\times 10^{-3}$ . Lane 1, no EGF, no glycolipid; lane 2, 100 ng of EGF, no glycolipid; lane 3, 100 ng of EGF, 7.0 nmol of GM<sub>1</sub>; lane 4, 100 ng of EGF, 14 nmol of GM<sub>1</sub>; lane 5, 100 ng of EGF, 7 nmol of GM<sub>3</sub>; lane 6, 100 ng of EGF, 14 nmol of GM<sub>3</sub>.

cell culture. These findings suggest that EGF receptor function of A5I cells is unrestricted because of the absence of GM<sub>3</sub>. Therefore, addition of EGF induces intracellular ATP exhaustion, which may lead to growth inhibition. In A1S cells, EGF receptor kinase activity is restricted by the presence of GM<sub>3</sub>; however, utilization of kinase through EGF stimulation is efficient and cell growth is stimulated, although the exact mechanism is still unexplored. The clear difference between the two mutant clones (presence vs. absence of GM<sub>3</sub>) indicates a significant role of GM<sub>3</sub> in modulation of EGF-dependent cell growth through modulation of transmembrane signaling by GM<sub>3</sub> (25). Experimental procedures involved in a series of studies of EGF-dependent cell growth and modification by gangliosides are described below.

### Cell Culture

Human oral epidermoid carcinoma KB cells and ovarian epidermoid carcinoma A431 cells were used throughout this study. KB cells have been characterized as having a moderate concentration of a high affinity EGF receptor at the cell surface and show clear EGF-dependent growth stimulation (33). A431 cells contain an unusually high content of the EGF receptor, but only a very small quantity of EGF can stimulate cell growth (30). These cells were grown in DME supplemented with 10% FCS. For experiments that required addition of purified glycolipids to the culture medium, 5% FCS was used (6,51). Purified glycolipids were added to the cell culture medium as follows. The glycolipid in C/M solution was transferred to a glass screw-cap test tube, evaporated under N<sub>2</sub> stream, and dissolved in 0.5 ml of distilled water with the aid of sonication. The glycolipid was then diluted with 5 ml of DME, passed through a 0.2-μm sterilizing filter (Gelman), and diluted with sterile medium to a final concentration of 50 nmol/ml.

### EGF-dependent Mitogenesis and its Inhibition by Exogenous Addition of GM<sub>3</sub>

This experiment was performed with KB cells, because mitogenic stimulation by EGF can be observed more clearly in a wider range of EGF concentrations than found in A431 cells. KB cells were grown in Falcon 24-well plates (15 mm diameter/well; Becton Dickinson, Oxnard, CA) in DME supplemented with 5% FCS with or without addition of ganglioside. Twenty-four hours before determination of mitogenesis, the medium was replaced with DME containing 1% human plasma-derived serum (2) to which different quantities of EGF were added, and the cells were cultured for 18 hr, followed by labeling with 1 μCi/ml [<sup>3</sup>H]thymidine for 2 hr. Cells were washed with PBS and extracted with 5% TCA. The insoluble residue filtered on a Millipore filter was counted.

### Phosphorylation Assays

Phosphorylation of membrane proteins by membrane kinases was carried out in a total volume of 40  $\mu$ l containing (at final concentration): 20 mM HEPES (pH 7.4), 100 mM NaCl, 0.2% Triton X-100, 10 mM MnCl<sub>2</sub>, 40  $\mu$ M adenosine 5'-( $\beta,\gamma$ -imino)triphosphate, 5 mM *p*-nitrophenyl phosphate, 10 nM [ $\gamma$ -<sup>32</sup>P]ATP (1000–3000 Ci/mmol), and approximately 30–40  $\mu$ g of membrane protein. In addition, the assay system contained 100 ng of EGF, plus 5  $\mu$ g of carrier BSA or 5  $\mu$ g of BSA alone for controls. To observe the effect of gangliosides and other lipids, various quantities (5–40 nmol) of gangliosides (GM<sub>1</sub>, GM<sub>3</sub>, NeuAcnLc<sub>4</sub>), Gb<sub>4</sub>, or 17–20  $\mu$ g of phospholipids in C/M solution were placed in individual test tubes and evaporated to dryness under N<sub>2</sub> stream. The lipids were solubilized in 25  $\mu$ l of the buffer containing 0.2% Triton X-100 as above with slight warming (37°C). To each 25- $\mu$ l solution of glycolipid or phospholipid in the above buffer solution was added 10  $\mu$ l of a membrane preparation containing 30–40  $\mu$ g of membrane protein, and this was incubated with or without 100 ng of EGF for 10 min at room temperature before the addition of [ $\gamma$ -<sup>32</sup>P]ATP (11). After 20 min, the assay tubes were cooled to 0°C in an ice bath. [ $\gamma$ -<sup>32</sup>P]ATP in 5  $\mu$ l of buffer solution was then added, and the reaction was allowed to proceed for 10 min at 0°C. The reactions were terminated by addition of 40  $\mu$ l Laemmli sample buffer and subsequently boiled for 3 min prior to application on an 8% polyacrylamide slab gel (36). For some experiments, in which phosphorylated products were purified by immunoadsorption, the reaction was terminated by addition of 0.5 ml of solubilization buffer followed by immunoadsorption as described below.

For some experiments, in which phosphorylation was performed on the isolated EGF receptor by immunoabsorption, the phosphorylation assay was performed identically to that with membrane preparation followed by addition of the anti-EGF receptor antibody-protein A-Sepharose complex (an amount equivalent to 30–40  $\mu$ g of total membrane protein) was used instead of cell membranes added. The beads were centrifuged and treated with 40  $\mu$ l of Laemmli sample buffer, boiled, and applied on slab gel electrophoresis (36) as above (see subsequent section).

To reduce serine or threonine O-phosphate, the gels were fixed and then treated with 1 N NaOH at 50°C for 1 hr according to the method of Cheng and Chen (9). The treated gel was then dried, and phosphoproteins were visualized by autoradiography. After visualization of the gel by autoradiography, the region containing the EGF receptor ( $M_r$  = 150,000–170,000) was excised from the gel, and the <sup>32</sup>P activity was determined with a liquid scintillation counter.

### Immunoabsorption of the EGF Receptor

Cell membranes were solubilized with 1 ml of solubilization buffer, consisting of 20 mM HEPES (pH 7.4), 150 mM NaCl, 1% Triton X-100, 10% glycerol,

0.03%  $\text{NaN}_3$ , and 0.1 unit/ml aprotinin (protease inhibitor, Sigma) for 1 hr on ice. Unsolubilized material was removed by centrifugation. The solubilized material was then incubated with 100  $\mu\text{l}$  of washed Blue Sepharose (Sigma) for 1 hr at 4°C on a rocking table in order to select for adenosine-binding proteins. The Blue Sepharose beads were then washed four times with 1 ml of solubilization buffer. The Blue Sepharose-bound proteins were eluted with 1 M NaCl in solubilization buffer for 1 hr at 4°C. After centrifugation, the eluate from the Blue Sepharose was used for isolation of the EGF receptor by immunoadsorption as described below.

The EGF receptor was immunoadsorbed from the above partially purified preparation by anti-EGF receptor MoAb 29.1 (62) linked to a protein A-Sepharose column (50). Approximately 25  $\mu\text{g}$  of the purified antibody was incubated with 2 mg of protein A-Sepharose 4B (Sigma) in 100  $\mu\text{l}$  of PBS at room temperature for 30 min. The beads were then centrifuged for 1 min in an Eppendorf centrifuge and washed with 0.5 ml of PBS. Aliquots of the Blue Sepharose eluate were diluted five times with PBS containing 0.1% BSA and 10% glycerol, and incubated with the antibody-protein A-Sepharose complex on a rocking table for 2 hr at 4°C. The beads were then washed four times with 1 ml of a solution containing 20 mM HEPES (pH 7.4), 150 mM NaCl, 0.2% Triton X-100, 10% glycerol, and 0.03%  $\text{NaN}_3$ . The washed beads were then used for phosphorylation assay.

### Inhibitory Effect of Lyso-GM<sub>3</sub>, and Stimulating Effect of De-N-Acetyl-GM<sub>3</sub> on EGF Receptor Phosphorylation

The effect of GM<sub>3</sub> on EGF receptor kinase activity is quite clear, as described in the previous section. Part of the GM<sub>3</sub> molecule may be modified, and subsequently exert a modulatory effect on various protein kinases. Two types of modified GM<sub>3</sub> have been detected in A431 cells and other types of cells. One is lyso-GM<sub>3</sub>, which was detected in A431 cells and showed a strong inhibitory effect on receptor kinase activity (24) as well as on PK-C (Igarashi Y, Hakomori S, unpublished). De-N-acetyl-GM<sub>3</sub> was detectable in various tumor cells, and showed a strong promoting effect on EGF receptor kinase activity. Exogenous addition of de-N-acetyl-GM<sub>3</sub> to 3T3 cells (which do not contain endogenous de-N-acetyl-GM<sub>3</sub>) induces cell growth (24). These findings indicate that not only GM<sub>3</sub> but also its breakdown products play important roles in positive or negative modulation of cell growth through receptor kinase or PK-C. The effect of lyso-GM<sub>3</sub> and de-N-acetyl-GM<sub>3</sub> on EGF receptor kinase activity is shown in Figs. 6 and 7.

More recently, we have demonstrated that the effect of GM<sub>3</sub> is greatly enhanced in the presence of lyso-phosphatidylcholine but not lyso-phosphatidylserine or lyso-phosphatidylethanolamine. It is possible that the inhibitory effect in membrane of GM<sub>3</sub> on various kinases is modulated by the presence of lyso-phospholipid. Studies along this line are in progress (Igarashi Y, Hakomori S, *unpublished*). Experimental details are described below.

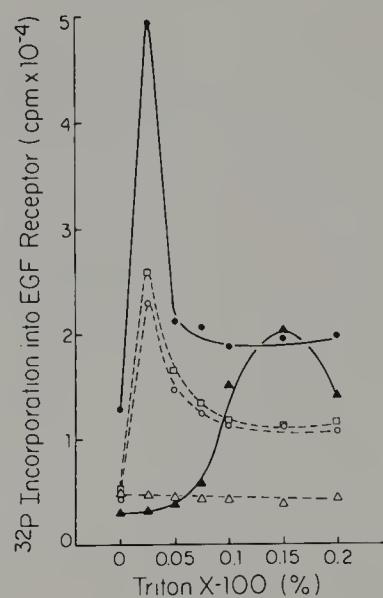


FIG. 6. *In vitro* phosphorylation assay was performed using membranes from A431 cells as described in the text. ○, control without ganglioside addition; □, with 500  $\mu$ M lyso-CDH (lactosylsphingosine); ▲, with 500  $\mu$ M GM<sub>3</sub>; ●, with 250  $\mu$ M de-N-acetyl-GM<sub>3</sub>; Δ, with 500  $\mu$ M lyso-GM<sub>3</sub>.



FIG. 7. The EGF receptor ( $M_r = 170,000$ ) bands were excised from the slab gel (examples as shown in Fig. 4). [<sup>32</sup>P]phosphoamino acids from the EGF receptor band were analyzed by two-dimensional thin-layer electrophoresis as described previously (12). Panel I: A, without EGF; B, with EGF; C, with EGF plus lyso-GM<sub>3</sub>. Panel II: A, with EGF; B, with EGF, C, with EGF and de-N-acetyl-GM<sub>3</sub>.

## Cells and Materials

A431 cells were cultured under the same conditions as described in the preceding section. Confluent cell cultures in 150-mm plastic dishes were scraped, pelleted in phosphate-buffered saline (800g), and resuspended in 7 ml of 20 mM HEPES buffer, pH 7.5, 1 mM EGTA, 0.5 mM phenylmethylsulfonyl fluoride in 250 mM sucrose, and homogenized in a Dounce homogenizer with a tight-fitting pestle (Wheaton Scientific, Millerville, NJ) and centrifuged (3000g, 10 min). The supernatant fraction was centrifuged (100,000g, 1 h), and the pellet was resuspended in 300  $\mu$ l of 20 mM HEPES buffer, pH 7.4. To keep the kinase activity stable, the membrane fraction thus prepared was aliquoted and stored in liquid nitrogen until use. Lyso-GM<sub>3</sub> was prepared by *N*-acetylation of neuraminyllactosylsphingosine when the amino group of sphingosine was protected by inclusion of the compound in liposome (44). Neuraminyllactosylsphingosine was prepared by alkaline hydrolysis of GM<sub>3</sub> in 1 N KOH in 90% *n*-butyl alcohol as described by Taketomi and Kawamura (55). Lactosylsphingosine was prepared by hydrolysis of lactosylceramide under the same conditions as described above (55).

## Phosphorylation Assay of EGF Receptor

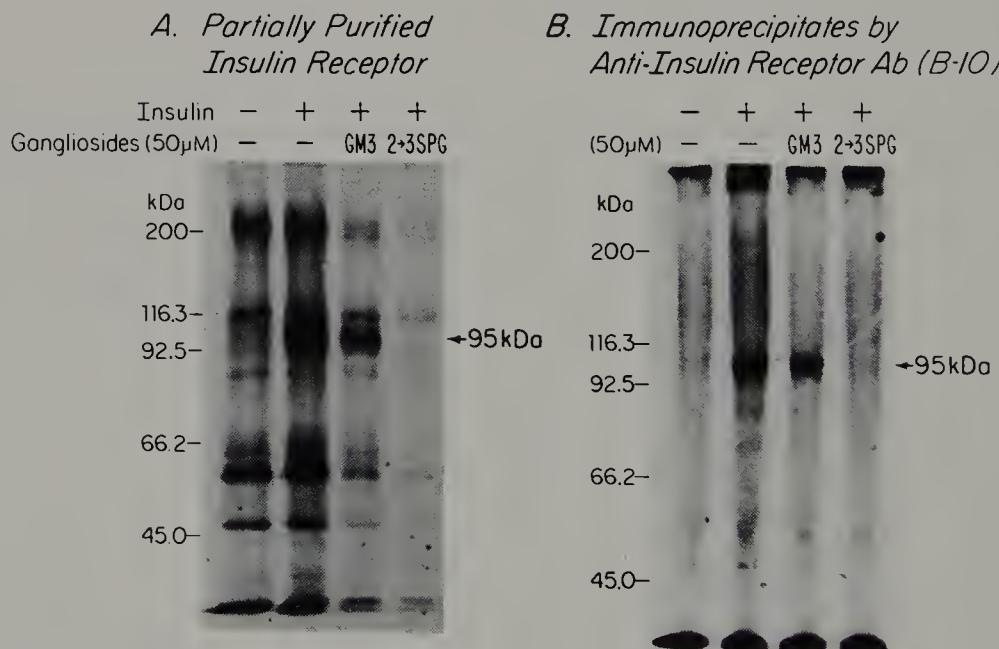
To observe the biphasic effect of GM<sub>3</sub> and lyso-GM<sub>3</sub>, and stimulatory effect of de-*N*-acetyl-GM<sub>3</sub> on EGF receptor phosphorylation, a high concentration of ATP (10  $\mu$ M) was used under various detergent concentrations, as follows. Cell membranes were incubated in the buffer (20 mM HEPES, pH 7.4, 1 mM MnCl<sub>2</sub>, 10  $\mu$ M ZnCl<sub>2</sub>, 30  $\mu$ M NaVO<sub>3</sub>) including 0.33  $\mu$ M EGF (receptor grade; Collaborative Research, Waltham, MA) plus 1.5  $\mu$ M carrier BSA and various concentrations of Triton X-100 (high purity detergent, Pierce Chemical Co.) in the presence or absence of gangliosides for 10 min at 25°C. The reaction was initiated by addition of 1.0 or 10  $\mu$ M [ $\gamma$ -<sup>32</sup>P]ATP (10  $\mu$ Ci) for 10 min at 0°C. The total reaction volume was 50  $\mu$ l, and the amount of membrane protein was 25  $\mu$ g. Reactions were terminated by addition of 50  $\mu$ l of Laemmli's sample buffer (36). Aliquots of the incubation mixture were subjected to 8% sodium dodecyl sulfate(SDS)-polyacrylamide gel electrophoresis. The gel was washed with 1 N NaOH for 15 min at 25°C, then treated with 1 N NaOH for 1 hr at 40°C to reduce serine or threonine O-phosphate (9) and dried, followed by visualization by autoradiography. The region containing the EGF receptor ( $M_r = 170,000$ ) was excised from the gel and the <sup>33</sup>P activity was determined with a liquid scintillation counter.

In a separate experiment, reaction mixtures containing various concentrations of [ $\gamma$ -<sup>32</sup>P]ATP (i.e., 0.1, 0.5, 2.0 and 10.0  $\mu$ M) were tested with a constant detergent concentration (0.05% Triton X-100). Since the specific activity of [ $\lambda$ -<sup>32</sup>P]ATP at the four ATP concentrations was 3300, 516, 119, and 25  $\mu$ Ci/mmol, respectively, decreased kinase activity was attained at the higher concentration of ATP. Although the physiological concentration of ATP is > 10  $\mu$ M, performing

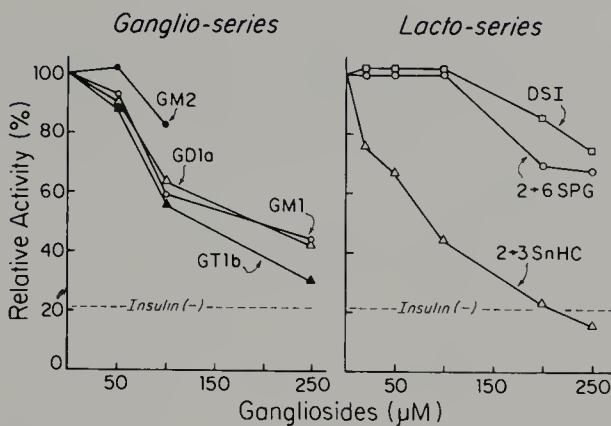
experiments with ATP concentrations  $> 10 \mu\text{M}$  was difficult in practice because of the great decrease in specific activity. Therefore, we used 1.0 or 10  $\mu\text{M}$  ATP concentration throughout these experiments.

### Effect of Gangliosides on Insulin Receptor Kinase Activity

Growth of essentially all animal cells is highly dependent on insulin, and the structure and function of the insulin receptor are well-characterized (29). Therefore, extended studies were directed toward effect of gangliosides on insulin-dependent cell growth. Results of these studies clearly indicate that 2 $\rightarrow$ 3SPG, but not other gangliosides, is the specific inhibitor of insulin-dependent cell growth. Receptor kinase activity was strongly inhibited by 2 $\rightarrow$ 3SPG, even at 1  $\mu\text{M}$  concentration. Results of these studies are shown in Figs. 8 and 9, and experimental details are described in the following four paragraphs.



**FIG. 8.** Autoradiogram of [ $^{32}\text{P}$ ]-labeled insulin receptor fraction partially purified on wheat germ agglutinin column (panel A), and immunoprecipitate by anti-insulin receptor antibody B-10 (panel B). Note that only the 95 kDa band ( $\beta$  subunit of insulin receptor) showed enhanced phosphorylation on addition of insulin. This autophosphorylation was inhibited slightly in the presence of 50  $\mu\text{M}$  GM<sub>3</sub> and completely in the presence of 50  $\mu\text{M}$  2 $\rightarrow$ 3SPG.



**FIG. 9.** Aliquots of insulin receptor fraction were incubated with [ $\gamma^{32}\text{P}$ ]ATP,  $10^{-6}$  M insulin, and various concentrations of several kinds of gangliosides. Incubation mixtures were subjected to polyacrylamide gel electrophoresis under reducing conditions, and the 95 kDa band was excised and counted. Relative activity was expressed as a percent of the same band in the absence of ganglioside.

#### Cells and Cell Culture

Human myeloma cell line IM-9 cells (16) were grown in Falcon 3045 tissue culture flasks (Becton Dickinson Labware, Lincoln Park, NJ) in RPMI 1640 medium supplemented with 10% heat-inactivated ( $56^\circ\text{C}$  for 30 min) FCS at  $37^\circ\text{C}$  in a humidified atmosphere of 5%  $\text{CO}_2$ .

#### Solubilization and Partial Purification of Insulin Receptor

Approximately  $5 \times 10^9$  IM-9 cells were collected and washed with PBS. The cells were solubilized with 40 ml of 1% (v/v) Triton X-100 in 50 mM HEPES buffer (pH 7.4) supplemented with 0.5 trypsin inhibitor units/ml aprotinin and 2 mM PMSF. The suspension was mixed by vortexing and allowed to stand at  $4^\circ\text{C}$  for 40 min with intermittent mixing. The detergent-insoluble material was sedimented by centrifugation at 200,000g for 40 min. The insulin receptors were further enriched by chromatography on a wheat germ agglutinin-agarose column and elution with 0.3 M N-acetylglucosamine in 50 mM HEPES buffer (pH 7.4) containing 0.1% (v/v) Triton X-100 as described by Kasuga et al. (20). Protein concentration was determined with the Bradford reagent (Bio-Rad Co., Richmond, CA) with BSA as a standard.

### Phosphorylation Assay

An appropriate amount of ganglioside was taken in microcentrifuge tubes and evaporated under nitrogen stream. Solubilized insulin receptor (approximately 10 µg protein) was added to the tubes and incubated with  $10^{-6}$  M insulin at 22°C for 1 hr in 55 µl of the buffer (50 mM HEPES, pH 7.4, 5 mM MnCl<sub>2</sub>) in the presence or absence of gangliosides.

Phosphorylation was initiated by adding 5 µl of 100 µM [ $\gamma$ -<sup>32</sup>P]ATP (25 µCi). The final concentration of Triton X-100 was 0.05%, which was reported to be optimal (27). The reaction mixtures were vortexed and incubated for 10 min at 22°C. The reaction was terminated by adding 15 µl of five-fold concentrated Laemmli's sample buffer (36) supplemented with 75 mg/ml dithiothreitol and heating this mixture in a boiling water bath for 3 min. The phosphoproteins were separated by 7.5% SDS-polyacrylamide gel electrophoresis according to the method of Laemmli (36), and visualized by autoradiography.

The phosphorylation corresponding to the  $\beta$  subunit of insulin receptor (95 kDa) was excised from the dried gel, and radioactivity was quantified by a liquid scintillation counter.

### Immunoprecipitation of Phosphorylated Insulin Receptor

The phosphoprotein of 95 kDA was confirmed to be the  $\beta$  subunit of the insulin receptor by immunoprecipitation with anti-insulin receptor antibodies.

The phosphorylation reaction was stopped by cooling the mixture to 4°C and adding 1 ml of 50 mM HEPES buffer (pH 7.4) containing 0.1% Triton X-100, 10 mM sodium fluoride, 4 mM EDTA, and 1 mM sodium vanadate.

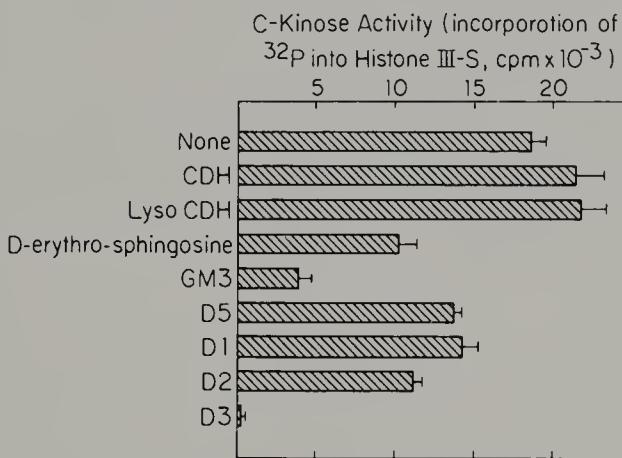
Anti-insulin receptor antibody designated B-10, which was obtained from a patient with an autoimmune form of insulin-resistant diabetes, was added to this mixture at a dilution of 1:300 and incubated at 4°C for 2 hr. The immune complex was precipitated from the solution by incubation with 200 µl of 10% Pansorbin (Calbiochem, Behring Diagnostics, La Jolla, CA) for at least 1 hr. The immunoadsorbed protein is solubilized by Laemmli's sample buffer containing dithiothreitol and resolved by 7.5% polyacrylamide slab gels in the presence of 0.1% SDS as described by Kasuga et al. (29). Resulting phosphoproteins were visualized by autoradiography.

### Effect of GM<sub>3</sub> and its Derivatives on PK-C Activity of A431 Cells

PK-C is a key regulatory element in signal transduction of essentially all animal cells (41). In view of the effect of *sn*-1,2-diacylglycerol and sphingolipids on PK-C, further studies were conducted in this laboratory using various ganglioside de-

rivatives. As shown in Fig. 10, lyso-GM<sub>3</sub> (D3) was the strongest inhibitor of PK-C, followed by GM<sub>3</sub>; D-*erythro*-sphingosine showed only weak inhibitory activity under comparable conditions. Further studies performed recently in this laboratory using sterically well-defined synthetic sphingosine derivatives showed that D-*erythro*- and D-*threo*-sphingosine did not inhibit PK-C activity at 25  $\mu$ M concentration, whereas L-*threo*-sphingosine showed a weak inhibitory activity at this concentration. By far the strongest inhibitory effect was produced by N,N-dimethyl-sphingosine (28). Experimental details are described in the following paragraphs.

A431 cells were grown in a mixture of DME and Ham's F-12 medium (weight ratio 1:1) supplemented with 10% FCS. Cells harvested from 50 150-cm diameter dishes were treated simultaneously for partial purification of PK-C, by the method of Kreutter et al. (35). Briefly, cells were scraped by rubber policeman, suspended in 50 ml of 20 mM Tris-HCl (pH 7.5), 2 mM EDTA, 0.5 mM EGTA, 0.15 U/ml aprotinin, and 0.25 M sucrose, and homogenized by 50 strokes at 4°C in a Dounce homogenizer with a tight-fitting pestle (Wheaton Scientific, Millerville, NJ). The homogenized cells were ultracentrifuged at 100,000g for 60 min. The supernatant



**FIG. 10.** PK-C activity was measured using Histone III-S as substrate under the standard assay conditions described in the text, in the presence (50  $\mu\text{M}$ ) or absence of various glycolipids. Values represent mean  $\pm$  S.E. of three separate experiments. Abbreviations on the ordinate are: CDH, lactosylceramide; lyso-CDH, lactosyl-sphingosine; D-*erythro*-sphingosine, synthetic D-*erythro*-1,3-dihydroxy-2-amino-4,5-*trans*-octadecene (synthetic compound). D1, D2, D3, and D5 are derivatives of GM<sub>3</sub>, identified as follows: D1, de-N-acetyl-GM<sub>3</sub> (amino group of sialic acid is free in GM<sub>3</sub>); D2, de-N-acetyl-lyso-GM<sub>3</sub> (amino groups of both sialic acid and sphingosine are free through complete hydrolysis of the N-acetyl group on sialic acid and N-fatty acyl group on sphingosine); D3, lyso-GM<sub>3</sub> (amino group of sphingosine is free); D5, sia-lyllactosyl-N-acetyl-sphingosine.

was purified by DE52 column equilibrated with 20 mM Tris-HCl (pH 7.5), 2 mM EDTA, and 0.5 mM EGTA (buffer B), and washed well with this buffer. The PK-C activity was eluted with buffer B containing 0.1 M NaCl. The activity in this fraction was 200–500 pmol P/min/mg protein. The fraction, which was free of A-kinase and other kinases, was aliquoted and kept at –80°C.

In view of the extremely variable results obtained in mixed micelle system, as described by Hannun and Bell (26), the standard liposome method described by Kraft and Anderson (34) was slightly modified, and the effect of gangliosides was studied under these conditions. In conical tubes (1.5 ml content, Sarstedt), phosphatidylserine (5 µg/tube) and 1,2-diolein (0.05 µg/tube), with or without an appropriate quantity of ganglioside or its derivatives, were added in organic solvent (ethanol or C/M), and the mixture evaporated under N<sub>2</sub> stream. The lipid mixture was sonicated in 30 µl of 20 mM Tris-HCl (pH 7.5) for 30 min. The liposomes in the tube were supplemented with the reaction mixture, consisting of 25 mM Tris-HCl (pH 7.5), 10 mM MgCl<sub>2</sub>, 400 µM EDTA, 50 µM EGTA, 500 µM CaCl<sub>2</sub>, 200 µg/ml Histone III-S, and 20 µM [ $\gamma$ -<sup>32</sup>P]-ATP ( $2 \times 10^6$  cpm); final volume was 90 µl.

The reaction was initiated by addition of 10 µl of PK-C fraction (containing 1–2 µg protein) prepared as described above, and the reaction mixture incubated for 10 min at 30°C. The reaction was terminated by addition of 1 ml of 25% TCA with 200 µl of 1% BSA in 1 mM ATP solution (pH 7.5). The precipitate was centrifuged, washed twice with 1 ml of 25% TCA, dissolved in 1 ml of 1 N NaOH containing 0.1% deoxycholate with slight heating (80°C for 10 min), and counted in a scintillation counter. The value without phosphatidylserine, 1,2-diolein, or Ca<sup>2+</sup> was used as a reference blank. The effects of various gangliosides and lysoglycolipids are shown in Fig. 10.

## ACKNOWLEDGMENT

This investigation was supported by NIH Outstanding Investigator Grant CA42505 and by funds from The Biomembrane Institute.

## REFERENCES

1. Barnes D, Sato G. Methods for growth of cultured cells in serum-free medium. *Anal. Biochem.* 1980;102:255–270.
2. Bowen-Pope DF and Ross R. Platelet-derived growth factor: II. Specific binding to cultured cells. *J. Biol. Chem.* 1982;257:5161–5171.
3. Bremer E, Hakomori S, Bowen-Pope DF, Raines E, Ross R. Ganglioside-mediated modulation of cell growth, growth factor binding, and receptor phosphorylation. *J. Biol. Chem.* 1984;259:6818–6825.
4. Bremer E, Schlessinger J, Hakomori S. Ganglioside-mediated modulation of cell

- growth: specific effects of GM<sub>3</sub> on tyrosine phosphorylation of the epidermal growth factor receptor. *J. Biol. Chem.* 1986;261:2434–2440.
- 5. Bremer EG, Hakomori S. GM<sub>3</sub> ganglioside induces hamster fibroblast growth inhibition in chemically defined medium: ganglioside may regulate growth factor receptor function. *Biochem. Biophys. Res. Commun.* 1982;106:711–718.
  - 6. Callies R, Schwarzmüller G, Radsak K, Siegert R, Weigandt H. Characterization of the cellular binding of exogenous gangliosides. *Eur. J. Biochem.* 1977;80:425–432.
  - 7. Chan K-FJ. Ganglioside-modulated protein phosphorylation: partial purification and characterization of a ganglioside-stimulated protein kinase in brain. *J. Biol. Chem.* 1987;262:5248–5255.
  - 8. Chan K-FJ. Ganglioside-modulated protein phosphorylation: partial purification and characterization of a ganglioside-inhibited protein kinase in brain. *J. Biol. Chem.* 1988;263:568–574.
  - 9. Cheng YSE, Chen LB. Detection of phosphotyrosine-containing 34,000-dalton protein in the framework of cells transformed with Rous sarcoma virus. *Proc. Natl. Acad. Sci. USA* 1981;78:2388–2392.
  - 10. Chorev M, Feigenbaum A, Keenan AK, Gilon C, Levitzki A. N-bromoacetyl-amino-cyanopindolol: a highly potent beta-adrenergic affinity label blocks irreversibly a non-protein component tightly associated with the receptor. *Eur. J. Biochem.* 1985;146:9–14.
  - 11. Cohen S, Carpenter G, King L. Epidermal growth factor receptor-protein kinase interactions: co-purification of receptor and epidermal growth factor-enhanced phosphorylation activity. *J. Biol. Chem.* 1980;255:4834–4842.
  - 12. Cooper JA, Sefton BM, Hunter T. Detection and quantification of phosphotyrosine in proteins. *Meth. Enzymol.* 1983;99:387–402.
  - 13. Critchley DR, Macpherson IA. Cell density-dependent glycolipids in NIL2 hamster cells derived from malignant and transformed cell lines. *Biochim. Biophys. Acta.* 1973;296:145–159.
  - 14. Dippold WG, Knuth A, zum Buschenfelde K-HM. Inhibition of human melanoma cell growth *in vitro* by monoclonal anti-GD<sub>3</sub>-ganglioside antibody. *Cancer Res.* 1984;44:806–810.
  - 15. Dohi T, Notes G, Hakomori S. An IgG<sub>3</sub> monoclonal antibody established after immunization with GM<sub>3</sub> lactone: immunochemical specificity and inhibition of melanoma cell growth *in vitro* and *in vivo*. *Cancer Res.* 1988;48:5680–5685.
  - 16. Fahey JL, Buell DN, Sox HC. Proliferation and differentiation of lymphoid cells: studies with human lymphoid cell lines and immunoglobulin synthesis. *Ann N. Y. Acad. Sci.* 1972;190:221–234.
  - 17. Fishman PH, Brady RO. Biosynthesis and function of gangliosides. *Science* 1976;194:906–915.
  - 18. Fishman PH, Simmons JL, Brady RO, Freese E. Induction of glycolipid synthesis by sodium butyrate in HeLa cells. *Biochem. Biophys. Res. Commun.* 1974;59:292–299.
  - 19. Gahmberg CG, Hakomori S. Surface carbohydrates of hamster fibroblasts: I. Chemical characterization of surface-labeled glycosphingolipids and a specific ceramide tetrasaccharide for transformants. *J. Biol. Chem.* 1975;250:2438–2446.
  - 20. Goldenring JR, Otis LC, Yu RK, DeLorenzo RJ. Calcium/ganglioside-dependent protein kinase activity in rat brain membrane. *J. Neurochem.* 1985;44:1229–1234.
  - 21. Hakomori S. Cell density-dependent changes in glycolipid concentrations in fibroblasts, and loss of this response in virus-transformed cells. *Proc. Natl. Acad. Sci. USA* 1970;67:1741–1747.

22. Hakomori S. Glycosphingolipids in cellular interaction, differentiation and oncogenesis. *Annu. Rev. Biochem.* 1981;50:733–764.
23. Hakomori S, Young, Jr WW, Patt LM, Yoshino T, Halfpap L, Lingwood CA. Cell biological and immunological significance of ganglioside changes associated with transformation. In: Svennerholm L, Dreyfus H, Urban P-F, eds. *Structure and function of gangliosides*. New York: Plenum Press, 1980; 247–261.
24. Hanai N, Dohi T, Nores GA, Hakomori S. A novel ganglioside, de-N-acetyl-GM<sub>3</sub> (II<sup>3</sup>NeuNH<sub>2</sub>LacCer), acting as a strong promoter for epidermal growth factor receptor kinase and as a stimulator for cell growth. *J. Biol. Chem.* 1988;263:6296–6301.
25. Hanai N, Nores GA, MacLeod C, Torres-Mendez CR, Hakomori S. Ganglioside-mediated modulation of cell growth: specific effects of GM<sub>3</sub> and lyso-GM<sub>3</sub> in tyrosine phosphorylation of the epidermal growth factor receptor. *J. Biol. Chem.* 1988;263:10915–10921.
26. Hannun YA, Bell RM. Lysosphingolipids inhibit protein kinase C: implications for the sphingolipidoses. *Science* 1987;235:670–674.
27. Hwang DL, Tay Y-C, Barseghian G, Roitman A, Lev-Ram A. Effect of Triton X-100 on insulin and epidermal growth factor receptor binding and autophosphorylation in Golgi fractions and partially purified receptors from rat liver. *J. Receptor Res.* 1985; 5:367–380.
28. Igarashi Y, Hakomori S, Toyokuni T, Dean B, Fujita S, Sugimoto M, Ogawa T, El-Ghendy K, Racker E. Effect of chemically well-defined sphingosine and its N-methyl derivatives on protein kinase C and src kinase activities. *Biochemistry* 1989;28:6796–6800.
29. Kasuga M, White MF, Kahn CR. Phosphorylation of the insulin receptor in cultured hepatoma cells and a solubilized system. *Meth. Enzymol.* 1985;109:609–621.
30. Kawamoto T, Sato JD, Le A, Polikoff J, Sato GH, Mendelsohn J. Growth stimulation of A431 cells by epidermal growth factor: identification of high-affinity receptors for epidermal growth factor by an anti-receptor monoclonal antibody. *Proc. Natl. Acad. Sci. USA* 1983;80:1337–1341.
31. Keenan TW, Schmid E, Franke WW, Weigandt H. Exogenous ganglioside suppresses growth rate of transformed and untransformed 3T3 mouse cells. *Exp. Cell Res.* 1975; 92:259–270.
32. Kijimoto S, Hakomori S. Enhanced glycolipid:alpha-galactosyltransferase activity in contact-inhibited hamster cells, and loss of this response in polyoma transformants. *Biochem. Biophys. Res. Commun.* 1971;44:557–563.
33. King AC, Cuatrecasas P. Resolution of high and low affinity epidermal growth factor receptors: inhibition of high affinity component by low temperature, cycloheximide and phorbol esters. *J. Biol. Chem.* 1982;257:3053–3060.
34. Kraft AS, Anderson WB. Phorbol esters increase the amount of Ca<sup>2+</sup>, phospholipid-dependent protein kinase associated with plasma membrane. *Nature* 1983;301:621–623.
35. Kreutter D, Kim JYH, Goldenring JR, Rasmussen H, Ukomadu C, DeLorenzo RJ, Yu RK. Regulation of protein kinase C activity by gangliosides. *J. Biol. Chem.* 1987; 262:1633–1637.
36. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;227:680–685.
37. Laine RA, Hakomori S. Incorporation of exogenous glycosphingolipids in plasma membranes of cultured hamster cells and concurrent change of growth behavior. *Biochem. Biophys. Res. Commun.* 1973;54:1039–1045.

38. Langenbach R, Kennedy S. Gangliosides and their cell density-dependent changes in control and chemically transformed C3H/10T1/2 cells. *Exp. Cell Res.* 1978;112:361–372.
39. Levitzki A. From epinephrine to cyclic AMP. *Science* 1988;241:800–806.
40. Lingwood C, Hakomori S. Selective inhibition of cell growth and associated changes in glycolipid metabolism induced by monovalent antibodies to glycolipids. *Exp. Cell Res.* 1977;108:385–391.
41. Nishizuka Y. The role of protein kinase C in cell surface signal transduction and tumour promotion. *Nature* 1984;308:693–698.
42. Nojiri H, Kitagawa S, Nakamura M, Kiritó K, Enomoto Y, Saito M. Neolacto-series gangliosides induce granulocytic differentiation of human promyelocytic leukemia cell line HL-60. *J. Biol. Chem.* 1988;263:7443–7446.
43. Nojiri H, Takaku F, Terui Y, Miura Y, Saito Y. Ganglioside GM<sub>3</sub>: an acidic membrane component that increases during macrophage-like cell differentiation can induce monocytic differentiation of human myeloid and monocytoid leukemic cell lines HL-60 and U937. *Proc. Natl. Acad. Sci. USA* 1986;83:782–786.
44. Nores GA, Hanai N, Levery SB, Eaton HL, Salyan MEK, Hakomori S. Synthesis and characterization of lyso-GM<sub>3</sub> (II<sup>3</sup>Neu5Ac lactosyl sphingosine), de-N-acetyl-GM<sub>3</sub> (II<sup>3</sup>NeuNH<sub>2</sub> lactosyl Cer), and related compounds. *Carbohydr. Res.* 1988;179:393–410.
45. Patt L, Itaya K, Hakomori S. Retinol induces density-dependent growth inhibition and changes in glycolipids and LETS. *Nature* 1978;273:379–381.
46. Pike JL, Bowen-Pope DF, Ross R, Krebs EG. Characterization of platelet-derived growth factor-stimulated phosphorylation in cell membranes. *J. Biol. Chem.* 1983;258:9383–9390.
47. Raines EW, Ross R. Platelet-derived growth factor: I. High yield purification and evidence for multiple forms. *J. Biol. Chem.* 1982;257:5154–5160.
48. Sakiyama H, Gross SK, Robbins PW. Glycolipid synthesis in normal and virus-transformed hamster cell lines. *Proc. Natl. Acad. Sci. USA* 1972;69:872–876.
49. Schengrund, C-L, Lausch RN, Rosenberg A. Sialidase activity in transformed cells. *J. Biol. Chem.* 1973;248:4424–4428.
50. Schneider C, Newman RA, Sutherland DR, Asser U, Greaves MF. A one-step purification of membrane proteins using high efficiency immunomatrix. *J. Biol. Chem.* 1982;257:10766–10769.
51. Schwarzmann G, Hoffmann-Bleithäuser P, Schubert J, Sandhoff K, Marsh D. Incorporation of ganglioside analogues into fibroblast cell membranes: a spin-label study. *Biochemistry* 1983;22:5041–5048.
52. Spiegel S, Fishman PH. Gangliosides as bimodal regulators of cell growth. *Proc. Natl. Acad. Sci. USA* 1987;84:141–145.
53. Spiegel S, Handler JS, Fishman PH. Gangliosides modulate sodium transport in cultured toad kidney epithelia. *J. Biol. Chem.* 1986;261:15755–15760.
54. Spiegel S, Panagiotopoulos C. Mitogenesis of 3T3 fibroblasts induced by exogenous ganglioside is not mediated by cAMP, protein kinase C, or phosphoinositide turnover. *Exp. Cell Res.* 1988;177:414–427.
55. Taketomi T, Kawamura N. Preparation of lysohematoside (neuraminy-galactosyl-glucosylsphingosine) from hematoside of equine erythrocyte and its chemical and hemolytic properties. *J. Biochem. (Tokyo)* 1970;68:475–485.
56. Tsuji S, Arita M, Nagai Y. GQ<sub>1b</sub>, a bioactive ganglioside that exhibits novel nerve

- growth factor (NGF)-like activities in the two neuroblastoma cell lines. *J. Biochem. (Tokyo)* 1983;94:303–306.
- 57. Tsuji S, Nakajima J, Sasaki T, Nagai Y. Bioactive gangliosides: IV. Ganglioside GQ<sub>1b</sub>/Ca<sup>++</sup> dependent protein kinase activity exists in the plasma membrane fraction of neuroblastoma cell line, GOTO. *J. Biochem. (Tokyo)* 1985;97:969–972.
  - 58. Tsuji S, Yamashita T, Nagai Y. A novel, carbohydrate signal-mediated cell surface protein phosphorylation: ganglioside GQ<sub>1b</sub> stimulates ecto-protein kinase activity on the cell surface of a human neuroblastoma cell line, GOTO. *J. Biochem (Tokyo)* 1988; 104:498–503.
  - 59. Usuki S, Hoops P, Sweeley CC. Growth control of human foreskin fibroblasts and inhibition of extracellular sialidase activity by 2-deoxy-2,3dehydro-N-acetylneuramnic acid. *J. Biol. Chem.* 1988;263:10595–10599.
  - 60. Usuki S, Lyu S-C, Sweeley CC. Sialidase activities of cultured human fibroblasts and the metabolism of GM<sub>3</sub> ganglioside. *J. Biol. Chem.* 1988;263:6847–6853.
  - 61. Vaheri A, Ruoslahti E, Nordline S. Neuraminidase stimulates division and sugar uptake in density-inhibited cell cultures. *Nature (New Biol.)* 1972;238:211–212.
  - 62. Yarden Y, Harari I, Schlessinger J. Purification of an active EGF receptor kinase with monoclonal antireceptor antibodies. *J. Biol. Chem.* 1985;260:315–319.
  - 63. Yogeeshwaran G, Hakomori S. Cell contact-dependent ganglioside changes in mouse 3T3 fibroblasts and a suppressed sialidase activity on cell contact. *Biochemistry* 1975; 14:2151–2156.

### **III. Post-receptor Phenomena**



# Neuronal Plasticity and Melanocortins

R. Gerritsen van der Hoop and W. H. Gispen

*• Division of Molecular Neurobiology*

*Rudolf Magnus Institute and Institute of Molecular Biology and Biotechnology  
University of Utrecht, Vondellaan 6, Utrecht, The Netherlands*

**M**elanocortins are peptides related to the pituitary hormones ACTH and  $\alpha$ -MSH. As pituitary hormones, both  $\alpha$ -MSH and ACTH mediate adaptive responses to changes in the environment, either by changes in skin color or alterations in energy metabolism. These same peptides are also known to be neuropeptides, that is, they are both synthesized in, and have effects on, neurons. The proposal that nerve tissue can be considered as a target for melanocortins was suggested by De Wied from studies on the effects of these agents on behavioral parameters (for a review see 18). Acute (short-term) effects on the electrical functions of motor neurons have been shown in the cat spinal cord by Krivoy et al. (42), frog neuromuscular preparations (40) and in the motor system of immature and hypophysectomized rats (30,53,55).

Many peptide hormones have long-term trophic influences on the target cells in which they also exert short-term functional effects. In nerve tissue, a trophic influence of melanocortins is evidenced by increased macromolecular synthesis under certain conditions (6,19). A vast array of effects of melanocortins on neural function have been described, all having in common "an enhancement of neural plasticity" (29), thus permitting behavioral adaptations to environmental change. At the simplest and most direct level, neural plasticity can be seen as the ability to overcome direct damage to the nervous system, and it is this aspect of melanocortin action which is emphasized here. The ability of melanocortins to facilitate repair after mechanical damage (cut or crush lesion) in experimental models has been demonstrated by measuring the recovery in functional, electrophysiological, and histological terms (see below). The potential applicability of the effects of melanocortins in experimental models to clinical problems requires careful consideration.

## MELANOCORTINS AND NEURONAL DEVELOPMENT

Postlesion neuronal repair is often viewed as a rapid replay of those cellular processes that govern neuronal development, maturation, and network formation.

Thus humoral factors that influence neuronal development may be of benefit to neuronal repair mechanisms in adult life. Recently, several authors have shown that ACTH/MSH-like peptides influence the growth and differentiation of central neurons in culture. Daval et al. (11) treated chick cerebral neurons with ACTH<sub>1-24</sub> and observed an enhancement of cell metabolism in general, and increased neurite formation. The effect appeared to be dose-dependent, with a maximum at 10 nM ACTH<sub>1-24</sub>. Azmitia and De Kloet (1) used cultures of dissociated rat hippocampal and raphe cells. They showed an enhancement of serotonergic maturation by various ACTH-like peptides (ACTH<sub>1-24</sub>, ACTH<sub>4-10</sub>, and Org 2766, a synthetic ACTH<sub>4-9</sub> analog with the structure-formula: Met-O<sub>2</sub>-Glu-His-Phe-D-Lys-Phe), provided that the raphe cells were cultured alone. In the presence of the natural hippocampal target cells of these raphe cells no effect of the peptides was observed, suggesting that they may exert their neurotrophic influence on raphe cells via the same mechanisms as the hippocampal-soluble protein that normally stimulates the outgrowth of raphe cells (1). Rigter-Landsberg et al. (50) studied the effect of ACTH<sub>4-10</sub> and ACTH<sub>1-24</sub> on the growth and development of embryonic rat cerebral cells in culture. Long-term peptide treatment resulted in an increase in the density of the neuronal network, in neuronal aggregates, and neurite bundles. At the same time a 30% increase in acetylcholinesterase activity was observed. All these changes were prominent in the presence of 10 nM peptide and reached a maximum during the second week of culture. Van der Neut et al. (10) used slices of fetal rat spinal cord in culture to establish neurotrophic effects of  $\alpha$ -MSH and ACTH<sub>4-10</sub>. After five and seven days, outgrowth was quantified with two different techniques, namely a visual scoring under phase contrast and by means of an ELISA for neurofilament protein. Both peptides stimulated in a dose-dependent way the formation of neurites by 30–40% in the subnanomolar range.

Earlier studies had already pointed to neurotrophic effects of melanocortins in neuronal development *in vivo*. In a series of experiments Swaab and co-workers (58) have obtained evidence that  $\alpha$ -MSH but no other ACTH-related peptides or Org 2766 affects intrauterine fetal rat body and brain growth. Interestingly, the trophic effect of  $\alpha$ -MSH was not detected when the peptide was given in the first weeks of postnatal life (see for review, 58). Van der Helm-Hylkema and De Wied (59) reported a stimulatory effect of ACTH-frgaments (ACTH<sub>1-39</sub>, 1-24, 1-18, 1-16) on eye opening of both male and female rat pups. The shorter peptides  $\alpha$ -MSH, ACTH<sub>1-10</sub>, ACTH<sub>4-10</sub>, or Org 2766 were not effective. How ACTH affects eye opening is not clear, although it is tempting to assume that it is brought about by a direct effect on the developing rat CNS. In another series of experiments Strand and co-workers reported a beneficial effect of ACTH<sub>4-10</sub> and Org 2766 on perinatal development of rat motor function and neuromuscular junction. Their neurophysiological data suggested an improvement by a neurotrophic effect of the peptide, although the effect on endplate maturation left the possibility of a myotrophic action (see ref. 56 for review).

## MELANOCORTINS AND CNS PLASTICITY

Early studies of the effect of melanocortins on recovery from CNS lesion gave conflicting results (for review see 2). Peptides with full endocrine activity were used, since the rationale behind the treatment was the reduction of reactive scar tissue, believed to limit CNS regeneration. However, studies on the peripheral nervous system (see below) have shown that the immunosuppressant and corticotrophic activities were not involved in the trophic response and may have disturbed the final outcome of previous CNS studies.

Presently, several reports have been published that suggest that the effects of damage in the central nervous system may be reduced by treatment with melanocortins. Part of such effects deal with compensatory mechanisms. Flohr and Luneberg (24) showed that systemic treatment with ACTH<sub>4-10</sub> improved the acquisition and maintenance of the compensated state after unilateral labyrinthectomy in *Rana temporaria*. These authors have also shown that similar effects could be obtained with  $\alpha$ -MSH, the synthetic ACTH analogs Org 2766 ACTH (4-9) and Org 5041 ACTH (4-16), ACTH<sub>4-7</sub>, ACTH<sub>4-10</sub> and ACTH<sub>1-10</sub> (45). Likewise, Igarashi et al. (34,37) reported that ACTH<sub>4-10</sub> modified the characteristics and time courses of vestibulo-spinal and vestibulo-oculomotor balance compensation after labyrinthectomy in the squirrel monkey. ACTH<sub>4-10</sub> appeared to contribute to the organic repair or remodelling of the neural network related to the motor output system (37).

Isaacson and Poplawsky (38,39) used the disappearance of hyperemotionality of rats, induced by septal area lesions, as an index of functional recovery from brain damage. The peptides ACTH<sub>4-10</sub> and Org 2766, given subcutaneously for four consecutive days beginning immediately after surgery, resulted in a smaller than usual lesion-induced increase in emotionality scores. The Org 2766 treatment also facilitated the return to normal values over subsequent days after surgery. Weeks after the daily test for emotionality, the animals were trained on a two-way active avoidance task. The typical increase in avoidance performance seen in animals with septal lesions was observed in previously Org 2766- or ACTH<sub>4-10</sub>-treated animals. Only the prior Org 2766 treatment reduced the number of intertrial responses that are typical of the septal lesion.

The beneficial effects of melanocortins after CNS lesions also became apparent from studies using lesions of the parafascicular nucleus in the rat (47). Animals had been treated daily with  $\alpha$ -MSH or Org 2766 for two weeks starting on the third day after surgery acquired a reversal learning deficit. This observation was corroborated by the fact that at the end of the treatment period, the abnormal grasping response that results from a parafascicular lesion was slightly ameliorated in  $\alpha$ -MSH-treated, but not in Org 2766-treated animals. Because acute treatment with Org 2766 or  $\alpha$ -MSH did not influence reversal performance in the animals with lesions, it was concluded that the beneficial effect of the neuropeptide treatment could be explained in terms of facilitation of recovery of cognitive functions

in rats with bilateral lesions in the nucleus parafascicularis (47). Recently, Wolterink and Van Ree (67) reported that daily intra-accumbal, subcutaneous, or oral administration of Org 2766 accelerated the functional recovery of impaired motor activity after bilateral destruction of the n. accumbens of the rat by local application of 6-hydroxydopamine. The reduced motor activity of such rats was notable at postlesion day 7 but not at day 21, suggesting a functional recovery of the behavioral impairment. The authors suggest that central dopamine systems are involved in the spontaneous and peptide-facilitated recovery of impaired motor activity induced by these accumbal lesions. The peptide treatment enhanced lesion-induced dopamine receptor supersensitivity in the n. accumbens and resulted in the re-appearance of more dopaminergic fibers within the lesional area (68).

Other experiments indicated that ACTH administration reduced the effect of amygdala damage in the performance of a two-way active avoidance task, and a reduction by Org 2766 of attentional and "working memory" deficits produced by hippocampal lesions was also observed (8). Recently, Spruijt et al. (54) have shown that postlesion, systemic treatment with Org 2766 of rats bearing a unilateral section of the fornix fimbria resulted in improved recovery of behavioral function as determined in a water-maze. Whether facilitation of function by melanocortins after central nervous system (CNS) damage depends on an enhancement of cellular responses involved in the repair mechanisms, as found in the peripheral system, remains to be determined. It is worth noting that treatment of rats with ACTH-like peptides similar to that used in the studies cited above is known to affect regional glucose uptake (46) and macromolecule (RNA and protein) synthesis (19) in the rat brain.

Like the importance of developmental mechanisms to the understanding of post-lesion-plasticity in the CNS, changes in the CNS related to age are also of significance. Several authors stress the importance of trophic factors in brain aging and disease. Thus, it seems appropriate to point to the findings of Landfield et al. (44), who showed that chronic treatment with Org 2766 resulted in diminished signs of hippocampal aging. The peptide-treated animals had an increased density of neural cells and fewer reactive astrocytes. In addition, the neuropeptide-treated animals displayed latency times in a reversal learning task that were similar to those of younger controls and less like the age-matched cohorts.

Reul et al. (49) demonstrated that in the hippocampus of aged rats there is a considerable reduction in type I-corticosteroid receptor levels. Chronic treatment with Org 2766 resulted in a restoration to normal levels. The authors concluded that type I-corticosteroid receptors in aged rats have maintained a considerable degree of plasticity that is sensitive to neurotrophic peptides.

## REGENERATION AFTER PERIPHERAL NERVE DAMAGE

In the mammalian central and peripheral nervous system the neuron has been shown to be the most vulnerable cell. In general, damage to cell bodies of these

very specialized and differentiated cells results in irreversible degeneration and death. Regeneration and resulting reinnervation of the target organ is possible, however, if the damage remains restricted to the neuronal processes (dendrites and axons). This repair is facilitated by numerous humoral and structural factors, both of neuronal, glial, and target cell origin.

In nontransecting neural damage like a crush lesion, in which the studied nerve is compressed for a fixed period of time, Wallerian degeneration of the complete distal part of the nerve (66) is rapidly followed by an appropriate regenerative process, due to the fact that the endoneurial tubes remain largely intact.

The return of sensory function after the use of a standardized crush procedure was measured by means of a foot reflex withdrawal test (3,14). Using this test, a range of ACTH-derived analogs like  $\alpha$ -MSH and Org 2766 was shown to facilitate recovery when given every 48 hr s.c. starting immediately after surgery (4,14). The structure-activity relationship suggested that the active amino acid sequence was similar to that required for melanotropic rather than corticotropic activity of the peptide (4). The recovery of motor function was measured by analyzing the free walking pattern of rats, comparing the foot print of the crushed paw with that of the control side (17). Treatment with Org 2766 or  $\alpha$ -MSH was shown to enhance normalization of the walking pattern of crushed rats (15). To obtain more insight in whether the enhanced return of function is caused by an action of the peptide on the speed of outgrowth or on the actual number of newly formed sprouts, a crush model of the tail nerves of the rat was developed. By recording the time needed for return of sensory function on various points of the tail, each located more distally with respect to the crush site, it was established that Org 2766 treatment enhanced the return of sensory function within the first two–three days, but that the subsequent rate of recovery was approximately the same in both peptide and saline-treated groups (26).

Electrophysiology supported the findings of functional tests. Treatment with ACTH<sub>4-10</sub> for a period of seven days after crush denervation of the extensor digitorum longus muscle in rats resulted in the formation of more and smaller motor units as compared with saline-treated controls (52). Repetitive stimulation with low frequency (10 Hz) lead to higher mean contraction amplitude in melanocortin-treated animals. The ability to maintain tetanic tension on high-frequency stimulation was also improved. Though these data can in part be explained by the fact that smaller motor units are less susceptible to fatigue (10), it is also known that melanocortins increase the excitability of motor units via influencing the spinal motoneurons (41). There is reason to believe that the action of melanocortins is located pre-synaptically, since the frequency of miniature endplate potentials (mepps) is increased by ACTH<sub>4-10</sub>, whereas the muscle resting membrane potential and mepp amplitude are not influenced (52). The measurement of sensory (SNCV) and motor nerve conduction velocities (MNCV) of the sciatic nerve after crush revealed that short-term melanocortin treatment results in complete recovery of these parameters to control values. In saline-treated rats, however, a deficit of 20% remained present even 200 days after crushing (15).

Histological data on the number and the degree of myelination of new sprouts showed that more sprouts were formed after melanocortin treatment, without an effect on the thickness of the myelin sheath (5). With the use of affinity-purified neurofilament-binding antibodies, outgrowing sprouts could be visualized as early as 48 hr after the lesion (64). It was demonstrated that the number of outgrowing sprouts was increased by Org 2766 treatment on various consecutive time-points, whereas the rate of outgrowth was not affected, since the percentage difference between axon counts in control and peptide-treated rat remained fairly constant with time and distance (14,26,64).

Apart from the regenerative sprouting from the proximal end of a damaged peripheral nerve, the capacity of neighbouring intact nerves also to some extent contributes in the form of collateral sprouting. This phenomenon is in part regulated by similar mechanisms that are active in the process of peripheral sprouting. Experiments in which rat soleus muscle was partly denervated by cutting the lumbar L5 root, showed collateral sprouting conveyed by remaining motor units innervated by the L4 and L6 roots. In saline-treated rats these motor units could increase their number of muscle fiber terminals five-fold, whereas in peptide-treated animals one motor unit could sprout even 7.5-fold (16).

Beneficial effects of melanocortin treatment have also been shown after transection and surgical reapproximation of the sciatic nerve in rats. Both systemic and local application of  $\alpha$ -MSH resulted in a significant shortening of the recovery period (43,22). The use of microporous polypropylene tube that can absorb high quantities of peptide hormones and an impermeable polythene sleeve, to minimize leaking, around the site of the suture further improved recovery (21).

Besides local administration of melanocortins, other routes of delivery have been investigated in the crush model as well. Both the use of an osmotic mini-pump and of microspheres that ensure a slow but constant release over a fixed period of time have been shown to be effective in reducing the time needed for recovery. Oral administration, even in 1000-fold higher doses, was never successful (12,61). Further experiments demonstrated the presence of a dose-response relationship in the form of a bell-shaped curve, indicating that regeneration could only be stimulated in a narrow dose range (1–10  $\mu$ g per rat) (14).

## THE MECHANISM OF ACTION

Several observations suggest that melanocortins mimic or amplify a naturally occurring peptide signal early in the repair process. Currently, two working hypotheses are being considered. One hypothesis suggests that the distal degenerating nerve segment produces an MSH-like peptide that facilitates sprouting from the proximal stump (20,21). The source of the peptide could be the neurofilament protein NF150 (20,63). Although, clearly, the distal degenerating nerve is producing MSH-like activity as judged from a bio-assay (20); immunocytochemically that factor has not yet been characterized as being  $\alpha$ -MSH (65).

The alternative or additional possibility is that the cell body of damaged peripheral nerves re-express proopiomelanocortin (POMC), the precursor of endorphin and melanocortins (33,34). Evidence is accumulating that embryonic motor neurons express POMC peptides (9). In the mature peripheral nervous system the POMC is depressed, with the exception of genetically dystrophic mice in which POMC expression is prolonged (35). Thus, it could be that POMC expression is related to the growth and maturation repertoire of damaged neurons.

In favor of both hypotheses is the observation that the efficacy of exogenous peptide treatment is typified by a critical period shortly after the damage (22,64) and that local administration of peptides at the site of injury is an effective route of administration (21,61). Recently, Dekker and Tonnaer (13) published some rather preliminary data suggesting that Org 2766 could also act at the level of the spinal cord. Bijlsma et al. (4,5) were unable to demonstrate effects of melanocortins on spinal cord neuronal protein synthesis in animals bearing a crush lesion in the nervus ischiadicus.

Both hypotheses need further experimental support before the neurotrophic effect of exogenous melanocortins can be fully understood. Yet the realization that the peptide treatment might mimic or amplify a naturally occurring signal in the regenerative repertoire of neurons had some important implications. For example, neurons may mobilize regenerative support under conditions other than in reaction to mechanical trauma. In other words, exogenous peptide treatment might also be tested under conditions other than nerve crush or transection. Therefore, the possible efficacy of melanocortin treatment in experimental models of peripheral nerve neuropathies was tested.

## MELANOCORTINS IN THE TREATMENT OF PERIPHERAL NEUROPATHIES

Acrylamide is an example of a cumulative toxin which causes a mainly peripheral neuropathy in man and animal. The intoxication causes a Wallerian degeneration of distal regions of nerve axons and many of the biochemical changes that occur resemble those observed after axotomy (7). The eventual recovery is the result of regeneration of the surviving proximal parts of the nerve (57). Rats treated with acrylamide (50 mg/kg, every 48 hr) for two weeks developed severe abnormalities in the walking pattern and the landing foot spread. Also, the SNCV was diminished. Concomitant treatment with Org 2766 did not affect the onset of the neuropathy, but whereas 88 days after the acrylamide intoxication, saline-treated animals still displayed a decreased SNCV, complete normalization had occurred in the peptide treated animals (23).

Efficacy was also studied in an animal model of diabetic neuropathy. The mechanisms underlying this neural disorder are not clear. It has been proposed that changes in the vessels that supply the nerves are changed due to the diabetic

condition, but disturbances in metabolic processes in which either sorbitol or myoinositol play a role have also been suggested (32). Rats made diabetic by a single injection of streptozotocin, which kills the insulin producing island cells in the pancreas, were shown to develop a peripheral neuropathy after 6–8 weeks. Concomitant treatment with Org 2766 did not prevent an initial decrease in SNCV and MNCV in diabetic rats, but prevented further deterioration seen in the saline-treated animals and even induced recovery in the late phase of the experiments. In the diabetic sciatic nerve, fewer large fibers were present in comparison to controls. Peptide treatment resulted in a normalization of the fiber diameter distribution (62).

Finally, the toxic neuropathy resulting from repetitive treatment with the anti-tumor drug cisplatin was studied. This drug, used in the treatment of ovarian and testicular cancer, causes a purely sensory neuropathy at cumulative dose levels of  $300 \text{ mg/m}^2$  or more, increasing in severity in a dose-dependent way and only reversible upon cessation of treatment in half of the afflicted patients (48). In an animal model, rats given cisplatin ( $1 \text{ mg/kg}$ ) twice a week developed a sensory neuropathy after seven weeks as indicated by a marked decrease in SNCV (15). In rats co-treated with Org 2766, no such decrease was observed even after 12 weeks of treatment (15). When treatment with Org 2766 was started once a cisplatin neuropathy already existed, the SNCV improved even during continuing cisplatin administration. In addition, it was shown that rats concomitantly treated with the peptide during a first period of cisplatin intoxication were less susceptible to a second treatment course when compared with saline-treated rats. The anti-tumor activity of cisplatin was not hampered by Org 2766 (15,25). Morphological analysis of sural nerves of rats that were given cisplatin for 12 weeks revealed a decrease in the number of thick myelinated nerve fibers, but not in the total number of fibers. Individual fiber measurements showed that the degree of myelination was less in cisplatin-treated animals than in controls. In rats that received Org 2766 treatment, a normal distribution of sensory nerve fibers was seen (27).

A multi-center, placebo-controlled clinical trial in 55 ovarian cancer patients treated with cisplatin was set up to study the possible preventive action of Org 2766 in human subjects. In this study, 2 doses of Org 2766 ( $0.25 \text{ mg/m}^2$  and  $1 \text{ mg/m}^2$ ) were compared with placebo treatment at the start, and after two, four, and six cycles of cytotoxic treatment. The vibration sense was used as the main parameter, since earlier studies indicated that this parameter was affected in a subclinical phase of the neuropathy (51). Vibration perception thresholds began to increase, implicating a gradual loss of function, in the placebo group after two treatment cycles and sharply deteriorated after four and six cycles. In the high dose treatment group, no change was seen after two and four cycles, whereas only a modest increase was seen after six cycles. The differences were statistically highly significant. The low-dose peptide group displayed a moderate loss of vibration sense intermediary between the other two groups. Symptoms and signs occurred in 50% of the patients in the placebo group after four courses, but were not seen in the high- or low-dose treatment groups, and though symptoms and signs were recorded

in the latter groups after six cycles, there still existed a substantial difference when compared with the placebo group at this point. The response to cisplatin treatment was similar in all three groups. These results suggest Org 2766 is capable of preventing or at least postponing cisplatin neuropathy in ovarian cancer patients (28).

## CONCLUSION

In summary, melanocortins exert a stimulatory influence in neuronal development and repair. As indicated by the data discussed, melanocortins appear to improve regeneration of peripheral nerves after mechanical trauma in rats and to beneficially affect neuropathies caused by various toxic and metabolic conditions. There now is evidence that suggests this neurotrophic activity seen in animals also extends to humans. Put into a broader perspective, clinical study of melanocortin treatment might be warranted in a number of different disorders that affect the peripheral nervous system.

## REFERENCES

1. Azmitia EC, De Kloet ER. Neuropeptide stimulation of serotonergic neuronal maturation in tissue culture: modulation by hippocampal cells. *Prog. Brain Res.* 1987; 72:311–318.
2. Berry M, Knowles J, Willis P, Riches AC, Morgans GP, Steers D. A reappraisal of the effect of ACTH on the response of the central nervous system to injury. *J. Anat.* 1979;128:859–871.
3. Bijlsma WA, Jennekens FGI, Schotman P, Gispen WH. Effects of corticotrophin (ACTH) on recovery of sensorimotor function in the rat: structure-activity study. *Eur. J. Pharmacol.* 1981;76:73–79.
4. Bijlsma WA, Schotman P, Jennekens FGI, Gispen WH, De Wied D. The enhanced recovery of sensorimotor function in rats is related to the melanotropic moiety of ACTH/MSH neuropeptides. *Eur. J. Pharmacol.* 1983;92:231–236.
5. Bijlsma WA, Van Asselt E, Veldman H, Jennekens FGI, Schotman P, Gispen WH. Ultrastructural study of the effect of ACTH(4–10) in nerve regeneration: axons become larger in number and smaller in diabetes. *Acta Neuropathol (Berlin)* 1983;62:24–30.
6. Bijlsma WA, Jennekens FGI, Schotman P, Gispen WH. Neurotrophic factors and regeneration in the peripheral nervous system. *Psychoneuroendocrinology* 1984;9:199–215.
7. Bisby MA, Redshaw JD. Acrylamide neuropathy: changes in the composition of proteins of fast axonal transport resemble those observed in regenerating axons. *J. Neurochem.* 1987;48:924–928.
8. Bush DF, Lovely R, Pagano RR. Injection of ACTH induces recovery from shuttle-box avoidance deficits in rats with amygdaloid lesions. *J. Comp. Psychol.* 1973;83:168–172.

9. Carr A, Haynes LW. Transient appearance of pro-opiomelanocortin (POMC) messenger RNA and N-terminal peptides in rat embryo spinal cord. *Neurosci. Res. Commun.* 1988;3:31–39.
10. Close R. Properties of motor units in fast and slow skeletal muscles of the rat. *J. Physiol.* 1967;193:45–55.
11. Daval JL, Louis JC, Gerard MJ, Vincendon G. Influence of adrenocorticotropic hormone on the growth of isolated neurons in culture. *Neurosci. Lett.* 1983;36:299–304.
12. Dekker AJAM, Princen MM, De Nijs H, De Leede LGJ, Broekkamp CLE. Acceleration of recovery from sciatic nerve damage by the ACTH(4–9) analog Org. 2766; different routes of administration. *Peptides* 1987;8:1057–1059.
13. Dekker AJAM, Tonnaer J. Binding of the neurotrophic peptide Org. 2766 to rat spinal cord fractions is affected by a sciatic nerve crush. *Brain Res.* 1989;477:327–331.
14. De Koning P, Brakkee JH, Gispen WH. Methods for producing a reproducible crush in the sciatic and tibial nerve of the rat and rapid and precise testing of return of sensory function: beneficial effects of melanocortins. *J. Neurol. Sci.* 1986;74:237–246.
15. De Koning P, Gispen WH. Org. 2766 improves functional and electrophysiological aspects of regenerating sciatic nerve in the rat. *Peptides* 1987;8:415–422.
16. De Koning P, Verhaagen J, Sloot W, Jennekens FGI, Gispen WH. Org. 2766 stimulates collateral sprouting in the rat soleus muscle following partial denervation. *Muscle & Nerve* 1989;12:353–359.
17. De Medicanelli L, Freed W, Wyatt RJ. An index of the functional condition of rat sciatic nerve based on measurements made from walking trades. *Exp. Neurol.* 1982;77:634–643.
18. De Wied D, Jolles J. Neuropeptides derived from proopiocortin: behavioral, physiological and neurochemical effects. *Physiol. Rev.* 1982;62:976–1059.
19. Dunn AJ, Schotman P. Effects of ACTH and related peptides on cerebral RNA and protein synthesis. *Pharmacol. Ther.* 1981;12:353–372.
20. Edwards PM, Van der Zee CEEM, Verhaagen J, Jennekens FGI, Gispen WH. Evidence that the neurotrophic actions of  $\alpha$ -MSH may derive from its ability to mimic the actions of a peptide formed in degenerating nerve stumps. *J. Neurol. Sci.* 1984;64:333–340.
21. Edwards PM, Gispen WH. Melanocortin peptides and neural plasticity. In: Traber J, Gispen WH, eds. *Senile Dementia of Alzheimer Type*. Berlin: Springer Verlag, 1985; 231–240.
22. Edwards PM, Kuiters RRF, Boer GJ, Gispen WH. Recovery from peripheral nerve transection is accelerated by local application of  $\alpha$ -MSH by means of Accurel polypropylene tubes. *J. Neurol. Sci.* 1986;74:171–176.
23. Edwards PM, Sporel E, Gerritsen van der Hoop R, Gispen WH. Recovery from acrylamide neuropathy is improved by treatment with an ACTH analogue, Org. 2766. *Human Toxicol.* 1989;8:159.
24. Flohr H, Luneburg U. Effects of ACTH<sub>4–10</sub> on vestibular compensation. *Brain Res.* 1982;248:169–173.
25. Gerritsen van der Hoop R, De Koning P, Neijt JP, Jennekens FGI, Gispen WH. Efficacy of the neuropeptide Org. 2766 in the prevention and treatment of cisplatin-induced neurotoxicity in rats. *Eur. J. Cancer Clin. Oncol.* 1988a;24:637–642.
26. Gerritsen van der Hoop R, Brakkee JH, Kappelle A, Samson M, De Koning P, Gispen WH. A new approach for the evaluation of recovery after peripheral nerve damage. *J. Neurosci. Methods* 1988;26:111–116.

27. Gerritsen van der Hoop R, Neijt JP, Jennekens FGI, Gispen WH. Both in normal and high-dose cisplatin treatment Org. 2766 protects from neurotoxicity in rats: electrophysiological and histological results. In: ENS Satellite Symposium: *peripheral nerve development and regeneration*. San Remo, 39.
28. Gerritsen van der Hoop R, Vecht ChJ, Van der Burg MEL, Elderson A, Boogerd W, Heimans JJ, Vries EP, Van Houwelingen JC, Jennekens FGI, Gispen WH, Neijt JP. Prevention of cisplatin neurotoxicity with an ACTH (4-9) analogue in patients with ovarian cancer. *N. Engl. J. Med.* 1990;322:89-94.
29. Gispen WH, Isaacson RL, Spruijt BM, De Wied D. Melanocortins, neural plasticity and aging. *Prog. Neuro-Psychopharmacol. Biol. Psychiatry* 1986;10:415-426.
30. Gonzales ER, Strand FL. Neurotropic action of MSH/ACTH<sub>4-10</sub> on neuromuscular function in hypophysectomized rats. *Peptides* 1981;2:107-113.
31. Hannigan JH, Isaacson RL. The effects of Org 2766 on the performance of sham, neocortical and hippocampal lesioned rats in a food search task. *Biochem. Behav.* 1984; 23:1019-1027.
32. Harati Y. Diabetic peripheral neuropathies. *Ann. Intern. Med.* 1988;107:546-549.
33. Haynes LW, Smith ME. The regulation by  $\beta$ -endorphin and related peptides of collagen-tailed acetylcholinesterase forms in the skeletal muscles of vertebrates. In: Stefano G, ed. *CRC Handbook of Comparative Aspects of Opioid and Related Neuropeptides Mechanisms*, Vol. 11. San Francisco, 1986;65-79.
34. Hughes S, Smith ME.  $\beta$ -Endorphin and  $\alpha$ -melanotropin immunoreactivity in degeneration and contralateral motoneurons of the rat. *J. Physiol.* 1987;338:52.
35. Hughes S, Smith ME. Effect of nerve section on  $\beta$ -endorphin and  $\alpha$ -melanotropin immunoreactivity in motor nerves of normal and dystrophic mice. *Neurosci. Lett.* 1988; 92:1-7.
36. Igarashi M, Ishihawa K, Ishii M. Effect of ACTH<sub>4-10</sub> on equilibrium compensation after unilateral labyrinthectomy in the squirrel monkey. *Eur. J. Pharmacol.* 1985; 119:239-242.
37. Igarashi M, Ishii M, Ishikawa K, Himi T. Comparative effect of some neurotropic agents on balance compensation after labyrinthectomy in the squirrel monkey. In: Flohr H, ed. *Postlesion Neural Plasticity*. Berlin: Springer Verlag, 1988;627-634.
38. Isaacson RL, Poplawsky A. An ACTH<sub>4-9</sub> analog (Org 2766) speeds recovery from septal hyperemotionality in the rat. *Behav. Neural Biol.* 1983;39:52-59.
39. Isaacson RL, Poplawsky A. ACTH<sub>4-10</sub> produces a transient decrease in septal hyperemotionality. *Behav. Neural Biol.* 1985;43:109-113.
40. Johnston MF, Kravitz EA, Meiri H, Rahamimoff R. Adrenocorticotropic hormone causes long-lasting potentiation of transmitter release from motor nerve terminals. *Science* 1983;220:1071-1072.
41. Krivoy WA, Zimmerman E. An effect of  $\alpha$ -melanocyte-stimulating hormone ( $\alpha$ -MSH) on motoneurons of rat spinal cord. *Eur. J. Pharmacol.* 1977;46:315-322.
42. Krivoy W, Couch JR, Stewart JA. Modulation of spinal synaptic transmission by  $\beta$ -melanocyte stimulating hormone ( $\beta$ -MSH). *Psychoneuroendocrinology* 1985;10:103-108.
43. Kuiters RRF, Gerritsen van der Hoop R, Van der Zee CEEM, Jennekens FGI, Gispen WH. Recovery from peripheral nerve transection in the rat accelerated by subcutaneous application of  $\alpha$ -MSH. *Neuro-orthop.* 1988;6:87-90.
44. Landfield PW, Baskin RK, Pitler TA. Brain aging correlates: retardation by hormonal pharmacological treatments. *Science* 1981;214:581-584.

45. Luneburg U, Flohr H. Effects of melanocortins on vestibular compensation. *Prog. Brain Res.* 1988;76:421–429.
46. McCulloch J, Kelly PAT, Van Delft AML. Alterations in local cerebral glucose utilization during chronic treatment with an ACTH(4–9) analog. *Eur. J. Pharmacol.* 1982;78:151–158.
47. Nyakas C, Veldhuis HD, De Wied D. Beneficial effect of chronic treatment with Org 2766 and  $\alpha$ -MSH on impaired reversal learning of rats with bilateral lesions of the parafascicular area. *Brain Res. Bull.* 1985;15:257–265.
48. Ozols PF, Ostchega Y, Myers CE, Young RL. High dose cisplatin in hypertonic saline in refractory ovarian cancer. *J. Clin Oncol.* 1985;3:1246–1250.
49. Reul JMHM, Tonnaer JADM, De Kloet ER. Neurotrophic ACTH analogue promotes plasticity of type I corticosteroid receptor in brain and senescent male rats. *Neurobiol. Aging* 1988;9:253–260.
50. Richter-Landsberg C, Bruns I, Flohr H. ACTH neuropeptides influence development and differentiation of embryonic rat cerebral cells in culture. *Neurosci. Res. Commun.* 1987;1:153–162.
51. Roelofs, RI, Hrushesky W, Rogin J, Rosenberg L. Peripheral sensory neuropathy and cisplatin chemotherapy. *Neurology* 1984;34:934–938.
52. Saint-Côme C, Strand FL. ACTH/MSH<sub>4–10</sub> improves motor unit reorganization during peripheral nerve regeneration in the rat. *Peptides* 1985;6:77–83.
53. Smith CM, Strand FL. Neuromuscular response of the immature rat to ACTH/MSH<sub>4–10</sub>. *Peptides* 1981;2:197–206.
54. Spruijt BM, Pitsikas N. Beneficial effects of Org. 2766 on regeneration after fimbria fornix lesions in the rat. *ENA, Turin* (abstract).
55. Strand FL, Smith CM. LPH, ACTH, MSH and motor systems. *Pharmacol. Ther.* 1980;11:509–533.
56. Strand FL, Smith CM. LPH; ACTH; MSH and motor systems. In: de Wied D, Gispen WH, van Wimersma Greidanus TJB, eds. *Neuropeptides and Behavior*. Oxford: Pergamon Press, 1986;245–272.
57. Suzuki K, Pfaff LD. Acrylamide neuropathy in rats. *Acta Neuropathol. (Berlin)* 1979; 24:197–213.
58. Swaab DF, Martin JT. Functions of  $\alpha$ -melanotropin and other opiomelanocortin peptides in labour, intrauterine growth and brain development: peptides of the pars intermedia. *CIBA Found. Symp.* 1981;81:196–217.
59. Van der Helm-Hylkema H, De Wied D. Effect of neonatally injected ACTH and ACTH analogs on eye-opening of the rat. *Life Sci.* 1976;18:1099–1104.
60. Van der Neut R, Bär PR, Sodaar P, Gispen WH. Trophic influences of alpha-MSH and ACTH<sub>4–10</sub> on neuronal outgrowth *in vitro*. *Peptides* 1988;9:1015–1020.
61. Van der Zee CEEM, Brakkee JH, Gispen WH.  $\alpha$ -MSH and Org. 2766 in peripheral nerve regeneration: different routes of delivery. *Eur. J. Pharmacol.* 1988;147:351–357.
62. Van der Zee CEEM, Gerritsen van der Hoop R, Gispen WH. Beneficial effect of the peptide Org 2766 (ACTH(4–9) analog) in the treatment of peripheral neuropathy in streptozotocin-diabetic rats. *Diabetes* 1989;38:225–230.
63. Verhaagen J, Edwards PM, Schotman P, Jennekens FGI, Gispen WH. Characterization of epitopes shared by  $\alpha$ -melanocyte stimulating hormone ( $\alpha$ -MSH) and the 150 kDa neurofilament protein (NF150); relationship to neurotrophic sequences. *J. Neurosci. Res.* 1986;16:589–600.
64. Verhaagen J, Edwards PM, Jennekens FGI, Schotman P, Gispen WH. Early effect of

- an ACTH(4–9) analog (Org. 2766) on regenerative sprouting demonstrated by the use of neurofilament-binding antibodies isolated from a serum raised by  $\alpha$ -MSH immunisation. *Brain Res.* 1987;404:142–150.
65. Verhaagen J, Edwards PM, Jennekens FGI, Gispen WH. Damaged rat peripheral nerves do not contain detectable amounts of  $\alpha$ -MSH. *J. Neurosci. Res.* 1988;19:14–18.
66. Waller AV. Experiments on the section of the glossopharyngeal and hypoglossal nerves of the frog, and observation on the alterations produced thereby in the structure of their primitive fibres. *Phil. Trans. R. Soc. London (Biol.)* 1850;140:423.
67. Wolterink G, Van Ree JM. Org 2766 accelerates functional recovery of impaired motor activity due to lesions in the nucleus accumbens of rats. In: Symposium on neuropeptides and brain function, Utrecht, P41 (abstract).
68. Wolterink G, Van Ree JM. Spontaneous and Org. 2766-induced recovery of 6-OHDA lesions in the nucleus accumbens of rats may be the result of the development of denervation supersensitivity. *Proc. 29th Dutch Federation Meeting*, Utrecht, 475.



# Effects of Gangliosides on the Growth and Differentiation of HL-60 Human Leukemia Cells

Robert K. Yu

Department of Biochemistry and Molecular Biophysics  
Medical College of Virginia  
Virginia Commonwealth University  
Richmond, VA 23298

**I**t is widely accepted that gangliosides have profound effects on cellular differentiation and proliferation. Thus, gangliosides added to certain neural culture systems have been shown to possess neurotogenic and neurotrophic effects (1–7). They are known to inhibit cellular growth by modulating growth factor receptor function (8,9). Although the underlying mechanisms for these ganglioside effects still remain obscure, there has been mounting evidence suggesting that these effects may be modulated by a variety of systems affecting protein phosphorylation. Thus, we first demonstrated that gangliosides added to a rat brain membrane preparation had profound effects, both stimulatory and inhibitory, in modulating several protein kinase systems (10,11). Thus,  $\text{Ca}^{2+}$ -gangliosides may stimulate the calmodulin-dependent kinase II system (11,12), whereas gangliosides can inhibit the calcium-phospholipid dependent kinase (protein kinase C) (13,14). Although a number of kinase systems have since been shown to be modulated by gangliosides *in vitro* (15–18), little is known about the mechanisms by which the ganglioside-mediated protein phosphorylation exerts its biological and physiological effects. Since it is well-known that the profiles of neutral and acidic glycosphingolipids (GSLs) undergo characteristic changes during cellular proliferation and differentiation, it would be informative to examine the effects of gangliosides and other inducers of differentiation on the biosynthesis of GSLs in relation to protein phosphorylation. For this reason, we have chosen the human promyelocytic cell line HL-60 as a model cell system.

The HL-60 cells are known to be induced to mature along either the granulocyte series (19) upon exposure to dimethylsulfoxide (DMSO), or the monocyte/macrophage pathway upon treatment with the tumor promotor 12-O-tetradecanoylphorbol-13-acetate (TPA) (20,21). Recently, Nojiri et al. (22) reported that ganglioside GM<sub>3</sub> also possessed the ability to induce the differentiation of

HL-60 cells, as well as that of another human leukemia cell line, U937. The ganglioside GM<sub>3</sub> is ubiquitous in that it is present in all well-differentiated cells, and it is commonly perceived as a marker for cellular differentiation. Thus, a characteristic increase in the level of GM<sub>3</sub> was observed during monocyte/macrophage-like differentiation of many human myelocytic and monocytic leukemia cell lines such as HL-60 (23), K562 (24), KG-1 (25), ML-1 (26), as well as HO melanoma cell differentiation (27), when these lines were treated with TPA. Furthermore, a corresponding increase in the activity of CMP-*N*-acetylneurameric acid: lactosylceramide sialyltransferase (ST1) accompanied that of GM<sub>3</sub> even in HL-60 cells treated with TPA (28). Another important biochemical event associated with the action of TPA involves its effects on PKC. In HL-60 cells, the TPA receptor and PKC activity co-purify (29–31). TPA directly activates this kinase (32) and induces the phosphorylation of several proteins in HL-60 cells (33–37). In addition, cellular adherence, a measure of monocytic maturation, is blocked by palmitoylcarnitine, an inhibitor of PKC (38). These findings suggest a role for PKC in the induction of cell differentiation by TPA.

Since ST1 represents a key glycosyltransferase in the biosynthesis of gangliosides, it is possible that a relationship exists between the activities of PKC and ST1 during the maturation of HL-60 cells induced by TPA, DMSO, and GM<sub>3</sub>. We have recently found that all three of these agents initiated cellular differentiation and enhanced PKC activity. TPA and GM<sub>3</sub>, which induced monocytic differentiation of HL-60 cells, markedly increased the extent of [<sup>14</sup>C]galactose-incorporation into neutral glycolipids and gangliosides. DMSO, which induced granulocytic maturation of HL-60 cells, significantly inhibited [<sup>14</sup>C]galactose incorporation into neutral glycolipid and gangliosides, except for GM<sub>3</sub>. TPA and DMSO also increased ST1 activity. The findings suggest that PKC activity may modulate the increment of ST1 activity, which may be of importance to the maturation of HL-60 cells regardless of whether they enter the granulocytic or monocytic/macrophagic pathways.

## MATERIALS AND METHODS

### Materials

DEAE-Sephadex A-25, Iatrobeads, and HPTLC plates were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden), Iatron Industries, Inc. (Tokyo, Japan), and E. Merck Co. (Darmstadt, West Germany), respectively. DEAE-Sepharose CL-6B-100, Octyl-Sepharose CL-4B, Triton X-100, TPA, DMSO, Type III-S histone, and phosphatidylserine (PS) were purchased from Sigma Chemical Co. (St. Louis, MO). D-[1-<sup>14</sup>C]galactose (specific activity, 51 mCi/mmol) was obtained from Amersham (Arlington Heights, IL). CMP-sialic acid ([4-<sup>14</sup>C]sialic acid]) (1.8 mCi/mmol) was purchased from New England Nuclear Corp. (Boston, MA) and

[ $\gamma$ -<sup>32</sup>P]ATP (300 Ci/mmol) was from Amersham. Gangliosides and sulfatides were isolated and purified from bovine or human brain by the method of Ledeen and Yu (39). Individual gangliosides and sulfatides were all demonstrated to be chromatographically pure on HPTLC plates. All other reagents were of at least analytical grade.

## Cell Culture

HL-60 leukemia cells were grown in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco), 100 IU/ml of penicillin, and 100  $\mu$ g/ml of streptomycin at 37°C, in a humidified atmosphere of 95% air-5% CO<sub>2</sub>. Cells were used between passages 30 and 50 and were seeded at a level of  $2 \times 10^5$  cells/ml in RPMI 1640 medium containing a reduced FBS concentration (5%) and either 10 nM TPA, 1.3% DMSO, 100  $\mu$ M GM3, 100  $\mu$ M GM1, 100  $\mu$ M BBG, or 100  $\mu$ M sulfatides.

## Extraction and Purification of Glycosphingolipids

After incubation for three days in the absence or presence of various agents, cells were harvested and washed with PBS at least three times. After the addition of 0.2–0.4 ml of H<sub>2</sub>O, cell pellets were sonicated for three min and the protein concentration was determined by the method of Lowry et al. (40) using bovine serum albumin as the standard.

The sonicate, containing 1 mg of protein with 50  $\mu$ g BBG added as a carrier, was resuspended in 6 ml of C:M 1:1 (v/v). The mixture was sonicated for 10 min and the solvent was adjusted to C:M:W 30:60:8 (v/v, solvent A) and further stirred overnight. After centrifugation, pellets were extracted with about 10 ml of solvent A and stirred overnight. The supernatants were combined and applied to a DEAE-Sephadex A-25 column (bed volume, 1.5 ml). The neutral glycolipid fraction was eluted with 15 ml of solvent A, and the acidic lipid fraction containing gangliosides, was eluted with 20 ml of C:M:aqueous 0.8 M sodium acetate 30:60:8 (v/v).

## Incorporation of [<sup>14</sup>C]Galactose in TCA-precipitable Material

Twenty-five ml of HL-60 cell suspension was cultured with 18 uCi of D-[1-<sup>14</sup>C]galactose/flask for different periods of time in the absence or presence of TPA, DMSO, GM<sub>3</sub>, GM<sub>1</sub>, or sulfatides. An aliquot of the cell suspension was removed each day and transferred into a tube containing 1 ml of 25% ice-cold TCA. After 10 min, the mixture was filtered (Millipore HA, 0.45  $\mu$ m) and the precipitate

was washed with 5 ml of 5% ice-cold TCA at least four times. The filters were placed in scintillation vials with 15 ml of scintillation fluid (Ecoscient, National Diagnostics, Inc., Manville, NJ) and the radioactivity incorporated into the cells was determined using an LKB Rackbeta scintillation spectrometer.

### **Analysis of [<sup>14</sup>C]-labeled Gangliosides**

The acidic lipid fraction was subjected to mild alkaline treatment (0.2 M NaOH in methanol at 37°C for 2 hr) to hydrolyze contaminating phospholipids, and then desalting by gel filtration on a Sephadex LH-20 column (bed volume, 20 ml). The recovered ganglioside fraction was separated on an HPTLC plate using C:M:aqueous 0.2% CaCl<sub>2</sub>·2H<sub>2</sub>O 55:45:10 (v/v) as the developing solvent system. After chromatography, plates were air-dried and exposed to x-ray film for five to eight days. Alternatively, plates were sprayed with resorcinol-HCl reagent (41) to locate gangliosides. The area corresponding to GM<sub>3</sub> was removed by scraping the band from the plate, and transferred into a scintillation vial containing 0.1 ml of water and 15 ml of scintillation fluid. Vials were sonicated for 5 min and the radioactivities therein determined. Labeling of other gangliosides was measured as described for GM<sub>3</sub>.

### **Analysis of [<sup>14</sup>C]-labeled Neutral Glycosphingolipids**

The neutral lipid fraction was treated with 0.2 M NaOH in methanol at 37°C for two hr to hydrolyze phospholipids, and then desalting by Folch partitioning (42). Neutral glycosphingolipids were separated on an HPTLC plate using C:M:W 60:35:8 (v/v) as the developing solvent. After chromatography, the plates were exposed to x-ray film for five to eight days, and then sprayed with the orcinol-H<sub>2</sub>SO<sub>4</sub> reagent (43). Labeling of GL<sub>1</sub>, GL<sub>2</sub>, and total neutral glycolipids by [<sup>14</sup>C]galactose was also determined in cells treated with various agents using a scintillation spectrometer as described above.

### **Protein Kinase C Assay**

After three days in culture in the presence or absence of various agents, the cells were harvested and washed twice with PBS. PKC was partially purified as described by Kreutter et al. (44). Briefly, the cell pellet was resuspended in lysis buffer (20 mM Tris-HCl, 0.1 mM EGTA, 10% sucrose, 50 mM 2-mercaptoethanol, pH 7.5) and disrupted by sonication for two min. Triton X-100 was added to a final concentration of 0.3%. The mixture was incubated on ice for 15 min to solubilize membrane-bound PKC, and the resulting solution was applied to a small DEAE-

cellulose column equilibrated in lysis buffer. The column was washed with lysis buffer and the enzyme was eluted with 0.1 M NaCl in lysis buffer. The enzyme was freshly prepared for each experiment. The activity of PKC was measured by the incorporation of  $^{32}\text{P}$  from [ $\gamma$ - $^{32}\text{P}$ ]ATP into histone III-S. The reaction mixture for assaying PKC activity contained 10 mM Mg acetate, 1 mM EGTA, 1.1  $\mu\text{M}$   $\text{Ca}^{2+}$ , 0.01 mM ATP, 20  $\mu\text{g}/\text{ml}$  of PS, 2  $\mu\text{g}/\text{ml}$  of 1,2-diolein, 200  $\mu\text{g}/\text{ml}$  of histone III-S, and 15 to 40  $\mu\text{g}/\text{ml}$  of PKC. Reactions were carried out at 37°C for 15 min, and the reaction was terminated by addition of 1.0 ml of 25% ice-cold trichloroacetic acid (TCA). After five min, the mixture was filtered (Millipore HA, 0.45  $\mu\text{m}$ ) and washed twice with 4 ml of 5% ice-cold TCA. The filters were placed in scintillation vials with 5 ml of Opti-Fluor scintillation fluid and  $^{32}\text{P}$  incorporation of histone III-S was determined using an LKB Rackbeta scintillation counter.

### CMP-N-Acetylneuraminic Acid:Lactosylceramide Sialyltransferase Assay

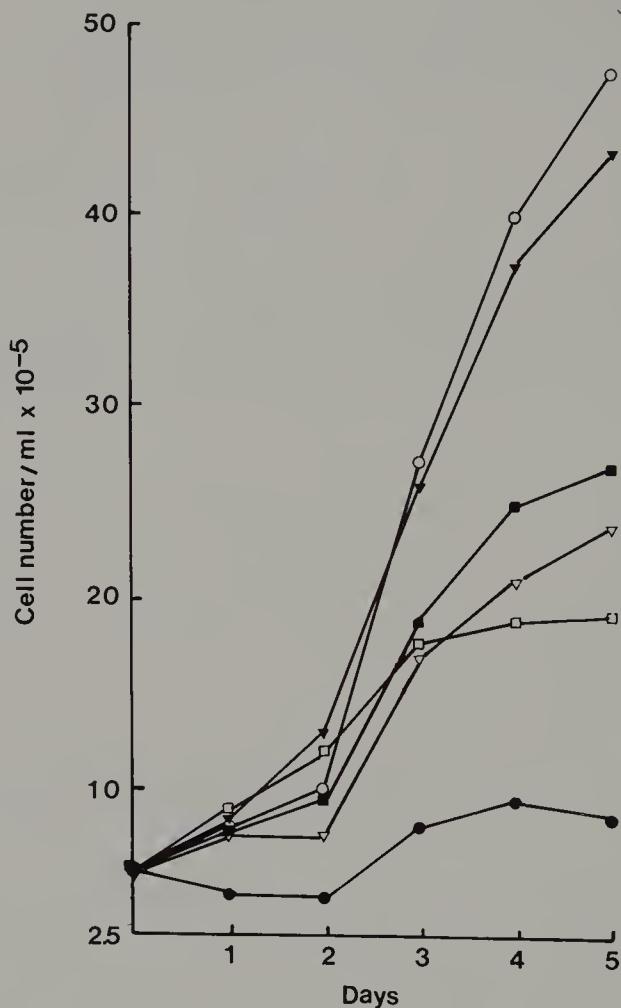
After three days in culture in the presence of various agents, cells were harvested and washed twice with PBS. The cell pellets were resuspended in distilled water and disrupted by sonication for two min. The sonicate was used as the enzyme source. The reaction mixture for the ST1 assay contained 40 nmol of LacCer, 80 nmol of CMP-[ $^{14}\text{C}$ ]NeuAc, 300  $\mu\text{g}$  of Triton CF-54, 2  $\mu\text{mol}$  of MnCl<sub>2</sub>, 500 nmol of cacodylate buffer (pH 6.3), and 150 to 800  $\mu\text{g}$  of protein in a final volume of 0.2 ml (45). The LacCer in C:M 1:1 (v/v), Triton CF-54 in methanol, and CMP-[ $^{14}\text{C}$ ]NeuAc in ethanol-water 1:1 (v/v) were dried in the incubation tube under N<sub>2</sub> prior to the addition of other reagents. The reaction was carried out at 37°C for one hr and was stopped by boiling the reaction mixture for 30 sec. Appropriate blanks were also processed with each assay which consisted of a) boiled enzyme, and b) no LacCer, to determine the activity due to endogenous substrate in the enzyme preparation.

### Protein Assay

Protein concentrations were determined by the method of Lowry et al. (40) or a modified Coomassie blue R-250 binding protein assay using crystalline bovine serum albumin as the standard (46).

## RESULTS

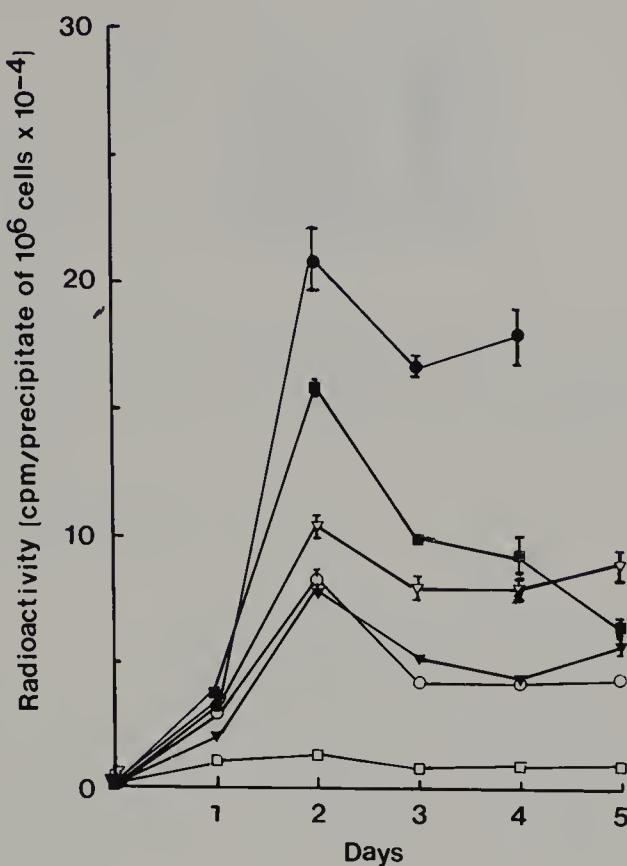
HL-60 leukemia cells were cultured in the presence of either TPA, DMSO, GM<sub>3</sub>, GM<sub>1</sub>, or sulfatides to measure the effects of these agents on cellular growth.



**FIG. 1.** Effects of exogenously added TPA, DMSO, gangliosides, and sulfatides on growth of HL-60 leukemia cells. HL-60 cells were grown in RPMI 1640 medium containing 5% FBS in the absence of added agents (○) or in the presence of either 10 nM TPA (●), 1.3% DMSO (□), 100  $\mu$ M GM<sub>3</sub> (■), 100  $\mu$ M GM<sub>1</sub> (▽), or 100  $\mu$ M sulfatides (▽). The number of cells was determined by using a Coulter ZM particle counter at various times. Each value represents the mean  $\pm$  SE of at least three separation determinations.

All of the tested materials, except for sulfatides, inhibited cellular replication to varying degrees (Fig. 1), with TPA being the most potent and sulfatides the least inhibitory.

Incorporation of [<sup>14</sup>C]galactose into TCA-precipitable material was measured in HL-60 cells incubated for different periods of time in the presence or absence of TPA, DMSO, GM<sub>3</sub>, GM<sub>1</sub>, or sulfatides. The incorporation of [<sup>14</sup>C]galactose into



**FIG. 2.** Effects of exogenously added TPA, DMSO, gangliosides, and sulfatides on the incorporation of  $[^{14}\text{C}]$ galactose into TCA-precipitable materials of HL-60 cells. The conditions were employed as for Fig. 1. Control (○); 10 nM TPA (●); 1.3% DMSO (□); 100 uM GM<sub>3</sub> (■); 100 uM GM<sub>1</sub> (▽); or 100 uM sulfatides (▼). Each value represents the mean  $\pm$  SE of four separate experiments. (Reproduced from ref. 60.)

cells treated with the various agents peaked on the second day (Fig. 2). TPA, GM<sub>3</sub> and GM<sub>1</sub> enhanced the incorporation of  $[^{14}\text{C}]$ galactose into the TCA-precipitable material of the cells, whereas DMSO inhibited incorporation and sulfatides had no effect.

The distribution of radioactivity in gangliosides was measured and the results were shown in Fig. 3. The labeling of gangliosides by  $[^{14}\text{C}]$ galactose was substantially modified by the presence of different agents in the culture medium. TPA, GM<sub>3</sub>, and GM<sub>1</sub> enhanced the incorporation of  $[^{14}\text{C}]$ galactose into gangliosides, while DMSO and sulfatides had an inhibitory effect (Fig. 4). Examination of GM<sub>3</sub> fraction indicated that the synthesis of this ganglioside was enhanced when cells were treated with TPA, DMSO, GM<sub>3</sub> or GM<sub>1</sub> (Fig. 5).

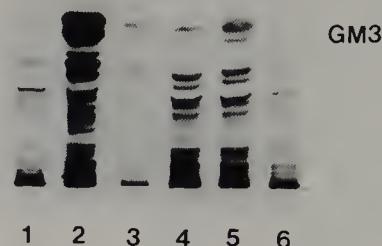


FIG. 3. HPTLC autoradiogram of [ $^{14}\text{C}$ ]-labeled gangliosides from HL-60 cells exposed to [ $^{14}\text{C}$ ]galactose. 1, Control; 2, TPA; 3, DMSO; 4, GM<sub>3</sub>; 5, GM<sub>1</sub>; 6, sulfatides. The developing solvent system was chloroform:methanol:0.2%  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  50:45:10 (v/v). (Reproduced from ref. 60.)

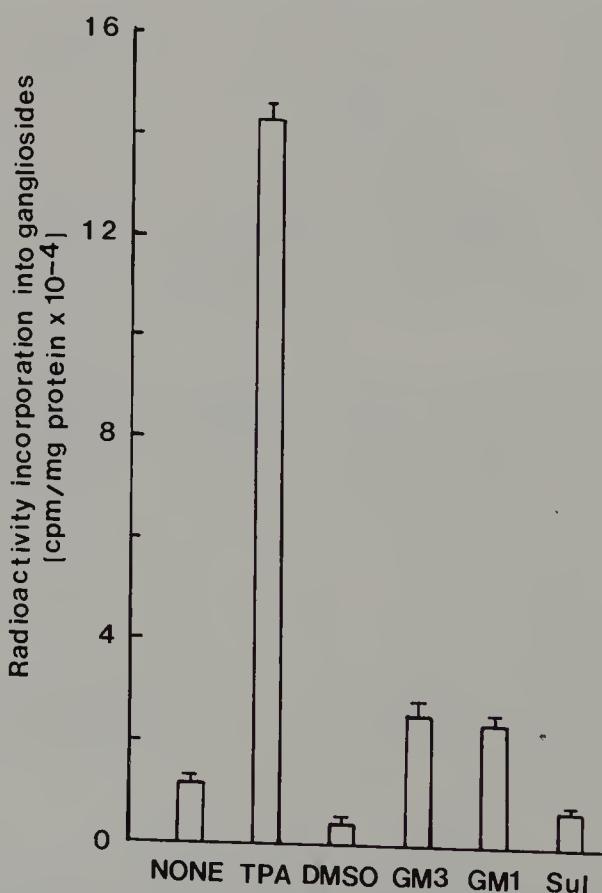
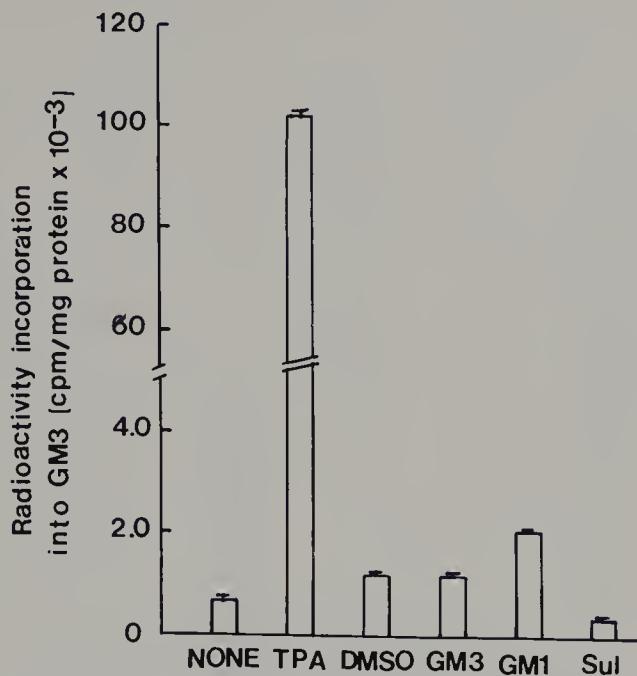
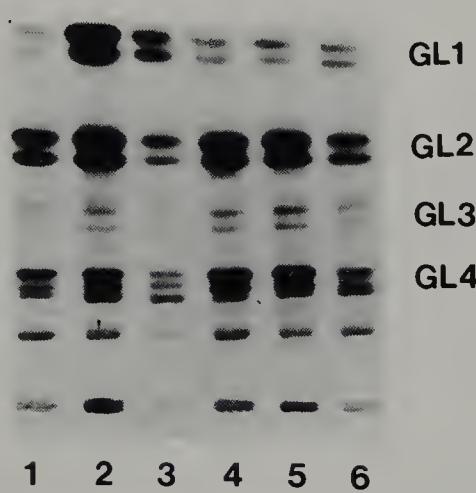


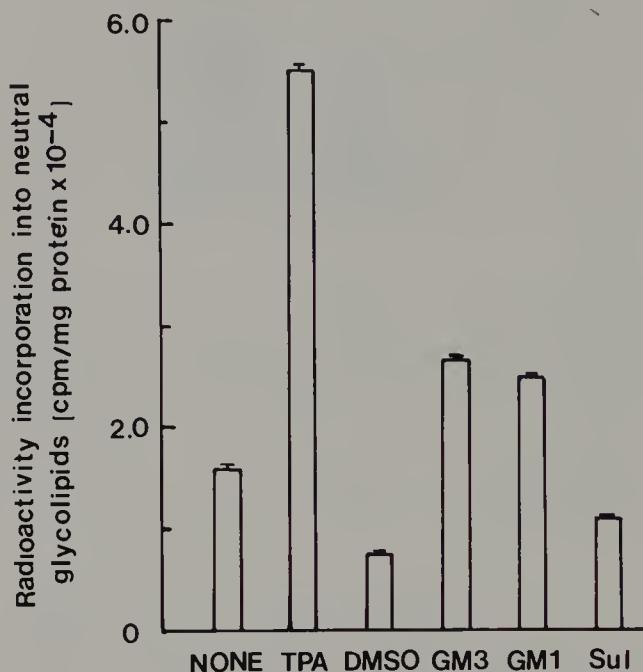
FIG. 4. Effects of exogenously added TPA, DMSO, gangliosides, and sulfatides on the synthesis of gangliosides. After HPTLC chromatography of gangliosides labeled with [ $^{14}\text{C}$ ]galactose, the area corresponding to gangliosides was removed by scraping the silica from plates. Radioactivity was determined as described in "Materials and Methods." Sul, sulfatides. Each value represents the mean  $\pm$  SE of at least three separate experiments. (Reproduced from ref. 60.)



**FIG. 5.** Effect of exogenously added TPA, DMSO, gangliosides, and sulfatides on the synthesis of GM<sub>3</sub>. After HPTLC chromatography of gangliosides labeled with [<sup>14</sup>C]galactose, the band corresponding to GM<sub>3</sub> was removed from plates and radioactivity was determined as described in "Materials and Methods." Sul, sulfatides. Each value represents the mean  $\pm$  SE of at least three separate experiments. (Reproduced from ref. 60.)



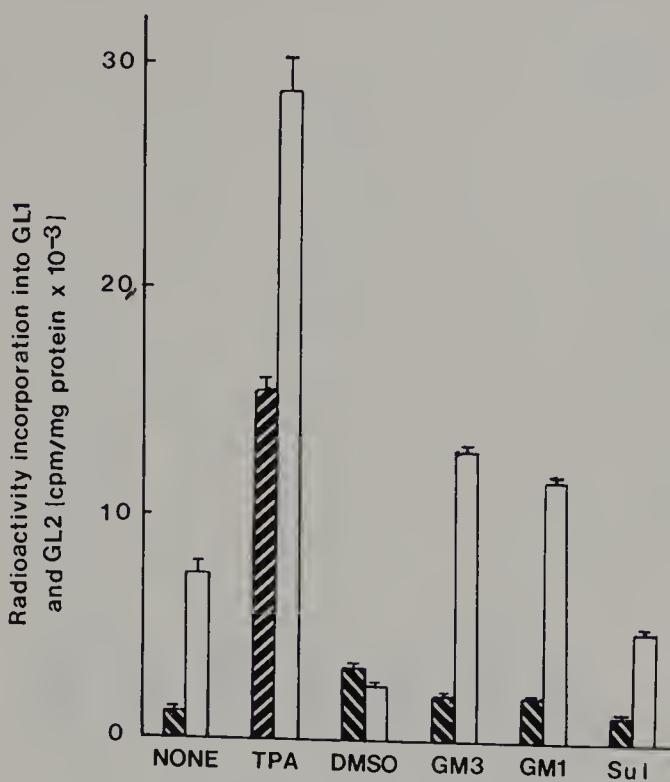
**FIG. 6.** HPTLC autoradiogram of [<sup>14</sup>C]-labeled neutral glycolipids from HL-60 cells exposed to [<sup>14</sup>C]galactose. 1, Control; 2, TPA; 3, DMSO; 4, GM<sub>3</sub>; 5, GM<sub>1</sub>; 6, sulfatides. HPTLC and autoradiography were performed as described in "Materials and Methods." (Reproduced from ref. 60.)



**FIG. 7.** Effect of exogenously added TPA, DMSO, gangliosides, and sulfatides on neutral glycolipid synthesis. After HPTLC chromatography, the area corresponding to neutral glycolipids was removed by scraping from the plate and radioactivity was determined as described in "Materials and Methods." Each value represents the mean  $\pm$  SE of at least three separate experiments. (Reproduced from ref. 60.)

Neutral GSLs were analyzed using HPTLC followed by autoradiography (Fig. 6). The total radioactivity of neutral GSLs of cells treated with TPA, GM<sub>3</sub>, and GM<sub>1</sub> was higher than that of the control cells (Fig. 7). However, treatment with DMSO or sulfatides decreased the biosynthesis of GSLs (Fig. 7). The radioactivity of GL<sub>1</sub> and GL<sub>2</sub> in cells treated with various agents was also examined (Fig. 8). TPA, DMSO, GM<sub>3</sub>, and GM<sub>1</sub> increased the incorporation of [<sup>14</sup>C]galactose into GL<sub>1</sub>, while sulfatides had no effect. Labeling of GL<sub>2</sub> was greater in cells treated with TPA, GM<sub>3</sub>, or GM<sub>1</sub> than in the corresponding untreated control, but lower than in the control of HL-60 leukemia cells cultured in the presence of DMSO or sulfatides.

To determine the effects of inducers of differentiation on PKC activity, enzyme activity was measured after cells were treated for three days, using partially purified enzyme. When 1,2-diolein was omitted from the reaction mixture, the PKC activity of undifferentiated HL-60 cells was not significantly different from that of their differentiated counterparts (Fig. 9). However, when 1,2-diolein was present, HL-60 cells that had been induced to differentiation by TPA, DMSO, or GM<sub>3</sub> showed

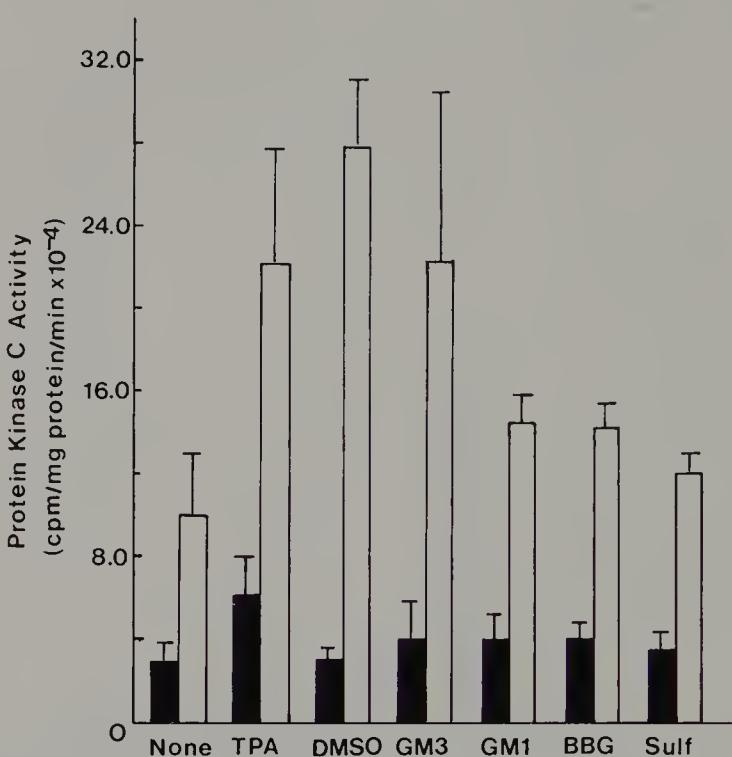


**FIG. 8.** Effects of exogenously added TPA, DMSO, gangliosides, and sulfatides on the synthesis of GL1 and GL2. Radioactivity in GL1 and GL2 from [<sup>14</sup>C]galactose was determined in bands corresponding to GL1 and GL2 after HPTLC separation. GL1, shaded bar; GL2, open bar. Each value represents the mean  $\pm$  SE of at least three separate experiments. (Reproduced from ref. 60.)

a pronounced increase in PKC activity from two- to three-fold (Fig. 9). The PKC activities of HL-60 cells treated with GM<sub>1</sub>, BBG, or sulfatides were about 1.6-, 1.4-, and 1.2-fold higher, respectively, than that of untreated cells; these elevations were considerably less than those of the differentiated leukemia cells.

The activity of ST1 was measured in sonicates of HL-60 cells; incorporation of [<sup>14</sup>C]NeuAc from CMP-[<sup>14</sup>C]NeuAc into the lipid acceptor LacCer was linear with concentrations of protein from 0.1 to 0.8 mg. Treatment with the various agents under study caused an increase in the activity of ST1, except for GM<sub>3</sub> (Fig. 10). The order of stimulatory efficacy was TPA > DMSO > sulfatides > GM<sub>1</sub>.

The effect of added exogenous GM<sub>3</sub> on the activity of ST1 was also examined. We found that the ST1 declined sharply at concentrations of GM<sub>3</sub> greater than 5 nmol in the reaction mixture, suggesting that the relatively high concentration of GM<sub>3</sub> present in HL-60 cells treated with this ganglioside might well inhibit ST1 activity (data not shown).

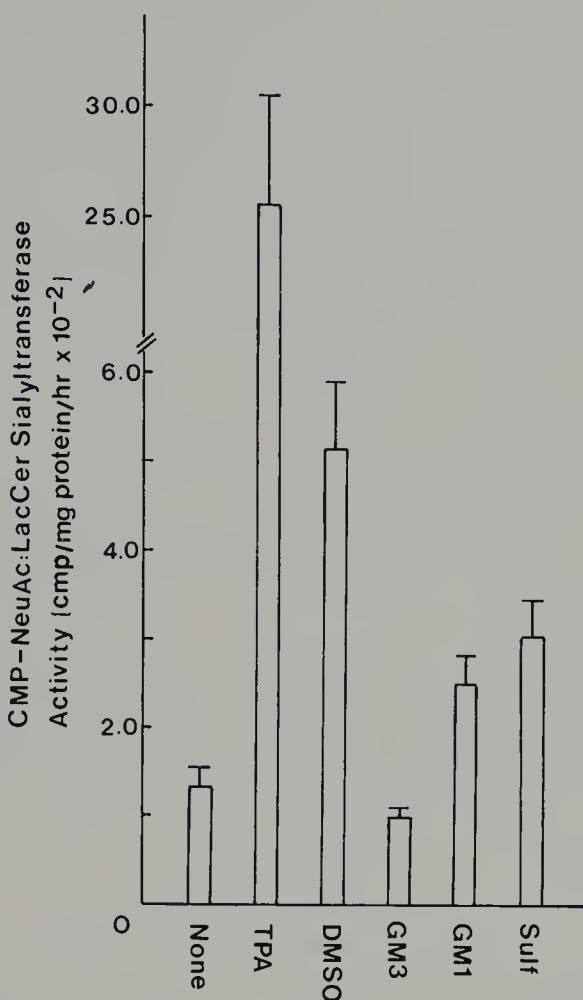


**FIG. 9.** Effect of various agents on diolein-stimulated PKC activity. Partially purified PKC was assayed in HL-60 leukemia cells treated for three days with either 10 nM TPA, 1.3% DMSO, 100  $\mu$ M GM<sub>3</sub>, 100  $\mu$ M GM<sub>1</sub>, 100  $\mu$ M BBG, or 100  $\mu$ M sulfatides using 200  $\mu$ g/ml histone III-2 as the substrate. The free Ca<sup>2+</sup> concentration was 1.1  $\mu$ M. Solid bars, 20  $\mu$ g/ml of phosphatidylserine; open bar, 2  $\mu$ g/ml of 1,2-diolein and 20  $\mu$ g/ml of phosphatidylserine. The data represent the mean  $\pm$  SEM. The number of determinations varied from 9 to 19. (Reproduced from ref. 59.)

## DISCUSSION

We have found that TPA, DMSO, and GM<sub>3</sub> inhibited the replication of HL-60 leukemia cells, in agreement with the reports of other laboratories (4,47). Under cell culture conditions used in our experiments, GM<sub>1</sub> also had an inhibitory effect; this observation is in contrast to the results of another group (4), which reported a stimulation of cell growth by this ganglioside. The reason for this difference is not known but may be related to differences in culture conditions.

TPA and GM<sub>3</sub> induce the differentiation of HL-60 cells along the monocytic pathway (2-4), and DMSO initiates maturation along the granulocytic route (1). Since all of these agents stimulated GM<sub>3</sub> synthesis, it has been suggested that an



**FIG. 10.** Effect of various agents on CMP-N-acetylneuraminic acid:lactosylceramide sialyltransferase activity. The enzyme activity from HL-60 leukemia cells treated for three days with either 10 nM TPA, 1.3% DMSO, 100  $\mu$ M GM<sub>3</sub>, 100  $\mu$ M GM<sub>1</sub>, or 100  $\mu$ M sulfatides was assayed as described in "Materials and Methods." Each value represents the means  $\pm$  SEM of at least three separate experiments. (Reproduced from ref. 59.)

enrichment of membrane GM<sub>3</sub> may be involved in the terminal differentiation process (48). GM<sub>3</sub> and GM<sub>1</sub> have been postulated to inhibit replication through cell-cell contact inhibition (48). In addition, the concentration-dependent binding of platelet-derived growth factor (PDGF) to certain cells has been reported to be altered by exogenous GM<sub>3</sub> and GM<sub>1</sub> (47-49). Thus, ganglioside-dependent cellular growth inhibition may be caused by an altered affinity of this receptor to PDGF

(47,49). GM<sub>1</sub> and GM<sub>3</sub> inhibited PDGF-stimulated phosphorylation of tyrosine (49), suggesting that at the level of the membrane, GM<sub>1</sub> and GM<sub>3</sub> may modulate receptor function by affecting the degree of tyrosine phosphorylation, thereby altering affinity for growth factors.

We have found that differentiated HL-60 leukemia cells exhibit modifications of glycolipid metabolism. Thus, the labeling of gangliosides, in particular GM<sub>3</sub>, and neutral glycolipids by [<sup>14</sup>C]galactose was increased by the presence of TPA, GM<sub>3</sub>, or GM<sub>1</sub>. In addition, the incorporation of [<sup>14</sup>C]galactose into GM<sub>3</sub> was also increased significantly by DMSO. The increased labeling of glycosphingolipids suggests an increase in sialyl- and glycosyltransferase activities rather than a decrease in the rate of glycolipid degradation, since no significant change in sialidase activity was found during the monocytic differentiation of HL-60 cells (50). Nojiri et al. (51) reported that after treatment of HL-60 cells with the granulocytic differentiating agent DMSO for three days, the sialidase activity was increased by 70%. However, except for GM<sub>3</sub>, the labeling of gangliosides by galactose in cells treated with DMSO was decreased. This result may also reflect a change in the enzymatic activities involved in the metabolism of gangliosides, and this effect might be part of the reason for the low incorporation of galactose into total gangliosides in HL-60 cells exposed to DMSO.

Our results with neutral GSLs were generally similar to those reported by Momoi et al. (5) and Buehler et al. (52). Their work (5,52) with TLC analysis and TLC-autoradiography of neutral glycolipids from HL-60 cells demonstrated that the major neutral glycolipids cochromatographed with LacCer (GL2) and nLc4Cer (GL4). Compounds comigrating with monohexosylceramide and trihexosylceramide have been detected as minor components.

We found that TPA and other agents tested in the present study were capable of enhancing the 1,2-diolein-stimulated PKC activity of HL-60 cells. Since TPA, DMSO, and GM<sub>3</sub>, which induce cellular differentiation in this system, caused a two- to three-fold increase in PKC activity, and whereas GM<sub>1</sub>, BBG, and sulfatides, which did not induce maturation, elicited a smaller enhancement of PKC activity, it is conceivable that the capacity of TPA, DMSO, and GM<sub>3</sub> to induce cellular differentiation is related at least in part to the magnitude of the enhancement of PKC activity. The mechanism by which PKC activity is increased by these agents may be due to several factors. First, DMSO, TPA, gangliosides, and sulfatides can be incorporated into the plasma membrane; such incorporation may produce a conformational change that leads to an increase in the affinity of the enzyme for Ca<sup>2+</sup> and phosphatidylserine. Second, TPA, DMSO, and GM<sub>3</sub> induce the differentiation of HL-60 cells, and the PKC activity of differentiated cells may be constitutively higher than that of their undifferentiated counterparts. Third, these agents may cause a change in the distribution of the enzymatic activity between the cytosol and the plasma membrane, which affects PKC activity.

We have also shown that the induced cellular differentiation produced by the tumor promotor TPA is accompanied by an increased ST1 activity; similar results have also been reported by Momoi et al. (28) in HL-60 cells. In addition, we have

also found that DMSO produces effects similar to those of TPA. These observations are consistent with the concept that the increase in GM<sub>3</sub> synthesis is directly correlated with the activation of PKC.

In contrast GM<sub>3</sub> did not enhance the sialyltransferase activity of these cells. It is possible that the GM<sub>3</sub> in the medium is inserted into the cellular membrane (53–55) as indicated in the present study, and the residual GM<sub>3</sub> decreased the activity of the sialyltransferase by end-product inhibition. This type of end-product inhibition has been previously shown by Yu et al. (56). This possibility is supported by our experiments in which the addition of exogenous GM<sub>3</sub> to the enzymatic system produced inhibition of sialyltransferase activity. Furthermore, GM<sub>1</sub> and sulfatides did not inhibit sialyltransferase activity, showing instead a slight activation of the enzyme.

The findings have led us to hypothesize that the PKC activity and the increase in GM<sub>3</sub> synthesis may be related events that are important to HL-60 cellular differentiation. It is possible that PKC may participate in regulating the activity of the sialyltransferase by a phosphorylation-dephosphorylation mechanism. In such a mechanism, the phosphorylated sialyltransferase may be envisioned to represent the active form of the enzyme, whereas the dephosphorylated form would represent the inactive catalyst. In support of such a concept, several lines of evidence have suggested that glycosyltransferases such as ST1 (57) and UDP-N-acetylgalactosaminyl-transferase (58) may be regulated by protein kinase systems. Further studies are being conducted to ascertain whether phosphorylated and nonphosphorylated forms of the sialyltransferase exist in HL-60 leukemia cells.

## SUMMARY

Gangliosides are known to play a crucial role in many dynamic cellular processes including the regulation of proliferation and differentiation. Recently, several lines of evidence have emerged suggesting that gangliosides may mediate these processes by modulating protein kinase systems. To study these complex effects in intact cells we have used the human leukemia cell line HL-60 as a model system because of its potential in differentiating into different cell types in response to different inducer agents. Thus, treatment of the human leukemia cells HL-60 with inducers of differentiation, such as phorbol esters (TPA), dimethylsulfoxide (DMSO), GM<sub>3</sub>, or GM<sub>1</sub> resulted in a marked inhibition of the growth of the cells. Treatment of HL-60 cells with TPA or GM<sub>3</sub> induced differentiation along the monocyte/macrophage lineage, while treatment with DMSO induced maturation along the granulocytic pathway. These effects were accompanied by more than a two-fold increase in protein kinase C (PKC) activity. In contrast, treatment with GM<sub>1</sub> or sulfatides caused less than a two-fold increase in PKC activity. Interestingly, these agents (with the exception of GM<sub>3</sub>) also caused an increase in sialyltransferase activity (ST1) for GM<sub>3</sub> synthesis. GM<sub>3</sub>, which also induced cellular differentiation,

inhibited ST1 activity, perhaps through an end-product inhibition mechanism. Our findings suggest that the direct or indirect modulation of PKC activity by some of these agents may be involved, at least in part, in the regulation of cellular growth and differentiation.

## ACKNOWLEDGMENTS

Financial support was provided by Grant NS-11853 from the National Institutes of Health. The work was performed with the collaboration of Dr. A. C. Sartorelli whose support is gratefully acknowledged. The technical assistance provided by Drs. X.-J. Xia, X.-B. Gu, and S. Ren is also acknowledged.

## REFERENCES

1. Roisen FJ, Bartfeld H, Nagele R, York G. Ganglioside stimulation of axonal sprouting *in vitro*. *Science* 1981;214:577-578.
2. Byrne MC, Ledeen RW, Roisen FJ, Yorke G, Sclafani JR. Ganglioside-induced neuritogenesis: verification that gangliosides are the active agents, and comparison of molecular species. *J. Neurochem.* 1983;41:1214-1222.
3. Dimpfel W, Moller W, Mengs U. Ganglioside-induced neurite formation in cultured neuroblastoma cells. In: Rapport MM, Gorio A, eds. *Gangliosides in Neurological and Neuromuscular Function, Development, and Repair*. New York: Raven Press, 1981; 119-134.
4. Cannella MS, Archer AJ, Ledeen RW. Stimulation of neurite outgrowth *in vitro* by a glycero-ganglioside. *Int. J. Dev. Neurosci.* 1988;6:319-326.
5. Carine K, Schengrund C-L. Effects of exogenous GM<sub>1</sub> and GD1a on S20Y neuroblastoma cells. *J. Neurosci. Res.* 1984;12:59-69.
6. Nakajima J, Tsuji S, Nagai Y. Bioactive gangliosides: analysis of functional structures of the tetrasialoganglioside GQ1b which promotes neurite outgrowth. *Biochim. Biophys. Acta*. 1986;876:65-71.
7. Rybak S, Ginzburg I, Yavin E. Gangliosides stimulate neurite outgrowth and induce tubulin mRNA accumulation in neural cells. *Biochem. Biophys. Res. Comm.* 1983; 116:974-980.
8. Bremer EG, Hakomori SI, Bowen-Pope DG, Raine E, Ross R. Ganglioside-mediated modulation of cell growth, growth factor binding and receptor phosphorylation. *J. Biol. Chem.* 1984;259:6818-6825.
9. Bremer EG, Schlessinger J, Hakomori SI. Ganglioside mediated modulation of cell growth. Specific affects of GM<sub>3</sub> on tyrosine phosphorylation of the epidermal growth factor receptor. *J. Biol. Chem.* 1986;261:2434-2440.
10. Yu RK, Goldenring JR, DeLorenzo RJ. Effects of gangliosides on rat brain membrane protein phosphorylation. *INSERM* 1984;126:335-354.
11. Goldenring JR, Otis LC, Yu RK, DeLorenzo RJ. Calcium/ganglioside-dependent protein kinase activity in rat brain membrane. *J. Neurochem.* 1985;44:1229-1234.

12. Yu RK. Regulation of protein phosphorylation by gangliosides. In: Ledeen RW, Hogan EL, Tettamanti G, Yates AJ, and Yu RK, eds. *New Trends in Ganglioside Research*. Padova: Liviana Press, 1989;461–471.
13. Kim JYH, Goldenring JR, DeLorenzo RJ, Yu RK. Gangliosides inhibit phospholipid sensitive  $\text{Ca}^{2+}$ -dependent kinase phosphorylation of rat myelin basic protein. *J. Neurosci. Res.* 1986;15:159–166.
14. Kreutter D, Kim JYH, Goldenring JR, Rasmussen H, Ukomadu C, DeLorenzo RJ, Yu RK. Regulation of protein kinase C activity by gangliosides. *J. Biol. Chem.* 1987;262:1633–1637.
15. Tsuji S, Nakajima J, Sasaki T, Nagai Y. Bioactive gangliosides. IV. Ganglioside GQ1b/ $\text{Ca}^{2+}$ -dependent protein kinase activity exists in the plasma membrane fraction of a neuroblastoma cell line, GOTO. *J. Biochem. (Tokyo)* 1985;97:969–972.
16. Chan K-FJ. Ganglioside-modulated protein phosphorylation. Partial purification and characterization of a ganglioside stimulated protein kinase in brain. *J. Biol. Chem.* 1987;262:5248–5255.
17. Chan K-FJ. Ganglioside-modulated protein phosphorylation. Partial purification and characterization of ganglioside-inhibited protein kinase in brain. *J. Biol. Chem.* 1987;263:568–574.
18. Yates AJ, Wood CJ, Halterman RK, Stock SM, Walter JD, Johnson JD. Effects of ganglioside, calmodulin, protein kinase C and copper on phosphorylation of protein in membranes of normal and transected sciatic nerve. In: Ledeen RW, Hogan EL, Tettamanti G, Yates AJ, Yu RK, eds. *New Trends in Ganglioside Research*, Padova: Liviana Press, 1988;495–511.
19. Collins SJ, Ruscetti FW, Gallagher RE, Gallo RC. Terminal differentiation of human promyelocytic leukemia cells induced by dimethyl sulfoxide and other polar compounds. *Proc. Natl. Acad. Sci. USA* 1978;75:2458–2462.
20. Rovera G, O'Brien TG, Diamond L. Induction of differentiation of human promyelocytic leukemia cells by tumor promoters. *Science* 1979;204:868–870.
21. Rovera G, Santoli D, Damsky C. Human promyelocytic leukemia cells in culture differentiate into macrophage-like cells when treated with phorbol ester. *Proc. Natl. Acad. Sci. USA* 1979;76:2779–2783.
22. Nojiri HK, Takaka F, Terui Y, Miura Y, Saito M. Ganglioside GM<sub>3</sub>: an acidic membrane component that increases during macrophage-like cell differentiation can induce monocytic differentiation of human myeloid and monocyte leukemic cell lines HL-60 and U937. *Proc. Natl. Acad. Sci. USA* 1986;83:782–786.
23. Momoi T, Yokota J. Alterations of glycolipids of human leukemia cell line HL-60 during differentiation. *J. Natl. Cancer Inst.* 1983;78:229–236.
24. Lozzio CB, Lozzio BB. Human chronic myelogenous leukemia cell-line with positive Philadelphia chromosome. *Blood* 1975;45:321–334.
25. Koeffler HP, Golde DW. Acute myelogenous leukemia: a human cell line responsive to colony-stimulating activity. *Science* 1978;200:1153–1154.
26. Takeda K, Minowada J, Bloch A. Kinetics of appearance of differentiation-associated characteristics in ML-1, a line of human myeloblastic leukemia cells, after treatment with 12-O-tetradecanoylphorbol-13-acetate, dimethyl sulfoxide or 1- $\beta$ -D-arabinofuranosylcytosine. *Cancer Res.* 1982;42:5152–5158.
27. Huberman E, Heckman C, Langenbach R. Stimulation of differentiated function in human melanoma cells by tumor-promoting agents and dimethyl sulfoxide. *Cancer Res.* 1979;39:2618–2624.

28. Momoi T, Shirmolo M, Kasuya J, Senoo H, Sazaki Y. Activation of CMP-N-acetylneurameric acid:lactosylceramide sialyltransferase during the differentiation of HL-60 cells induced by 12-O-tetradecanoylphorbol-13-acetate. *J. Biol. Chem.* 1986; 261:16270–16273.
29. Niedel JE, Kuhn L, Vandenberk GR. Phorbol diester receptor copurifies with protein kinase C. *Proc. Natl. Acad. Sci. USA* 1983;80:35–40.
30. Vandenberk GR, Kuhn L, Niedel JE. Possible mechanism of phorbol diester-induced maturation of human promyelocytic leukemia cells. *J. Clin. Invest.* 1984;73:448–457.
31. Leach KL, James ML, Blumberg PM. Characterization of a specific phorbol ester aporeceptor in mouse brain cytosol. *Proc. Natl. Acad. Sci. USA* 1983;80:4208–4212.
32. Castagna M, Takai K, Kaibuchi K, Sano K, Kikkawa U, Nishizuka Y. Direct activation of calcium activated, phospholipid-dependent protein kinase by tumor promoting phorbol esters. *J. Biol. Chem.* 1982;257:7847–7851.
33. Feuerstein N, Cooper HL. Rapid protein phosphorylation induced by phorbol ester in HL-60 cells. *J. Biol. Chem.* 1983;258:10786–10793.
34. Feuerstein N, Cooper HL. Rapid phosphorylation-dephosphorylation of specific proteins induced by phorbol ester in HL-60 cells. Further characterization of the phosphorylation of 17-kilodalton and 27-kilodalton proteins in myeloid leukemic cells and human monocytes. *J. Biol. Chem.* 1984;259:2782–2788.
35. Feuerstein N, Cooper HL. Studies of the differentiation of promyelocytic cells by phorbol ester. II. A methylation inhibitor, 3-deazaadenosine, inhibits the induction of specific differentiation proteins. Lack of effect on early and late phosphorylation events. *Biochim. Biophys. Acta.* 1984;781:247–256.
36. Mita S, Nakaki T, Yamamoto S, Kato R. Phosphorylation and dephosphorylation of human promyelocytic leukemia cell (HL-60) proteins by tumor promotor. *Exp. Cell Res.* 1984;154:492–499.
37. Morin MJ, Kreutter D, Rasmussen H, Sartorelli AC. Disparate effects of activators of protein kinase C on HL-60 promyelocytic leukemia cell differentiation. *J. Biol. Chem.* 1984;262:11758–11763.
38. Nakaki T, Mita S, Yamamoto S, Kato R. Inhibition by palmitoylcarnitine of adhesion and morphological changes in HL-60 cells induced by 12-O-tetradecanoylphorbol-13-acetate. *Cancer Res.* 1984;44:19908–19912.
39. Ledeen RW, Yu RK. Gangliosides: structure, isolation and analysis. *Methods Enzymol.* 1982;83:139–191.
40. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurements with the Folin phenol reagent. *J. Biol. Chem.* 1951;193:265–275.
41. Svennerholm L. Quantitative estimation of sialic acid. II. A colorimetric and resorcinol hydrochloric acid method. *Biochim. Biophys. Acta.* 1957;24:604–611.
42. Folch J, Lees M, Sloane-Stanley GH. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* 1957;226:497–509.
43. Sewell AC. An improved thin-layer chromatographic method for urinary oligosaccharide screening. *Clin. Chim. Acta.* 1979;92:411–414.
44. Kreutter D, Caldwell AB, Morin MJ. Association of protein kinase C activation from phorbol ester-induced maturation of HL-60 leukemia cells. *J. Biol. Chem.* 1985; 260:5979–5984.
45. Yu RK, Lee SH. *In vitro* biosynthesis of sialosylgalactosylceramide ( $G_7$ ) by mouse brain microsomes. *J. Biol. Chem.* 1976;251:198–203.

46. Ahmad H, Saleemudin MA. A Coomassie blue-binding assay for the microquantitation of immobilized proteins. *Anal. Biochem.* 1985;148:533–541.
47. Bremer EG, Hakomori SI, Bowen-Pope DF, Raines E, Ross R. Ganglioside-mediated modulation of cell growth, growth factor binding, and receptor phosphorylation. *J. Biol. Chem.* 1984;259:6818–6825.
48. Hakomori S-I. Glycosphingolipids in cellular interactions, differentiation and oncogenesis. *Annu. Rev. Biochem.* 1981;50:710–764.
49. Bremer EG, Schlessinger J, Hakomori SI. Ganglioside-mediated modulation of cell growth. Specific effects of GM<sub>3</sub> on tyrosine phosphorylation of the epidermal growth factor receptor. *J. Biol. Chem.* 1986;261:2434–2440.
50. Nojiri H, Takaku F, Tetsuka T, Motoyoshi K, Miura Y, Saito M. Characteristic expression of glycosphingolipid profiles in the bipotential cell differentiation of human promyelocytic leukemia cell line HL-60. *Blood.* 1984;64:534–541.
51. Nojiri H, Takaku F, Tetsuka T, Saito M. Stimulation of sialidase activity during cell differentiation of human promyelocytic leukemia cell line HL-60. *Biochem. Biophys. Res. Commun.* 1982;104:1239–1246.
52. Buehler J, Qwan E, DeGregorio MW, Macher BA. Biosynthesis of glycosphingolipids by human myeloid leukemia cells. *Biochemistry* 1985;24:6978–6984.
53. Laine RA, Hakomori S. Incorporation of exogenous glycosphingolipids in plasma membranes of cultured hamster cells and concurrent change of growth behavior. *Biochem. Biophys. Res. Commun.* 1973;54:1039–1045.
54. Icard-Liepkalns C, Liepkalns VA, Yates AJ, Stephens RE. Cell cycle phases of a novel human neural cell line and the effect of exogenous gangliosides. *Biochem. Biophys. Res. Commun.* 1982;105:225–230.
55. Keenan TW, Schmid E, Franke WW, Weigandt H. Exogenous glycosphingolipids suppress growth rate of transformed and untransformed 3T3 mouse cells. *Exp. Cell Res.* 1975;92:259–270.
56. Yu RK, Itoh T, Yohe HC, Macala LJ. Characterization of some minor gangliosides of Tay-Sachs brain. *Brain Res.* 1983;275:47–52.
57. Burczak JD, Soltysiak RM, Sweeley CC. Regulation of membrane-bound enzymes of glycosphingolipid biosynthesis. *J. Lipid Res.* 1984;25:1541–1547.
58. Scheideler MA, Dawson G. Direct demonstration of the activation of UDP-N-acetylgalactosamine:[GM<sub>3</sub>]N-acetylgalactosaminyltransferase by cyclic AMP. *J. Neurochem.* 1986;46:1639–1643.
59. Xia X-J, Gu X-B, Sartorelli AC, Yu RK. Effects of inducers of differentiation on protein kinase C and CMP-N-acetylneurameric acid:lactosyl-ceramide sialyltransferase activities of HL-60 leukemic cells. *J. Lipid Res.* 1989;30:181–188.
60. Xia X-J, Ren S, Sartorelli AC, Yu RK. Effects of phorbol esters, dimethylsulfoxide, and gangliosides on the biosynthesis of glycolipids by HL-60 promyelocytic leukemia cells. In: Oettgen HT, ed. *Gangliosides and Cancer*. Weinheim, FDR: VCH Publishers, 1989; 341–353.



# Nerve Growth Factor-sensitive Phosphorylations and the Action of K-252a on PC12 Cells

Shinichi Koizumi, Tatsuro Mutoh, Alexey Ryazanov\*,  
Brian B. Rudkin and Gordon Guroff

Section on Growth Factors

National Institute of Child Health and Human Development

National Institutes of Health

Bethesda, MD 20892

\* Institute of Protein Research

Academy of Sciences of the U.S.S.R.

142292 Pushchino, Moscow Region, U.S.S.R.

**N**erve growth factor (NGF) is a peptide that is required for the survival and development of neurons in the sympathetic and sensory nervous systems (21,7). It also has effects on certain populations in the central nervous system (6,12), on the adrenal medulla (1,40), on some tumors (4,41), and on specific classes of blood cells (22,23). Although the phenotypic changes produced by NGF on its target cells have been described in some detail (32,5), the molecular mechanism(s) by which these changes are produced is known only in outline. There is evidence that NGF does not act directly inside the cell (13), but a number of second messenger systems have been studied (8,18,34,36) and none has proven to be the sole mediator of NGF action.

Some years ago, it was shown that NGF treatment of PC12 cells produces a change in the phosphorylation of several cellular proteins (9,44). Since that time other NGF-sensitive phosphorylations have been identified (Table 1). Now it is clear that many of the phenotypic changes seen upon treatment of cells with NGF may be due to changes in the phosphorylation, and, in turn, in the function, of key proteins in the cell. There are some who believe that a mechanism, or the mechanism, by which NGF acts is by altering these phosphorylative events in its target cells.

Our interest has been to dissect these phosphorylations biochemically in order to illuminate the molecular mechanism by which NGF acts. To this end, we have prepared cell-free systems from PC12 cells that reflect the prior treatment of those cells with NGF. These "footprint" systems, then, remember that the cells from which they were prepared were treated with NGF, and reflect, in altered phos-

**TABLE 1.** Short-term actions of nerve growth factor on specific phosphorylations in PC12 cells

Substrate	Localization	Kinase	Mechanism	Reference
Tyrosine hydroxylase	Cytosol	N-kinase	Unknown	35
Ribosomal protein S6	Ribosomes	S6 kinase	cAMP pathway	9, 24
EF-2 (Nsp100)	Cytosol	CaM-PK III	Kinase C pathway	39, 10
pp250	Cytoskeleton	Cofactor-independent kinase	cAMP pathway	29, 30
Histones H1A, H2A, H3	Nuclear	Unidentified	Unknown	19
HMG 17	Nuclear and cytoplasmic	Unidentified	cAMP pathway	9
SMP	Nuclear	Unidentified	Unknown	44, 31

phorylation, the same changes seen in whole cells. We have been able, in some cases, to study these phosphorylations in detail, purifying kinase and substrate, and to define, biochemically, the changes NGF has caused.

The phosphorylation of a nuclear protein, called by us SMP (slow migrating protein) is markedly increased in sympathetic neurons and in PC12 cells treated with NGF (44). This increase can be seen within one hour after the addition of NGF and can be observed even after several hours of NGF treatment. The protein is phosphorylated exclusively on serine residues. Epidermal growth factor and cAMP analogs also increase the phosphorylation of SMP in PC12 cells (44), as does fibroblast growth factor (38). The protein has an apparent molecular weight of about 30,000 and appears to be a member of the low-mobility group of proteins. Although SMP is one of the more strongly phosphorylated of the nuclear proteins, it is a minor constituent of the nuclei. SMP appears to be identical to the protein called BRP by Rosenfeld and his colleagues (26,27); the phosphorylation of BRP in GH4 cells is stimulated by thyrotropin-releasing hormone and it has been suggested that BRP phosphorylation is correlated with the transcription of the prolactin gene. Nuclei prepared from cells treated with NGF and incubated with radioactive ATP show an increased phosphorylation of SMP (31), but this nuclear preparation has resisted further fractionation, so the identity of the NGF-sensitive SMP kinase is not yet known.

NGF-treated PC12 cells show a decrease in the phosphorylation of a soluble protein with an apparent molecular weight of 100,000 (3), which we have called Nsp100. The phosphorylation in this case is threonine-specific and the decrease in phosphorylation is apparent within a few minutes after the addition of NGF. A cell-free preparation from NGF-treated PC12 cells, incubated with radioactive ATP, also shows a decreased phosphorylation of this protein (3), and by fractionation of this cell-free system (39) it has been possible to identify a kinase of apparent molecular weight between 110,000 and 130,000 that phosphorylates the 100,000 substrate on threonine and whose activity is decreased in extracts from cells treated

with NGF. Studies from this laboratory indicate that the decrease in the activity of the Nsp100 kinase is caused by a phosphorylation of that kinase by protein kinase C (10).

It is now clear that Nsp100 is, in fact, elongation factor 2 (EF-2) (17) and the kinase phosphorylating it is the kinase identified by Palfrey and his colleagues as calcium/calmodulin kinase III (29). This enzyme demonstrates a clear requirement for calcium and calmodulin at pH 7.4 (29), but a clear independence from these cofactors at 6.2 (39). In contrast to the data from this laboratory, implicating kinase C (26), Nairn et al. (30) have presented data indicating that the decrease in the activity of this kinase produced by NGF treatment of PC12 cells involves a cAMP-dependent mechanism.

The phosphorylation of the S6 protein of the ribosomes is also sensitive to NGF treatment of the cells (9). When cell-free extracts from treated cells are incubated with 40S ribosomal subunits from rat liver, the phosphorylation of S6 is much enhanced. The NGF-sensitive S6 kinase has been identified and partially purified (24). The kinase has an apparent molecular weight of some 45,000 and is unlike any of the already identified cellular kinases. The phosphorylation of S6 is serine-specific and appears to require only  $Mg^{2+}$  as cofactor. The increased activity of the S6 kinase is accompanied by a shift in the isoelectric point of the kinase to a slightly more acidic pH. It has been shown that the increased activity of the kinase and the change in its isoelectric point can both be reversed by treatment with alkaline phosphatase (24), suggesting that the increased activity is due to a phosphorylation of the S6 kinase. The increase in the enzymatic activity produced by NGF is mimicked by treatment of the cells with dibutyryl cyclic AMP (dBcAMP) and the effects of NGF and dBcAMP on S6 kinase activity are not additive. These data can be interpreted to indicate that the increase in S6 kinase activity is due to a phosphorylation of S6 kinase by a cAMP-dependent kinase.

It is of interest that both NGF and EGF increase the phosphorylation of S6 in PC12 cells (9). Since NGF and EGF have very different effects on PC12 cells, the former differentiating them and inhibiting their proliferation, the latter stimulating their proliferation, the physiological meaning of the S6 phosphorylation has been hard to discern. Recent studies have shown, however, that there are, in fact, at least two different S6 kinase activities in PC12 cells, one activated by NGF, the other by EGF (28). The kinase activated by NGF is sensitive to beta-glycerophosphate; the kinase activated by EGF is resistant to beta-glycerophosphate. The two kinases have different time courses of activation and have different apparent molecular weights. This finding opens the possibility that the phosphorylation of S6 by these two kinases is different, and, further, that this difference in S6 phosphorylation causes a difference in S6 function. It is even possible that the different functions of the S6 protein participate in the choice the cell makes to divide or to differentiate.

Overall, it can be suggested that the action of NGF is mediated by the phosphorylation of key proteins in various parts of the cell, altering the functions of those proteins and, thereby, changing the phenotype of the cells. For example,

the phosphorylations of EF-2 and S6 most certainly alter the protein synthetic capabilities of the cells either qualitatively or quantitatively. It can be postulated that the phosphorylation of the nuclear protein SMP alters the transcription of specific genes or groups of genes. Because the changes in phosphorylation are caused by changes in the activities of specific kinases and those changes appear due, in turn, to changes in the phosphorylation of the kinases themselves, it can be suggested that NGF acts through a kinase cascade. Finally, since different kinases appear to be involved, kinase C in the phosphorylation of EF-2 and cAMP-dependent kinase in the phosphorylation of S6, it can be suggested that different kinase cascades are involved. Thus, NGF might work through a series of parallel kinase cascades, initiated by the combination of NGF with its receptor.

Even if this postulate proves true, an important question remains. We do not know the identity of the proximal link between the receptor and the kinase cascades. One tool that has recently become available is the kinase inhibitor K-252a (Fig. 1). K-252a is an alkaloid isolated from the culture broth of *Nocardiopsis* sp. and reported to be an inhibitor of kinase C (14) and other kinases (15,33) by virtue of its ability to inhibit the binding of ATP in a competitive fashion. In whole cell systems K-252a has been found to be a potent inhibitor of the contraction of rabbit artery strips induced by several agents (43) and of phorbol ester-induced serotonin release in platelets (42). K-252a also inhibits superoxide anion production by polymorphonuclear neutrophils stimulated with diacylglycerol (37).

In the PC12 cell system, K-252a has the intriguing property of inhibiting, selectively, the actions of NGF on these cells (11,16). For example, K-252a inhibits the NGF-induced neurite outgrowth displayed by PC12 cells, but does not inhibit the neurite outgrowth induced by fibroblast growth factor. The induction of ornithine decarboxylase by NGF is blocked, but the inductions of ornithine decar-

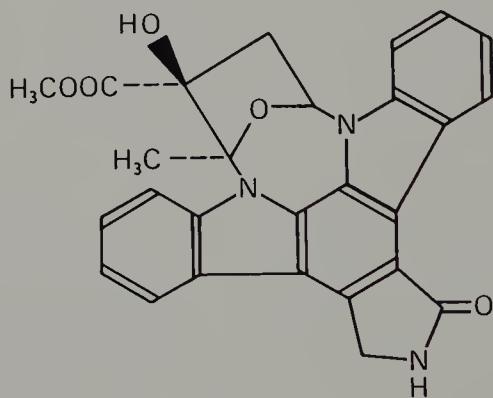


FIG. 1. Structure of K-252a.

boxylase by EGF, fibroblast growth factor, dBcAMP, or phorbol myristate acetate (PMA), in parallel cultures under the same conditions, is not only not blocked, it is generally stimulated. K-252a also selectively blocks the NGF-induced increase in phosphatidylinositol turnover, the NGF-induced increase in intracellular calcium, and the NGF-induced increase in the transcription of *c-fos* (20). Finally, it has been shown (2) that K-252a inhibits the induction, by NGF, of a specific set of genes in PC12 cells, but does not inhibit the induction of the same set of genes by fibroblast growth factor. For all practical purposes, K-252a inhibits all the actions of NGF on PC12 cells, but does not inhibit comparable actions of other compounds on the same cells. K-252a has also been shown to inhibit the actions of NGF on sensory neurons (25).

The mechanism by which K-252a acts on PC12 cells is not known, but its actions as a kinase inhibitor in cell-free systems suggests that it is acting here in the same fashion. It also seems likely that it acts intracellularly, because when K-252a and NGF are added simultaneously, only partial inhibition of the short-term actions of NGF is seen; complete inhibition requires that K-252a be added somewhat before the addition of NGF (20). It is quite clear that it is not acting as a receptor antagonist (16), but the fact that it inhibits all the actions of NGF suggests that it is acting on some event very close to receptor binding. It is clear that it is not inhibiting one of the well-known kinases, because the actions of PMA and dBcAMP on the cells are unaffected, while the actions of NGF are completely blocked. Since there is no kinase activity associated with the NGF receptor itself, it is reasonable to suggest that K-252a inhibits a protein kinase that is in the signal transduction pathway and closely associated with the NGF receptor, or inhibits a kinase that regulates the function of a protein having an important role in that transduction pathway. Thus, discovery of the target of K-252a in PC12 cells may very well reveal a key mediator of NGF action. The search for that target is presently underway in several laboratories.

## SUMMARY

Nerve growth factor treatment of PC12 cells in culture causes them to change from a rapidly dividing chromaffin-like phenotype to a nondividing cell type virtually identical to a mature sympathetic neuron. The biochemical events underlying this conversion are not fully known, but it is clear that there are a number of alterations in the phosphorylation of key proteins in several compartments in the cells, and that these alterations are caused by the stimulation or inhibition of a number of different kinases. These changes in phosphorylation are indicative of changes in the functions of these proteins and may, in aggregate, be the basis of the alteration in phenotype. The initial reactions linking the nerve growth factor receptor to these several kinases are not known, but a new kinase inhibitor, K-252a, may provide a tool by which these reactions can be uncovered. K-252a has

been found to be absolutely specific for the actions of nerve growth factor on PC12 cells, and does not inhibit the comparable actions of other ligands on these same cells.

## REFERENCES

1. Aloe L, Levi-Montalcini R. Nerve growth factor-induced transformation of immature chromaffin cells *in vivo* into sympathetic neurons: effect of antiserum to nerve growth factor. *Proc. Natl. Acad. Sci. USA* 1979;76:1246-1250.
2. Cho K-O, Skarnes WC, Minsk B, Palmieri S, Jackson-Grusby L, Wagner JA. Nerve growth factor regulates gene expression by several different mechanisms. *Mol. Cell. Biol.* 1989;9:135-143.
3. End D, Tolson N, Hashimoto S, Guroff G. Nerve growth factor-induced decrease in the cell-free phosphorylation of a soluble protein in PC12 cells. *J. Biol. Chem.* 1983; 258:6549-6555.
4. Fabricant RN, De Larco JE, Todaro GJ. Nerve growth factor receptors on human melanoma cells in culture. *Proc. Natl. Acad. Sci. US* 1977;74:565-569.
5. Fujita K, Lazarovici P, Guroff G. Regulation of the differentiation of PC12 pheochromocytoma cells. *Environ. Health Perspec.* 1989;80:127-142.
6. Gnahn H, Hefti F, Heumann R, Schwab ME, Thoenen H. NGF-mediated increase in choline acetyltransferase (ChAT) in neonatal rat forebrain: evidence for a physiological role of NGF in the brain. *Brain Res.* 1983;285:45-52.
7. Greene LA, Shooter EM. Nerve growth factor: biochemistry, synthesis, and mechanism of action. *Annu. Rev. Neurosci.* 1980;3:353-402.
8. Gunning PW, Landreth GE, Bothwell MA, Shooter EM. Differential and synergistic actions of nerve growth factor and cyclic AMP in PC12 cells. *J. Cell Biol.* 1981;89:240-245.
9. Halegoua S, Patrick J. Nerve growth factor mediates phosphorylation of specific proteins. *Cell* 1980;22:571-581.
10. Hama T, Huang K-P, Guroff G. Protein kinase C as a component of a nerve growth factor-sensitive phosphorylation system in PC12 cells. *Proc. Natl. Acad. Sci. USA* 1986;83:2353-2357.
11. Hashimoto S. K-252a, a potent protein kinase inhibitor, blocks nerve growth factor-induced neurite outgrowth and changes in the phosphorylation of proteins in PC12h cells. *J. Cell Biol.* 1988;107:1531-1539.
12. Hefti F, Hartikka J, Eckenstein F, Gnahn H, Heumann R, Schwab M. Nerve growth factor increases choline acetyltransferase but not survival or fiber outgrowth of cultured fetal septal cholinergic neurons. *Neuroscience* 1985;14:55-68.
13. Heumann R, Schwab M, Thoenen H. A second messenger required for nerve growth factor biological activity? *Nature (London)* 1981;292:838-840.
14. Kase H, Iwahashi K, Matsuda Y. K-252a, a potent inhibitor of protein kinase C from microbial origin. *J. Antibiot.* 1986;39:1059-1065.
15. Kase H, Iwahashi K, Nakanishi S, Matsuda Y, Yamada K, Takahshi M, Murakata C, Sato A, Kanako M. K-252a compounds, novel and potent inhibitors of protein kinase C and cyclic nucleotide-dependent protein kinases. *Biochem. Biophys. Res. Commun.* 1987;142:436-440.

16. Koizumi S, Contreras ML, Matsuda Y, Hama T, Lazarovici P, Guroff G. K-252a: a specific inhibitor of the action of nerve growth factor on PC12 cells. *J. Neurosci.* 1988; 8:715–721.
17. Koizumi S, Ryazanov A, Hama T, Chen HC, Guroff G. Identification of Nsp100 as elongation factor 2 (EF-2). *FEBS Lett.* 1989;253:55–58.
18. Landreth G, Cohen P, Shooter EM.  $\text{Ca}^{2+}$  transmembrane fluxes and nerve growth factor action on a clonal cell line of rat pheochromocytoma. *Nature (London)* 1980; 283:202–204.
19. Landreth G, Rieser GD. Nerve growth factor- and epidermal growth factor-stimulated phosphorylation of a PC12 cytoskeletal-associated protein *in situ*. *J. Cell Biol.* 1985; 100:677–683.
20. Lazarovici P, Levi B, Lelkes PI, Koizumi S, Fujita K, Matsuda Y, Ozato K, Guroff G. K-252a inhibits the increase in c-fos transcription and the increase in intracellular calcium produced by nerve growth factor in PC12 cells. *J. Neurosci. Res.* 1989;23:1–8.
21. Levi-Montalcini R, Angeletti PU. Nerve growth factor. *Physiol. Rev.* 1968;48:534–569.
22. Matsuda H, Coughlin MD, Bienenstock J, Denburg JA. Nerve growth factor promotes human hemopoietic colony growth and differentiation. *Proc. Natl. Acad. Sci. USA* 1988;85:6508–6512.
23. Matsuda H, Switzer J, Coughlin MD, Bienenstock J, Denburg JA. Human basophilic cell differentiation promoted by 2.5S nerve growth factor. *Int. Arch. Allergy Appl. Immunol.* 1988;86:453–457.
24. Matsuda Y, Guroff G. Purification and mechanism of activation of a nerve growth factor-sensitive S6 kinase from PC12 cells. *J. Biol. Chem.* 1987;262:2832–2844.
25. Matsuda Y, Fukuda J. Inhibition by K-252a, a new inhibitor of protein kinase, of nerve growth factor-induced neurite outgrowth of chick embryo dorsal root ganglion cells. *Neurosci. Lett.* 1988;87:11–17.
26. Murdoch GH, Rosenfeld MG, Evans RM. Eukaryotic transcriptional regulation and chromatin-associated protein phosphorylation by cyclic AMP. *Science* 1982;218:1315–1317.
27. Murdoch GH, Waterman M, Evans RM, Rosenfeld MG. Molecular mechanisms of phorbol ester, thyrotropin-releasing hormone, and growth factor stimulation of prolactin gene transcription. *J. Biol. Chem.* 1985;260:11852–11858.
28. Mutoh T, Rudkin BB, Koizumi S, Guroff G. Nerve growth factor, a differentiating agent, and epidermal growth factor, a mitogen, increase the activities of different S6 kinases in PC12 cells. *J. Biol. Chem.* 1988;263:15853–15856.
29. Nairn AC, Palfrey HC. Identification of a major M<sub>r</sub> 100,000 substrate for calmodulin-dependent protein kinase III in mammalian cells as elongation factor-2. *J. Biol. Chem.* 1987;262:17299–17303.
30. Nairn AC, Nichols RA, Brady MJ, Palfrey HC. Nerve growth factor treatment for cyclic AMP elevation reduces  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase III activity. *J. Biol. Chem.* 1987;262:14265–14272.
31. Nakanishi N, Guroff G. Nerve growth factor-induced increase in the cell-free phosphorylation of a nuclear protein in PC12 cells. *J. Biol. Chem.* 1985;260:7791–7799.
32. Nakanishi N, Guroff G. Growth factors for the nervous system. In: Marangos PJ, Campbell L, Cohen R, eds. *Neuronal and Glial Proteins: Structure, Function, and Clinical Application*. New York: Academic Press, 1988; 159–207.

33. Nakanishi S, Yamada K, Kase H, Nakamura S, Nonomura Y. K-252a, a novel microbial product, inhibits smooth muscle myosin light chain kinase. *J. Biol. Chem.* 1988;263:6215–6219.
34. Nikodijevic B, Nikodijevic O, Yu MW, Pollard H, Guroff G. The effect of nerve growth factor on cyclic AMP levels in superior cervical ganglia of the rat. *Proc. Natl. Acad. Sci. USA* 1975;72:4769–4771.
35. Rowland EA, Muller TH, Goldstein M, Greene LA. Cell-free detection and characterization of a novel nerve growth factor-activated protein kinase in PC12 cells. *J. Biol. Chem.* 1987;262:7504–7513.
36. Schubert D, LeCorbiere M, Whitlock C, Stallcup W. Alterations in the surface properties of cells responsive to nerve growth factor. *Nature (London)* 1978;273:718–723.
37. Smith RJ, Justin JM, Sam LM. Effects of protein kinase C inhibitor, K-252a, on human polymorphonuclear neutrophil responsiveness. *Biochem. Biophys. Res. Commun.* 1988;152:1497–1503.
38. Togari A, Dickens G, Kuzuya H, Guroff G. The effect of fibroblast growth factor on PC12 cells. *J. Neurosci.* 1985;5:307–316.
39. Togari A, Guroff G. Partial purification and characterization of nerve growth factor-sensitive kinase and its substrate from PC12 cells. *J. Biol. Chem.* 1985;260:3804–3811.
40. Unsicker K, Krisch B, Otten U, Thoenen H. Nerve growth factor-induced outgrowth from isolated rat adrenal chromaffin cells: impairment by glucocorticoids. *Proc. Natl. Acad. Sci. USA* 1978;75:3498–3502.
41. Waris T, Rechardt L, Waris P. Differentiation of neuroblastoma cells induced by nerve growth factor *in vitro*. *Experientia* 1973;29:1128–1129.
42. Yamada K, Iwahashi K, Kase H. K-252a, a new inhibitor of protein kinase C, concomitantly inhibits 40K protein phosphorylation of serotonin secretion in phorbol ester-stimulated platelets. *Biochem. Biophys. Res. Commun.* 1987;144:35–40.
43. Yamada K, Tanaka H, Kubo K, Kase H. Inhibition by K-252a, a microbial product, of contraction of isolated rabbit arteries. *Jpn. J. Pharmacol.* 1987;43:284.
44. Yu MW, Tolson NW, Guroff G. Increased phosphorylation of specific nuclear proteins in superior cervical ganglia and PC12 cells in response to nerve growth factor. *J. Biol. Chem.* 1980;255:10481–10492.

# How Do Neurites Grow? Clues from NGF-regulated Cytoskeletal Phosphoproteins

John M. Áletta, Hensin Tsao and Lloyd A. Greene

Laboratory of Cellular and Molecular Neurobiology  
Columbia University College of Physicians and Surgeons  
New York, NY 10032

Differentiation and neurite outgrowth are complex landmarks in the developmental history of the neuron. PC12 cells, a clonal line derived from a rat phenochromocytoma, provide a useful model system in which to study the molecular mechanisms underlying these events (21). When these cells are treated with nerve growth factor (NGF), their mitotic activity ceases, virtually all extend neurites, and they assume many of the characteristics of sympathetic neurons (22). Also, because PC12 cells are a clonal line, they provide a homogeneous source of cell protein. NGF, thus, provides a reproducible trigger that activates the cellular machinery necessary for producing neurites, and PC12 cell cultures offer an accessible, uniform population from which protein compositions can be compared and correlated with neurite initiation and elongation.

The dramatic changes in cell shape that occur after NGF treatment imply a basic restructuring of the cytoskeleton. For the purpose of this paper, therefore, discussion will focus on NGF-regulated cytoskeletal proteins. Stimulation of specific, transcription-dependent synthesis is, first of all, required for the formation and maintenance of neuritic processes (13). However, re-generation of neurites from PC12 cells, though dependent upon NGF, is independent of new RNA transcription. Thus, nongenomic actions, including protein modifications, are also likely to be important for neurite growth. For instance, when NGF is withdrawn from neurite-bearing cells and then re-added several hours later, changes in shape (2,47) and motility (2) at the specialized growing tips of the neurites, the growth cones, proceed with a short latency of seconds to minutes. The rapid return of NGF-dependent morphological features and growth is consistent with post-translational regulation of preexisting proteins. Phosphorylation is now recognized as a key regulator of many cellular functions. The phosphorylation of cytoskeletal elements during process outgrowth has therefore been extensively examined in our studies and will be summarized here.

Normal neurite elongation is likely to progress via a sequence of biochemically mediated events including motility, axoplasmic transport, membrane addition at

the neurite ending, interactions among cytoskeletal elements and, ultimately, stabilization of the newly added length of neurite. Each of these steps may be regulated by extracellular and developmental cues. To begin to catalog and characterize some of the mechanisms of these controls, we shall describe here several cytoskeletal phosphoproteins that have been studied during controlled exposure of PC12 cells to a variety of growth factors, including NGF and other compounds thought to activate various signaling mechanisms *in vivo*. Each of these phosphoproteins exhibits a unique set of characteristics with regard to: phosphorylation time course after NGF treatment, localization in the cell, the dependence of phosphorylation on microtubule integrity, and sensitivity to specific kinases. The results obtained from correlating neurite outgrowth with the chemical composition of differentiating cells provides several insights into the mechanism underlying trophic factor-promoted nerve growth.

## MICROTUBULE-ASSOCIATED PROTEINS

Microtubules are composed of  $\alpha$ - and  $\beta$ -tubulin subunits along with a diverse set of proteins known as MAPs—microtubule-associated proteins. MAPs are thought to play several possible functional roles that are important for neurite growth, including regulating the assembly of tubulin dimers into microtubules (36), stabilizing microtubules (36), crosslinking microtubules to each other and to other cytoskeletal elements (31,45,48), and organelle transport along microtubules (39). The effect of NGF on tubulin polymerization in PC12 cells is correlated with neurite outgrowth (10,18). An increase in total tubulin polymer and in the proportion of cellular tubulin that is polymerized begins at three–four days after treatment, rising sharply thereafter. This effect may be due at least in part to enhancement of microtubule stability after exposure to NGF (10).

### $\beta$ -Tubulin

NGF increases the relative amount of PC12 cell tubulin (soluble dimer plus insoluble tubulin polymer) by about two-fold with little time lag (18). It is thus unlikely that this increase alone drives neurite formation. NGF treatment also results in a large increase in the phosphorylation of  $\beta$ -tubulin (3,10). However, this occurs with a time course similar to that for tubulin polymerization and neurite growth as described above. Because this effect can be observed in long-term (3 wks) NGF-treated PC12 cells in which microtubules had been depolymerized with nocodazole, an anti-microtubule agent, it appears that NGF-promoted  $\beta$ -tubulin phosphorylation is not merely a consequence of enhanced microtubule stability, nor that it absolutely requires the presence of polymerized microtubules (3).

## Chartins

A relatively new class of MAPs first identified in neuroblastoma and non-NGF-treated PC12 cells (37) has recently been given the name "chartins" by Solomon and colleagues (34). These proteins can be categorized as three families that migrate at apparent molecular weights of approximately 64, 72, and 80 kilodaltons (3,10,34,37). Each family can be resolved on 2-D IEF  $\times$  SDS PAGE gels as a series of spots that are distinct from each other, but that are related as shown by peptide mapping (10). The chartins are clearly different from tau proteins based on their isoelectric points, precipitation by high temperature, and recognition by specific immunochemical reagents (3,34,40).

With long-term NGF treatment, the more acidic isoforms of each chartin family are much more abundant (3,10). The absolute protein levels within each chartin family relative to total cell protein, however, are not substantially increased by NGF. These shifts in isoelectric point that occur with NGF exposure are characteristic of changes in protein phosphorylation. This has been confirmed by metabolic radiolabeling of the chartins with either [<sup>35</sup>S]methionine or [<sup>32</sup>P]orthophosphate. The increase in phosphorylation begins at about four days after NGF-treatment and increases rapidly afterwards—a time course similar to NGF's effect on tubulin polymerization and neurite outgrowth.

It is well known that neurite growth requires intact microtubules (16,52). The effect of microtubule depletion on the chartins in intact neurite-bearing cells was, therefore, examined. Exposure of such cells to nocodazole or podophyllotoxin, microtubule-depolymerizing drugs, for 2–24 hr resulted in a shift of the isoform distribution in all three chartin families toward the more basic members. Such a pattern is more typical of non-NGF-treated PC12 cells (3). This suggests that chartin phosphorylation is in some manner dependent on the presence of intact microtubules.

Surprisingly, the neurites of these drug-treated cells did not deteriorate for at least 24–36 hr. Although individual neurites did not elongate, they did not retract significantly either (3). Therefore, in well-established neurites, microtubules do not appear necessary for short-term neurite support, but are required for elongation. Electron microscopy confirmed that microtubules had been lost from the drug-treated neurites. However, at the same time, other filamentous structures became apparent (Fig. 1). It is unclear at this time whether these filaments are induced by the drugs or are simply less evident in the non-drug-treated neurites because their cytoplasm is so densely packed with microtubule bundles. These structures are discussed further below.

Treatments that inhibit neurite outgrowth and regeneration from PC12 cells such as lithium chloride (14) and the kinase A activators, forskolin and cholera toxin (25), also diminish the phosphorylation of chartins. These effects occur without disrupting the integrity of microtubules. Taxol, an alkaloid that increases microtubule polymerization in intact cells (17), does not drive phosphorylation of the chartins. On the contrary, taxol interferes with the normal association between



FIG. 1. Electron micrograph of a PC12 cell neurite after treatment with 50  $\mu\text{M}$  nocodazole for 20 hr. The cell was exposed to NGF for three weeks prior to drug treatment. Tannic acid was present during fixation of the tissue. Bundles of intermediate filaments (*arrows*) were detectable in drug-treated, but not in control neurites (not shown).

chartins and microtubules (3,8). Differential effects of this drug on cytoplasmic versus microtubule-bound chartins are apparently related to phosphate turnover and the ability of chartins to interact with the stabilized tubule. These proteins, thus, may normally interact only intermittently with the microtubule. The most important functional aspect of these interactions then, could be their reversible nature. In this light, it is interesting that taxol also interferes with normal neurite elongation (15,32), although it is still not clear how this effect is generated.

## MAP 1.2

Greene, Liem, and Shelanski first characterized a high-molecular-mass MAP in PC12 cells based upon immunological and microtubule-cycling experiments (26). They demonstrated that the relative levels of phosphate incorporation into this protein were dramatically increased during process outgrowth. On the basis of the protein's relative mobility in polyacrylamide gels compared with those of high-molecular-mass MAPs from brain, it was designated MAP1.2. It is likely that this molecule corresponds to what other investigators have termed MAP 1B (11) or MAP5 (12). It was unclear at the time of the first study to what extent NGF's influence was due to either increased levels of the protein or to enhancement of its phosphorylation. An initial study reported that after NGF-treatment, MAP1 protein levels rise 15- to 20-fold as determined by quantitative immunoblotting (18). A second report indicated that the message levels for MAP1 increase only several-fold (28). Considering the large NGF-induced increase in phosphorylated MAP1.2 and the relatively modest effect of NGF on its message level, the actions of the factor on both steady-state protein levels of MAP1.2 and the extent of its phosphorylation were reexamined.

Increases in both phosphorylation and steady-state protein levels of MAP1.2 were observed (5). An increase in steady-state level, relative to total cellular protein, was observed at three days but not after two hours of NGF. The increase in phosphorylation, however, began within 15 minutes after NGF-treatment followed by a further sustained increase days to weeks later. In contrast to the case for chartins, this phosphorylation is not dependent upon intact microtubules (3). The increase in MAP1.2 protein measured after two weeks of NGF-treatment was 3.5-fold, whereas the average increase in phosphate incorporation per molecule at this time was estimated to be approximately 4.5-fold. The relative average increase in phosphorylation per MAP1.2 molecule at various times of NGF treatment is diagrammed in Fig. 2. The biphasic nature of the plot raises the possibility of separate functional effects of this phosphorylation event.

As with the chartins, phosphorylation of MAP1.2 is accompanied by a mobility

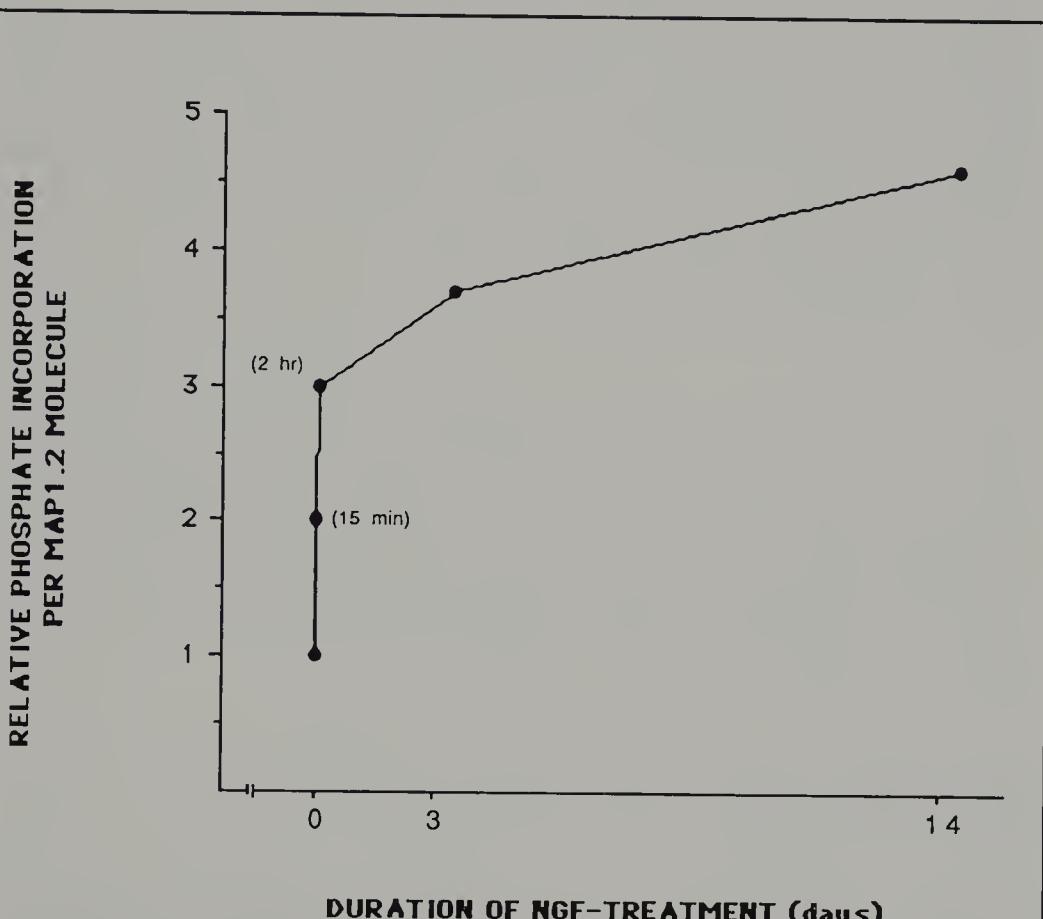


FIG. 2. Changes in the average relative phosphorylation of MAP1.2 in PC12 cells during NGF treatment. Phosphate incorporation per molecule is expressed relative to time zero, when no NGF was present.

shift in the migration of the protein on polyacrylamide gels. Based on alkaline phosphatase treatment of MAP1.2, this change in mobility has been shown to be dependent, at least to some degree, upon the extent of the molecule's phosphorylation (5). This indicates that not only does NGF affect the degree of MAP1.2 phosphorylation, but that it also thereby dramatically shifts the distribution of MAP1.2 isoforms in the cells. One explanation of earlier results (18) suggesting a much larger increase in the amount of MAP1.2 in PC12 cells involves the antibody preparation used for immunoblotting. If this monoclonal reagent specifically recognizes a phosphoepitope, it would result in an underestimation of the amount of MAP1.2 in non-NGF-treated cells because the basal level of phosphorylation is low. Direct tests of this hypothesis have recently confirmed this hypothesis by use of the same monoclonal ascites preparation (7) on Western blots treated with alkaline phosphatase (J. Aletta, *unpublished observations*).

It is currently unclear whether NGF's effect on the phosphorylated levels of MAP1.2 is due to an activated kinase or to inhibition of a phosphatase. Preliminary experiments in a cell-free assay system, however, have begun to examine this issue further. When hypotonic extracts of PC12 cells (non-treated versus NGF-treated for 1–15 min) are incubated for 15 min with radioactive ATP, the material from NGF-treated cells exhibits significantly greater levels of labeled MAP1.2 (H. Tsao, *unpublished observations*). This observation indicates that an NGF-regulated kinase is the more likely mechanism utilized *in vivo*. In a first attempt to identify the kinase responsible for the rapid increase in MAP1.2 phosphorylation, intact PC12 cells were treated for 15 min with known activators of different kinases: 40mM K<sup>+</sup>, which indirectly activates Ca<sup>++</sup>-dependent kinases; 8-chlorophenylthio-cAMP, a potent activator of kinase A; tetradecanoyl-phorbol-13-acetate (TPA), a kinase C agonist; and epidermal growth factor (EGF), which activates a tyrosine kinase. Although these agents affected a variety of other phosphorylations in PC12 cells, none mimicked the effect of NGF on MAP1.2 phosphorylation. The only other agent thus far tested that is capable of triggering the rapid phosphorylation of MAP1.2 in PC12 cells is fibroblast growth factor (FGF). Interestingly, this is the only other substance of those noted above that, like NGF, can produce long-term stable neurite outgrowth from PC12 cells (44,50).

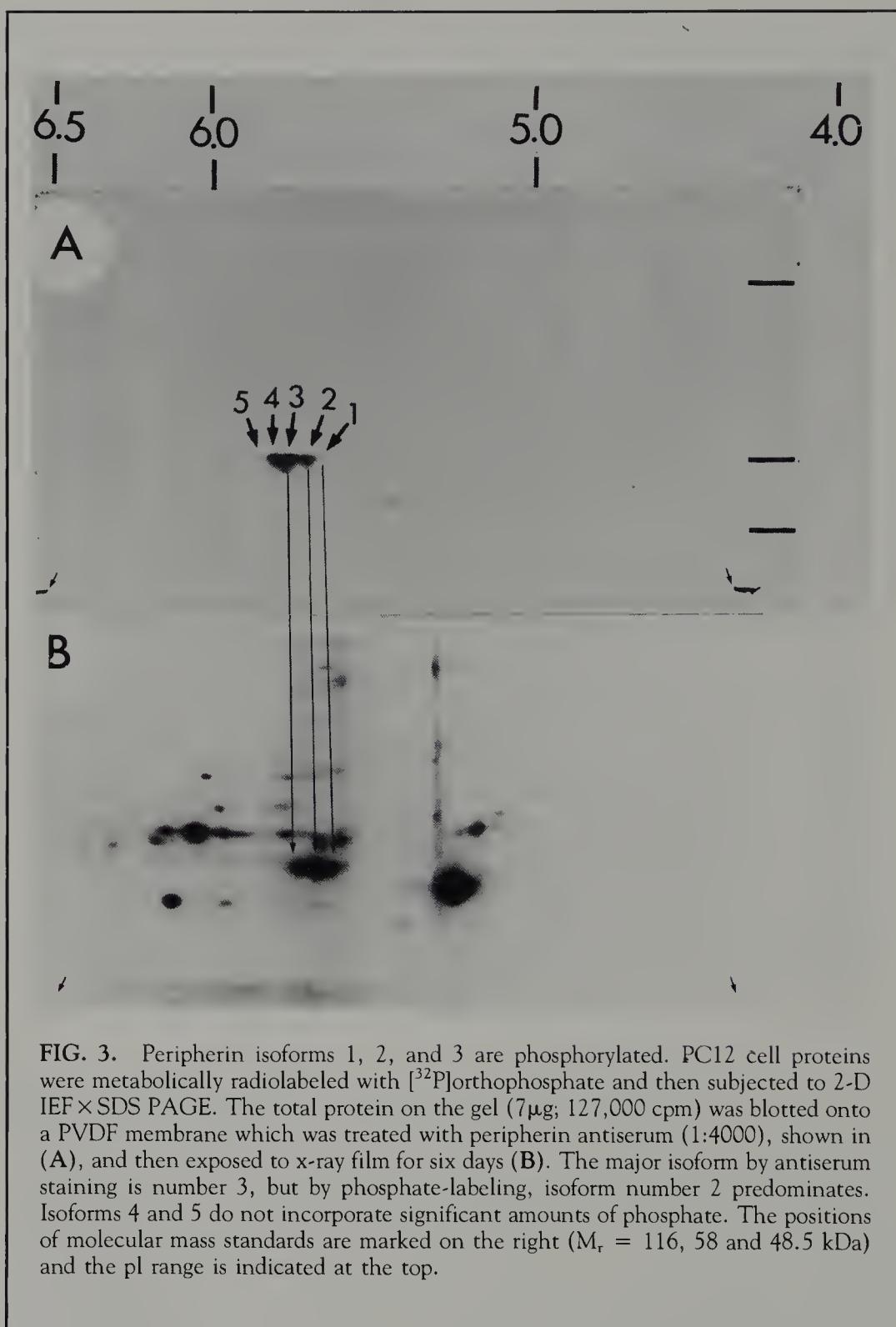
## PERIPHERIN: A NOVEL NEURONAL INTERMEDIATE FILAMENT PROTEIN

Leonard et al. (29) have studied NGF-regulated mRNAs from PC12 cells. Among the regulated messages that were detected, one (clone 73 mRNA) was found to be present in significant quantities, by Northern blot analysis, in sympathetic ganglia and at much lower levels in brain. It was undetectable in a variety of nonneuronal tissues. The cDNA from this clone was subsequently sequenced and compared with those present in data bases (30). The comparison revealed an

extensive sequence homology with the rod domain of all known intermediate filament proteins. Unique sequences in the NH<sub>2</sub> and COOH termini, however, indicated that clone 73 mRNA encodes a novel neuronal intermediate filament (IF) protein. These initial studies and a more recent analysis of the clone 73 gene (49) show that the protein it encodes belongs to the type III IF family. This family includes desmin, vimentin, and glial fibrillary protein and is distinct from the type IV neurofilament protein family. In addition, clone 73 mRNA was detected by *in situ* hybridization in neurons of the rat sympathetic, dorsal root, and ciliary ganglia, as well as in ventral horn motor neurons, cranial nerve nuclei, and several discrete brainstem nuclei (30).

A combination of experiments utilizing a polyclonal antiserum, directed against a synthetic 19-mer peptide identical to the deduced COOH terminus encoded by clone 73 mRNA, and microsequencing of gel-purified material have led to the identification of the NGF-induced clone 73 gene product (4). This protein is abundant in PC12 cells and is, as expected, significantly elevated in the cells after NGF-treatment (4,42). It is, in fact, the major IF protein found in PC12 cells (6). Although neurofilament proteins are also found in PC12 cells and are NGF-inducible (33), they are present in much lower abundance. The clone 73 product is, thus, likely to be the major constituent of the 10 nm filaments illustrated in Fig. 1. Based upon the protein's physical properties (molecular weight, isoelectric point, and Triton-insolubility) and its elevated levels after long-term NGF-treatment, it corresponds to peripherin, an IF protein first described and named by Portier and her co-workers (41,42). Similar observations of putative IF proteins of similar molecular weight and charge have been reported by others for PC12 cells (19,38) and for F9 embryonal carcinoma cells (27).

In PC12 cells and neuronal tissue, peripherin occurs in multiple isoelectric forms (6). The polyclonal antiserum noted above specifically recognizes five isoelectric variants at 58 kilodaltons ranging in pl from 5.6 to 5.8. Peripherin is a phosphoprotein, as are all other known intermediate filament proteins (6). However, only isoforms 1, 2, and 3 incorporate detectable amounts of phosphate (Fig. 3). Although protein apparently corresponding to isoform 3 is detectable in a Triton-extract of PC12 cells by immunoblotting and to forms 3 and 5 by Coomassie-staining, no phosphate-label is detectable in the soluble peripherin from cells cultured with or without long-term NGF exposure. Long-term NGF-treatment leads to a marked (~17-fold) increase in phosphorylated peripherin relative to total cytoskeletal phosphoprotein. This appears to be due to both an increase in peripherin synthesis and to the extent of phosphorylation per molecule. The increase in synthesis commences only after several days of NGF exposure and rapidly increases at the time when the cells extend neurites (29). In contrast, the onset of enhanced phosphorylation is apparent after one to two hours of exposure to NGF. By three hours of treatment there is a relative enhancement of two- to three-fold. Several different treatments other than NGF, known to activate protein kinases, are also capable of enhancing peripherin phosphorylation over a three-hour time course. Depolarizing levels of K<sup>+</sup>, cAMP derivatives, and TPA each produce effects



**FIG. 3.** Peripherin isoforms 1, 2, and 3 are phosphorylated. PC12 cell proteins were metabolically radiolabeled with [<sup>32</sup>P]orthophosphate and then subjected to 2-D IEF × SDS PAGE. The total protein on the gel (7 $\mu$ g; 127,000 cpm) was blotted onto a PVDF membrane which was treated with peripherin antiserum (1:4000), shown in (A), and then exposed to x-ray film for six days (B). The major isoform by antiserum staining is number 3, but by phosphate-labeling, isoform number 2 predominates. Isoforms 4 and 5 do not incorporate significant amounts of phosphate. The positions of molecular mass standards are marked on the right ( $M_r = 116, 58$  and 48.5 kDa) and the pI range is indicated at the top.

similar in magnitude to those of NGF, that is, about two- to three-fold. Peripherin, therefore, can serve as a substrate for a variety of different kinases;  $\text{Ca}^{++}$ -dependent kinases, kinase A, and kinase C, respectively.

Peripherin phosphorylation can also be regulated by various substances in neurite-bearing PC12 cells (6). This has been tested in cell cultures washed several times to remove NGF and then exposed to radiolabeled phosphate for 2–3 hr in the presence of different agents. Re-addition of NGF produces a modest (~50%), but reproducible increase in phosphorylation. TPA, cAMP derivatives, and elevated  $\text{K}^+$  each stimulated peripherin phosphorylation in the neurite-bearing cells to the same degree that they did in non-NGF-treated cells. Concentrations of EGF or insulin known to affect PC12 cells in other ways, however, have no effect on peripherin phosphorylation.

Further experiments indicate that the various effectors of peripherin phosphorylation described above use independent initial pathways (6). In kinase A-deficient PC12 cell variants (51), phosphorylation of peripherin is enhanced by NGF, TPA, and elevated  $\text{K}^+$ , but cAMP derivatives are without effect. Down-regulation of protein kinase C (35) renders TPA ineffective but does not impair the capacity of NGF to increase peripherin phosphorylation. Finally, nitrendipine, which blocks L-type  $\text{Ca}^{++}$  channels, suppresses the ability of elevated  $\text{K}^+$  to enhance peripherin phosphorylation in neurite-bearing cells, but does not inhibit the effects of cAMP derivatives or TPA. Peripherin is, thus, a major neuronal cytoskeletal element that is likely to be phosphorylated by diverse signaling mechanisms found in developing and mature neurons *in vivo*.

## POSSIBLE RELATIONSHIPS BETWEEN CYTOSKELETAL PHOSPHORYLATIONS AND FUNCTION

The evidence that we have presented in this review demonstrates that during NGF-mediated neurite outgrowth, NGF triggers multiple regulatory cascades with regard to several different cytoskeletal elements. Each of the proteins discussed (chartins,  $\beta$ -tubulin, MAP1.2, and peripherin) exhibits a unique set of responses to NGF treatment. Many of these are summarized in Table 1. By careful examination of these characteristics, we have drawn the following implications about the possible functional roles of these molecules.

The chartins and  $\beta$ -tubulin undergo an NGF-promoted increase in phosphorylation, which is temporally correlated with both tubulin polymerization and neurite outgrowth. Although the mechanism by which polymerized microtubules promote neuritic growth is not fully understood, these phosphorylations offer temporal attributes that are consistent with a possible role for phosphorylation in one or more of the following events: (a) promoting the addition of new tubulin subunits to distal tips of tubule bundles, (b) facilitation of microtubule interactions with other cytoskeletal elements, or (c) with membranous components that are necessary

TABLE 1. Properties of several NGF-regulated cytoskeletal phosphoproteins

Protein	Elevated protein levels <sup>1</sup>	Phosphorylation correlated with neurite outgrowth	Microtubule dependent phosphorylation	Kinase Activators				Phosphoforms enriched in neurites <sup>2</sup>
				NGF	cAMP	TPA	Ca <sup>2+</sup>	
chartins	-	+	+	+	-	? <sup>3</sup>	?	+
β-tubulin	+	+	-	+	-	?	?	+
MAP1.2	+	-/+ <sup>4</sup>	-	+	-	-	-	+
peripherin	+	-	-	+	+	+	-	-

(+), positive relation

(-), negative relation

(?), not investigated in PC12 cells

<sup>1</sup> in response to NGF, relative to total cellular protein<sup>2</sup> relative to total cellular phosphoprotein<sup>3</sup> for fibroblasts, see Shaw et al., *J. Biol. Chem.* 1988;263:1459.<sup>4</sup> biphasic response; see text

for extending neurite length. In addition, these phosphoproteins are also present in the correct spatial localization in the cell expected for growth-related molecules. Separation of neurites from clusters of cell bodies (23) reveals that the highly phosphorylated forms of chartins and phospho- $\beta$ -tubulin are  $\sim$ three-fold more abundant in neurites than in cell bodies (1). In the case of chartins, the phosphorylation is dependent upon intact microtubules, and treatments which interfere with neurite growth also inhibit chartin phosphorylation. Treatment of PC12 cells with lithium ion blocks chartin phosphorylation in neurite-bearing cells and also the regeneration of neurites (14). Preliminary studies indicate that the lithium-induced dephosphorylation of the acidic isoforms of the chartins does not collapse microtubules, but that no further growth occurs (J. Aletta, *unpublished observations*). Because these isoforms are enriched in neuritic processes, we suspect that chartin phosphorylation is thus necessary to increase microtubule length there.

In contrast to the chartins, MAP1.2 and  $\beta$ -tubulin can be phosphorylated in a microtubule-independent fashion. In addition to the NGF-induced increase in phosphate incorporation, the steady-state protein levels of these molecules, relative to total cell protein, rise modestly (two- to four-fold) after NGF-treatment. They are similar to chartins, however, in the relative enrichment of their phosphorylated forms in neurites versus cell bodies. With regard to MAP1.2, which has been studied more extensively, the increases in steady-state protein levels and phosphorylation that occur over days to weeks of NGF treatment are correlated with, and therefore are likely to be involved in, neurite elongation. The striking shift in the distribution of MAP1.2 isoforms after NGF-induced neurite growth signifies a fundamental change in the cell's complement of this MAP which in turn may alter microtubule function as alluded to above. Because the time course of MAP1.2 phosphorylation is so sharply biphasic (Fig. 2), a separate function for the very rapid rise may exist. One possibility is that it may in some way be related to the cell's commitment to undergo neuronal differentiation. This is one of the earliest NGF-induced phosphorylations that has been detected and, more importantly, FGF is the only other treatment known to trigger this effect on MAP1.2. Both NGF and FGF are capable of producing stable neurite outgrowth from PC12 cells.

Peripherin presents quite a different set of properties. Although the protein levels, relative to total cytoskeletal protein, are elevated by NGF-treatment, the time course of its phosphorylation is not correlated with the time course of outgrowth. Since peripherin can be phosphorylated *in vivo* by multiple independent pathways, including an NGF-dependent route, a more general role for this phosphorylation in cellular architecture may be inferred. The possible control over peripherin's function exerted by these varied mechanisms could provide the means to regulate equally varied aspects of neurite maintenance and repair. Unlike the phosphorylation of other intermediate filament proteins which is correlated with rendering these proteins polymer-incompetent (reviewed in ref. 6), the phosphoforms of peripherin are entirely associated with the Triton-insoluble cytoskeleton. Stability of the filament is therefore one possible implication of the phosphorylation, but this hypothesis must be tempered by the presence of nonphosphorylated

peripherin isoforms in the insoluble cytoskeleton as well. The role of peripherin phosphorylation in membrane-cytoskeleton interactions and, perhaps, even nuclear signaling, should also be explored.

Changes in cell shape during process outgrowth are, of course, most evident at the growing tip of the neurite. Figure 4 schematizes a working hypothesis of how

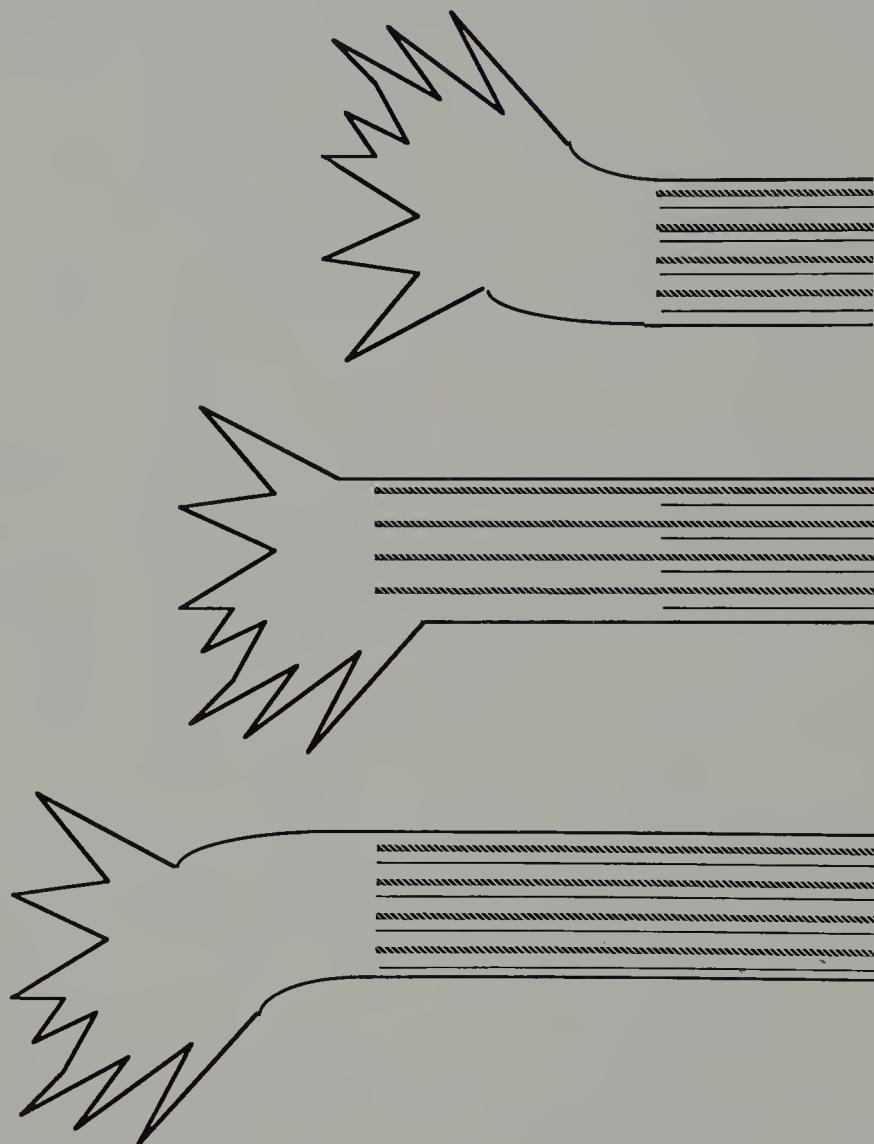


FIG. 4. Schematic representation of the neurite ending during growth. The thicker lines represent microtubules and the thinner lines intermediate filaments. See text for further explanation.

the microtubule and intermediate filament systems might be reorganizing during neurite growth at sites just proximal to the advancing growth cone. Evidence has been presented elsewhere that a "consolidation event" occurs at the growth cone, which prevents resorption of newly extended lamellipodia (2). The conversion of those lamellipodia into less motile domains of the growth cone is accompanied by an influx of cytoplasm and organelles into those areas (2,20). We propose that the mechanism that mediates this event involves the extension of microtubules and the phosphorylation of MAPs. This idea is consistent with Goldberg and Burmeister's (20) observation of rapid transport of organelles into newly consolidated growth cone areas. Interactions between intracellular vesicles and assembled microtubules are now thought to account for such fast axoplasmic movement (46). On the other hand, intermediate filaments and their post-translational modification are more likely to serve a long-term support function. In this regard, it may be significant that virtually all of the phosphorylated peripherin protein is found in an insoluble form. In addition, it should be recalled that only 3 of the 5 peripherin isoforms are phosphorylated, raising the likelihood of other modifications. Also, although the role of actin-associated proteins has not been dealt with here, it deserves serious consideration, particularly with regard to growth cone motility.

In conclusion, for each segment of new neurite growth, separate reaction pathways, at least one for microtubules and MAPs and another for intermediate filaments, must be accurately coordinated. Further insight into how such orchestration is mediated by NGF, and by other peptide factors, is now being actively pursued.

## ACKNOWLEDGMENTS

The work described here was supported in part by NIH grant NS 16036 and by a basic research grant from the March of Dimes Birth Defects Foundation.

## REFERENCES

1. Aletta JM, Greene LA. NGF-sensitive phosphoproteins from PC12 cells are differentially localized between cell bodies and neurites. *Soc. Neurosci. Abstr.* 1985;11:759.
2. Aletta JM, Greene LA. Growth cone configuration and advance: a time-lapse study using video-enhanced differential interference contrast microscopy. *J. Neurosci.* 1987; 8:1425-1435.
3. Aletta JM, Greene LA. Sequential phosphorylation of chartin microtubule-associated proteins is regulated by the presence of microtubules. *J. Cell. Biol.* 1987;105:277-290.
4. Aletta JM, Angeletti R, Liem RKH, Purcell C, Shelanski ML, Greene LA. Relationship between the nerve growth factor-regulated clone 73 gene product and the 58-kilodalton neuronal intermediate filament protein (Peripherin). *J. Neurochem.* 1988;51:1317-1320.
5. Aletta JM, Lewis SA, Cowan NJ, Greene LA. Nerve growth factor regulates both

- the phosphorylation and steady-state levels of microtubule-associated protein 1.2 (MAP1.2). *J. Cell Biol.* 1988;106:1573–1581.
- 6. Aletta JM, Shelanski ML, Greene LA. Phosphorylation of the peripherin 58-kDa neuronal intermediate filament protein: regulation by nerve growth factor and other agents. *J. Biol. Chem.* 1989;264:4619–4627.
  - 7. Asai DJ, Thompson WC, Wilson L, Dresden CF, Schulman H, Purich DL. Microtubule-associated proteins (MAPs): a monoclonal antibody to MAP 1 decorates microtubules *in vitro* but stains stress fibers and not microtubules *in vivo*. *Proc. Natl. Acad. Sci. USA* 1985;82:1434–1438.
  - 8. Black MM. Taxol interferes with the interaction of microtubule-associated proteins with microtubules in cultured neurons. *J. Neurosci.* 1987;7:3695–3702.
  - 9. Black MM, Green LA. Changes in colchicine susceptibility of microtubules associated with neurite outgrowth: studies with nerve growth factor-responsive PC12 pheochromocytoma cells. *J. Cell Biol.* 1982;95:379–386.
  - 10. Black MM, Aletta JM, Greene LA. Regulation of microtubule composition and stability during nerve growth factor-promoted neurite outgrowth. *J. Cell Biol.* 1986;103:545–557.
  - 11. Bloom GS, Schoenfeld TA, Vallee RB. Widespread distribution of the major polypeptide component of MAP 1 (microtubule associated protein 1) in the nervous system. *J. Cell Biol.* 1984;98:320–330.
  - 12. Brugg B, Matus A. PC12 cells express juvenile microtubule-associated proteins during nerve growth factor-induced neurite outgrowth. *J. Cell Biol.* 1988;107:643–650.
  - 13. Burstein DE, Greene LA. Evidence for RNA synthesis-dependent and -independent pathways in stimulation of neurite outgrowth by nerve growth factor. *Proc. Natl. Acad. Sci. USA* 1978;75:6059–6063.
  - 14. Burstein DE, Seeley PJ, Greene LA. Lithium ion inhibits nerve growth factor-induced neurite outgrowth and phosphorylation of nerve growth factor-modulated microtubule-associated proteins. *J. Cell Biol.* 1985;101:862–870.
  - 15. Corvaja N, DiLuzio A, Biocca S, Cattaneo A, Calissano P. Morphological and ultrastructural changes in PC12 pheochromocytoma cells induced by a combined treatment with NGF and taxol. *Exp. Cell Res.* 1982;142:385–395.
  - 16. Daniels MP. Colchicine inhibition of nerve fiber formation *in vitro*. *J. Cell Biol.* 1972;53:164–176.
  - 17. De Brabender M, Geuens G, Nuydens R, Willebrords R, De Mey J. Taxol induces the assembly of free microtubules in living cells and blocks the organizing capacity of the centrosomes and kinetochores. *Proc. Natl. Acad. Sci. USA* 1981;78:5608–5612.
  - 18. Drubin DG, Feinstein SC, Shooter EM, Kirschner MW. Nerve growth factor-induced neurite outgrowth in PC12 cells involves the coordinate induction of microtubule assembly and assembly-promoting factors. *J. Cell Biol.* 1985;101:1799–1807.
  - 19. Franke WW, Grund C, Achstatter T. Co-expression of cytokeratins and neurofilament proteins in a permanent cell line: cultured rat PC12 cells combine neuronal and epithelial features. *J. Cell Biol.* 1986;103:1933–1943.
  - 20. Goldberg DJ, Burmeister DW. Stages in axon formation: observations of growth of Aplysia axons in culture using video-enhanced contrast-differential interference contrast microscopy. *J. Cell Biol.* 1986;103:1921–1931.
  - 21. Greene LA, Tischler AS. Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc. Natl. Acad. Sci. USA* 1976;73:2424–2428.

22. Greene LA, Tischler AS. PC12 pheochromocytoma cultures in neurobiological research. *Adv. Cellular Neurobiol.* 1982;3:373–414.
23. Greene LA, Aletta JM, Rukenstein A, Green SH. PC12 pheochromocytoma cells: culture, nerve growth factor treatment, and experimental exploitation. *Meth. Enzymol.* 1987;147 Part B:207–216.
24. Greene LA, Burstein DE, Black MM. The role of transcription-dependent priming in nerve growth factor promoted neurite outgrowth. *Dev. Biol.* 1982;91:305–316.
25. Greene LA, Drexler SA, Connolly JL, Rukenstein A, Green SH. Selective inhibition of responses to nerve growth factor and of microtubule-associated protein phosphorylation by activators of adenylyl cyclase. *J. Cell Biol.* 1986;103:1967–1978.
26. Greene LA, Liem RKH, Shelanski ML. Regulation of a high molecular weight microtubule-associated protein in PC12 cells by nerve growth factor. *J. Cell Biol.* 1983;103:76–83.
27. Lampron C, Royal A. Expression of new proteins of the intermediate filament protein family in differentiating F9 embryonal carcinoma cell cytoskeleton. *J. Biol. Chem.* 1987;262:4893–4898.
28. Lewis SA, Sherline P, Cowan NJ. A cloned cDNA encoding MAP1 detects a single copy gene in mouse and a brain-abundant mRNA whose level decreases during development. *J. Cell Biol.* 1986;102:2106–2114.
29. Leonard DGB, Ziff EG, Greene LA. Identification and characterization of mRNAs regulated by nerve growth factor in PC12 cells. *Molec. Cell Biol.* 1987;7:3156–3167.
30. Leonard DGB, Gorham JD, Cole P, Greene LA, Ziff EB. A nerve growth factor-regulated messenger RNA encodes a new intermediate filament protein. *J. Cell Biol.* 1988;106:181–193.
31. Leterrier J-F, Liem RKH, Shelanski ML. Interactions between neurofilaments and microtubule-associated proteins: a possible mechanism for intraorganelle bridging. *J. Cell Biol.* 1982;95:982–986.
32. Letourneau PC, Ressler AH. Inhibition of neurite initiation and growth by taxol. *J. Cell Biol.* 1984;98:1355–1362.
33. Lindenbaum MH, Carbonetto S, Mushynski WE. Nerve growth factor enhances the synthesis, phosphorylation and metabolic stability of neurofilament proteins in PC12 cells. *J. Biol. Chem.* 1987;262:605–610.
34. Magendantz M, Solomon F. Analyzing the components of microtubules: antibodies against chartins, associated proteins from cultured cells. *Proc. Natl. Acad. Sci. USA* 1986;82:6581–6585.
35. Matthies HJG, Palfrey HC, Hirning LD, Miller RJ. Down regulation of protein kinase C in neuronal cells: effects on neurotransmitter release. *J. Neurosci.* 1987;7:1198–1206.
36. Olmsted JB. Microtubule-associated proteins. *Annu. Rev. Cell Biol.* 1986;2:421–457.
37. Pallas D, Solomon F. Cytoplasmic microtubule-associated proteins: phosphorylation at novel sites is correlated with their incorporation into assembled microtubules. *Cell* 1982;30:407–414.
38. Parysek LM, Goldman RD. Characterization of intermediate filaments in PC12 cells. *J. Neurosci.* 1987;7:781–791.
39. Paschal BM, Shpetner HS, Vallee RB. MAP 1C is a microtubule-activated ATPase that translocates microtubules *in vitro* and has dynein-like properties. *J. Cell Biol.* 1987;105:1273–1282.

40. Peng I, Binder LI, Black MM. Cultured neurons contain a variety of microtubule-associated proteins. *Brain Res.* 1985;361:200–211.
41. Portier M-M, Croizat B, Gros F. A sequence of changes in cytoskeletal components during neuroblastoma differentiation. *FEBS Lett.* 1982;146:283–288.
42. Portier M-M, Brachet P, Croizat B, Gros F. Regulation of peripherin in mouse neuroblastoma and rat PC12 pheochromocytoma cell lines. *Dev. Neurosci.* 1983/84;6:215–226.
43. Portier M-M, de Néchaud B, Gros F. Peripherin, a new member of the intermediate filament protein family. *Dev. Neurosci.* 1983/84;6:335–344.
44. Rydel RE, Greene LA. Acidic and basic fibroblast growth factors promote stable neurite outgrowth and neuronal differentiation in cultures of PC12 cells. *J. Neurosci.* 1987;7:3639–3653.
45. Sattilaro RF, Dentler WL, Le Cluyse EL. Microtubule-associated proteins (MAPs) and the organization of actin filaments *in vitro*. *J. Cell Biol.* 1981;90:467–473.
46. Schnapp BJ, Vale RD, Sheetz MP, Reese TS. Single microtubules from squid axoplasm support bidirectional movement of organelles. *Cell* 1985;40:455–462.
47. Seeley PJ, Greene LA. Short latency local actions of nerve growth factor at the growth cone. *Proc. Natl. Acad. Sci. USA* 1983;80:2789–2793.
48. Selden SC, Pollard TD. Phosphorylation of microtubule-associated proteins regulates their interactions with actin filaments. *J. Biol. Chem.* 1983;258:7064–7071.
49. Thompson MA, Ziff EB. Structure of the gene encoding peripherin, an NGF-regulated neuronal-specific type III intermediate filament protein. *Neuron* 1989;2:1043–1053.
50. Togari A, Baker D, Dickens G, Guroff G. The neurite-promoting effect of fibroblast growth factor on PC12 cells. *Biochem. Biophys. Res. Comm.* 1983;114:1189–1193.
51. Van Buskirk R, Corcoran T, Wagner JA. Clonal variants of PC12 pheochromocytoma cells with defects in cAMP-dependent protein kinases induce ornithine decarboxylase in response to nerve growth factor but not to adenosine agonists. *Molec. Cell Biol.* 1985;5:1984–1992.
52. Yamada KM, Spooner BS, Wessells NK. Axon growth: roles of microfilaments and microtubules. *Proc. Natl. Acad. Sci. USA* 1970;66:1202–1212.

# Consequences to CNS Cholinergic Neurons of *In Vivo* Nerve Growth Factor Administration

Silvio Varon, Theo Hagg, James Conner, Barry Fass,  
H. Lee Vahlsing and Marston Manthorpe

Department of Biology, School of Medicine  
University of California, San Diego,  
La Jolla, CA 92093

Nerve growth factor (NGF) is the well-established prototype for a family of neuronotrophic proteins regulating maintenance, growth, and functional performances of selected nerve cells (24). Until recently, most of the information concerning NGF has been derived from the observation of peripheral sensory and sympathetic ganglionic neurons during development *in vivo* and *in vitro* (20). A new chapter in the history of NGF has begun with the more recent discoveries that NGF can also address certain cholinergic neurons in the central nervous system (CNS) of adult as well as developing mammals (reviewed in 25). Specifically: (a) the NGF protein and its messenger RNA are present in the innervation territories of basal forebrain cholinergic neurons—the hippocampal formation for medial septum and diagonal band neurons, and the neocortex for nucleus basalis neurons; (b) NGF injected into those territories is retrogradely transported to the respective projecting cholinergic neurons; (c) NGF receptors and their mRNA are demonstrable in cholinergic cell bodies of basal forebrain and neostriatum; (d) *in vitro* cultures of prenatal CNS cholinergic neurons respond to NGF treatment with increases in their choline acetyltransferase (ChAT) activity; and (e) *in vivo* administration of exogenous NGF to adult rat brain affects various features of CNS cholinergic neurons.

Developmental and *in vitro* investigations of NGF-sensitive peripheral neurons have documented four main responses of these neurons to NGF: (1) numerical increases in neuronal survival; (2) stimulation of neuritic growth; (3) somal and neuritic hypertrophy; and (4) enhanced production of neurotransmitter enzymes. As development proceeds, some of these responses (e.g., survival and neuritic growth) are no longer recognized in the animal, unless experimental or other lesions are applied, but transmitter enzyme stimulation remains a robust effect of NGF treatment.

Similar responses have been recognized in adult rat CNS cholinergic neurons upon intraventricular administration of exogenous NGF. This paper will first review several recent findings and some of the questions raised by them for future investigations. We will then focus on one particular feature of adult CNS cholinergic neurons, namely the susceptibility to modulation of their *cell body size*.

## ACUTELY INJURED RATS

A well-studied model for an acute lesion of adult CNS cholinergic neurons is the septo-hippocampal system. Cholinergic neurons in the medial septum (MS), on each side of the brain midline, project most of their axons to the ipsilateral hippocampal formation via a discrete tract, the fimbria-fornix. A fimbria-fornix transection (FFT) axotomizes these neurons and thus interrupts this presumptive retrograde supply of hippocampal endogenous NGF, resulting in the disappearance of ChAT or acetylcholinesterase (AChE) positive (i.e., cholinergic) cell bodies in the corresponding MS nucleus (8). The responsiveness of these axotomized MS neurons to unilateral intraventricular administration of NGF has been examined by several research groups (9,10,15,18,21,27). Despite several differences in the experimental protocols used, all of those studies have demonstrated that the axotomy-induced disappearance of MS neurons was largely *prevented* by the NGF treatment. In addition, NGF was noted to cause an increase in ChAT activity in partially deafferented hippocampal tissue (16), and to elicit the appearance of a cholinergic fiber plexus in the septum ipsilateral to a complete FFT (10,27). Taken at face value, these results proposed that at least three of the classic responses to NGF (neuronal survival, neuritic growth, and production of transmitter enzymes) could be displayed by acutely injured cholinergic neurons in the adult brain.

A re-evaluation of some of these conclusions, however, is warranted in the light of more recent observations (10,11,14). A unilateral FFT (whose completeness was verified by the disappearance of AChE-positive innervation in the ipsilateral hippocampus) leads to the disappearance of about 50% and 75% of the ipsilateral ChAT-stainable MS neurons after one and two weeks, respectively. Continuous intraventricular NGF infusion *starting after* their disappearance caused the *reappearance* of all or most of the apparently lost neurons. The counting of cholinergic neurons depends on their ability to express enough cholinergic enzyme for its detection. Thus: (1) the disappearance of cholinergic MS neurons after FFT is largely due to their loss of cholinergic markers below detectability rather than their actual death, since they can be detected again upon an NGF treatment delayed by even three months after the FFT treatment (14), and (2) the recognized effects of NGF on cholinergic neurons involve the stimulation of new cholinergic marker expression (e.g., ChAT). Such conclusions assume that the increased number of ChAT-positive neurons after delayed NGF administration represents "retrieval" of the missed cells rather than ChAT induction in a different set of neurons—because

no appearance of supernumerary ChAT-positive cells follows NGF treatments started at or shortly after the FFT time.

One might be tempted to view the ChAT response to NGF as a sufficient (and thus possibly the only) effect of the factor in this adult CNS model—just as transmitter enzyme stimulation is the main recognized response to NGF in adult peripheral ganglionic neurons. This is not the case, because FFT also induces disappearance and NGF induces reappearance of other markers for the adult MS cholinergic neurons, e.g., the lower-affinity NGF receptor antigen (3,11,14) and a newly recognized intra-neuronal laminin-like antigen (13)—additional evidence that NGF-rescued cells are the original cholinergic MS neurons.

## CHRONICALLY INJURED RATS

Cholinergic deficits are known to accompany, and possibly underlie, the cognitive behavioral deficits that result from poorly understood, chronic, involutive processes during brain aging (1), and chronic degenerative damage in Alzheimer's disease (5). The linkage between cholinergic and cognitive deficits is supported by the behavioral consequences of acute basal forebrain lesions (17), which may also be alleviated by NGF treatment (26).

In a study on aged (two-year old) rats, animals were screened for spatial memory impairment, continuously infused intraventricularly with either vehicle alone or vehicle + NGF, and re-tested thereafter on the same behavioral task (6). Four weeks of NGF treatment led to a reversal of the cognitive impairment. Histologic analyses revealed that NGF also reduced, by 20–30%, the age-related hypotrophy of AChE-positive neurons in the nucleus basalis and striatum (but not medial septum).

Such findings provide demonstration that NGF action on cholinergic CNS neurons can lead to functional benefits in chronically impaired animals and suggestions that NGF treatment might similarly benefit behavioral deficits in Alzheimer patients. They also make a number of additional points. One is that cholinergic damage need not always or necessarily imply cholinergic neuron death. Another point is the further confirmation that cholinergic neurons may remain susceptible to NGF action long after the onset of injury. Yet a third point is that NGF can affect neuronal size, and not only neuronal content of transmitter enzymes and other cell constituents.

## “NON-INJURED” ADULT RATS

All the studies reviewed thus far have involved NGF treatment of “impaired” adult CNS cholinergic neurons. It was possible, therefore, to speculate that “normal” neurons may be sufficiently supported by endogenous NGF to display maximal

performances, and that only adverse circumstances reducing NGF availability—such as axotomy or aging (19)—could allow for recognizable effects of exogenous NGF administration (23). Two new studies force a reassessment of such views.

Cholinergic neurons in the *neostriatum* and the *nucleus accumbens*, unlike those in the basal forebrain, are non-projective interneurons, and their cell bodies are within the same territory in which axons impinge on postsynaptic partners. In such a situation, endogenous NGF sources are not readily segregated from the NGF target neurons. Fimbria-fornix transection and intraventricular cannulations do not impose direct mechanical injuries on these two structures. Their neurons, therefore, must be deemed to perform under normal regulation (including normal local supply of endogenous NGF) in the standardly lesioned experimental animal. A recent study (12), however, has revealed that intraventricular infusion of exogenous NGF does lead to changes in the cholinergic neurons of both striatum and nucleus accumbens. In the striatum, the neuronal responses decrease with increased distance from the infused ventricle, suggesting gradients of NGF in the periventricular parenchyma (laterally into the striatum, medially into the septum, and medio-ventrally to the nucleus accumbens). The neuronal responses include: (a) a marked intensification of ChAT-immunoreactivity in both nerve cell bodies and parenchyma, and (b) an increase in the size (cross-sectional area) of cholinergic neurons, 40% in the striatum and 20% in the nucleus accumbens. There was no indication that NGF affected NGF-receptor immunoreactivity, which was undetectable in both structures with or without NGF treatment.

Cavicchioli et al. (2) and Fusco et al. (7) have examined *medial septum* cholinergic neuronal features in neonatal and adult rats receiving intraventricular administration of NGF but no FFT. They measured the presence of messenger RNA for NGF receptor (4) in the extract of whole septum, and ChAT enzymatic activity in the extract of hippocampal tissue from the same rat. NGF administration caused a substantial increase in both features over the levels of control animals. Thus, the ability of "normal" cholinergic neurons to be stimulated by an exogenous NGF supplement may be a general property of adult (as well as developing) NGF-receptive CNS nerve cells.

## EXTRINSIC REGULATION OF SOMAL SIZE IN CNS CHOLINERGIC NEURONS

A major role of trophic factors has long been assumed to be the stimulation of the growth capability of their target cells, with local circumstances and/or other microenvironment-derived signals dictating whether such a growth stimulus would result in (a) population growth (cell proliferation), (b) hypertrophy (increase in cell size), (c) extension of processes (neuritic growth), or even (d) enhanced production of functional cell products (transmitter enzymes and their secretory products) (cf. 22). The ability of NGF to affect cholinergic neurons in the adult CNS provides new opportunities to examine the regulation of neuronal size and the pu-

tative role of NGF in it. Table 1 and Figure 1 summarize several observations pertaining to this question in the rat. Cross-sectional neuronal areas have been determined by use of a computerized morphometric procedure (14), which yields basal values consistent with those reported by others (e.g., 6,9).

We have already seen that striatal (and accumbens) cholinergic neurons of an adult rat respond to NGF treatment with a 30–40% increase in size independently of an FFT lesion (12). The increase results from a shift in overall size distribution, rather than the appearance of a special subset of hypertrophic cells. No significant increase was recognized with the medial septum cholinergic neurons in these "normal" rats (9,12). It is noteworthy that behaviorally impaired, aged rats also exhibited NGF-induced size increases in the striatum (and nucleus basalis) but not the medial septum neurons (6).

The size of *medial septum* cholinergic neurons has been more recently evaluated in three-month old rats subjected to fimbria-fornix transection without or with NGF treatments (14). As seen in Table 1, the 25% of cholinergic neurons that remained detectable (i.e., ChAT-positive) two weeks after FFT differed significantly in size from the original population, with an average cross-sectional area reduction of about 25%. A normal size, on the other hand, was retained by those neurons (100% of the original number) that remained detectable at that time under a continuous NGF protection. A two-week delayed NGF treatment (for an additional 14 days), which restored the number of ChAT-positive MS neurons to about 75% of the unlesioned level, also increased their average size. With this treatment, however, the average size increase went beyond normal levels by almost 20%—a genuine hypertrophic effect. These changes in average size of medial septum cholinergic neurons, like those of striatal cells, were found to reflect shifts in overall size distribution, rather than an exclusion (under FFT) or reinstatement (under FFT + delayed NGF) of size-selective neuronal subjects.

**TABLE 1.** Size changes in adult CNS medial septum cholinergic neurons induced by FFT and NGF infusion *in vivo*.

	Size ( $\mu\text{m}^2$ ) $\pm$ SEM
<i>No Lesion</i>	
no treatment	$165 \pm 2$ (3) (82)
NGF (14 d)	$162 \pm 7$ (4) (84)
<i>FFT Lesion</i>	
no treatment (14 d)	$124 \pm 5^*$ (5) (22)
NGF (0–14 d)	$172 \pm 4$ (5) (84)
delayed NGF (14–28 d)	$193 \pm 7^*$ (5) (62)

FFT, complete unilateral fimbria-fornix transection. NGF, intraventricular infusion (ipsilateral to FFT) over the indicated periods. Asterisks, differences from control (no FFT, no NGF) at  $p \leq 0.0005$  \*(Student *t*-test).

Sprague-Dawley 3 month old female rats. Presented are the average cross-sectional sizes ( $\mu\text{m}^2$ )  $\pm$  SEM.

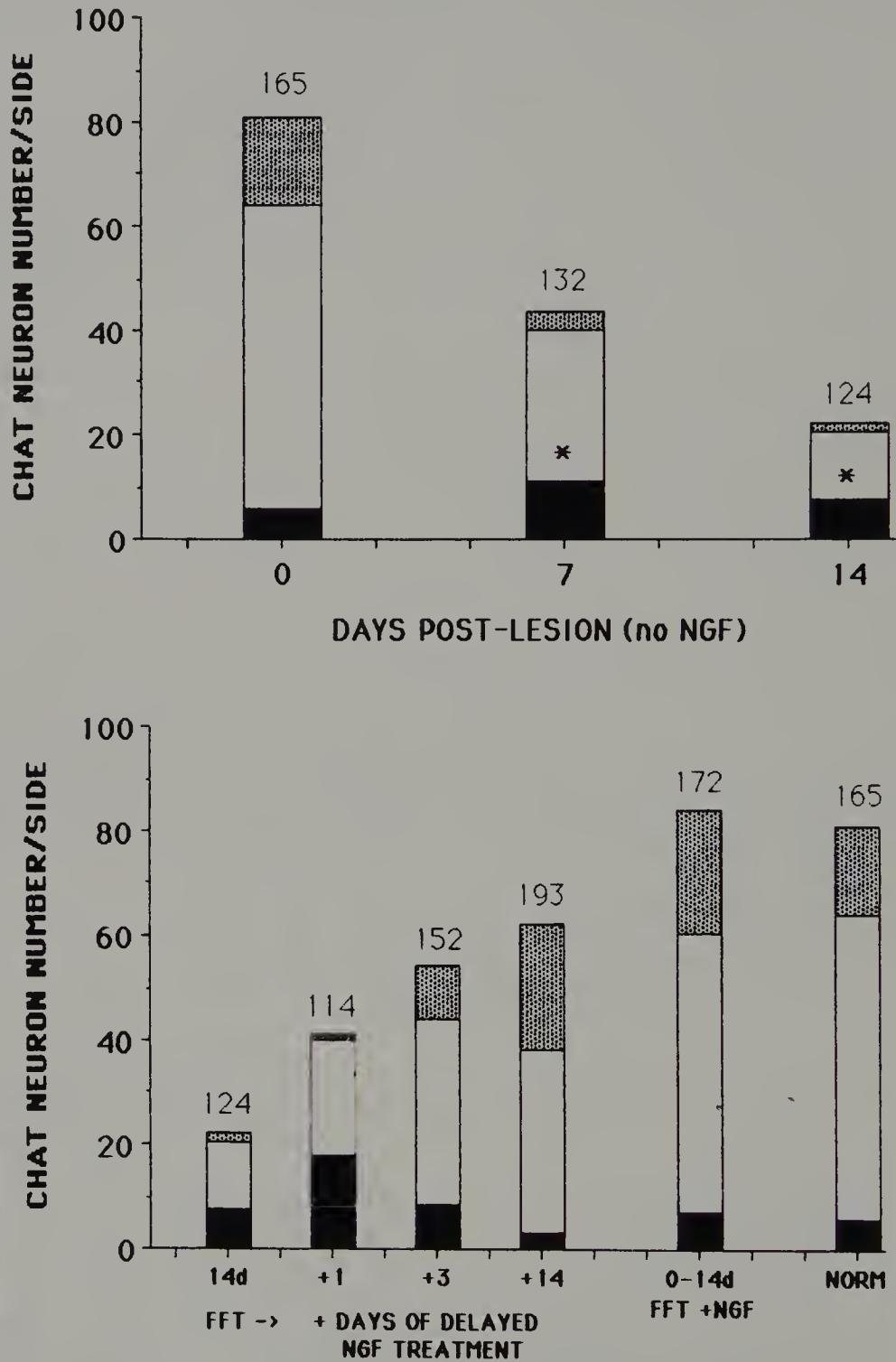


FIG. 1.

Figure 1 displays actual numbers of detectable (ChAT-positive) medial septum cholinergic neurons at different times post-lesion without NGF treatment (upper panel) or at different times after a 14-day delayed onset of NGF administration (lower panel). In all cases, the total number was subdivided into three subsets according to cross-sectional neuronal sizes: below  $100 \mu\text{m}^2$  (*solid bars*), between  $100$  and  $200 \mu\text{m}^2$  (*open bars*) and above  $200 \mu\text{m}^2$  (*stippled bars*). In the absence of exogenous NGF, the FFT lesion caused a progressive numerical decline in "large" and "midsize" neurons, while the number of "small" cells actually increased, by over two-fold at seven days and about 1.5-fold at 14 days. This recruitment into the small neuron subset demonstrates that the other subsets have undergone size reductions, even though the temporal details of the FFT-induced hypotrophy are obscured by the concurrent "disappearance" of ChAT-positivity.

At 14-day postlesion (the starting time for delayed NGF infusion), only about one quarter of the initial ChAT-positive neurons were still detectable and, of those, nearly 30% were small ones. Within one day of NGF treatment (14 + 1 day in the lower panel), the total number had about doubled—a reflection of the rapid increase of ChAT above detection threshold—and the numbers of both small and midsize neurons had increased. The absolute number of small neurons was now three times that seen in an intact animal, a clear demonstration that the lesion had brought about an excess of small cells (at the expense of the larger ones). Many of these small cells, additionally, had lost their detectability until addressed by exogenous NGF. With further exposure to NGF (14 + 3 and 14 + 14 days), the total number of ChAT-positive cells reached their maximal recovery (14) to about 75% of the original population. The small cell number declined below its original level, demonstrating a reverse shift into the midsize and the large subsets. The large cell subset expanded to exceed even the absolute numbers of an intact animal (lower panel, NORM), revealing an actual hypertrophy of some of the axotomized medial septum neurons. A similar hypertrophy was recognizable in animals for which a 14-day NGF treatment had started concurrently with the FFT, even though the total number of ChAT-positive cells remained at control levels.

These experiments demonstrate the concurrence of two overlapping processes induced by the FFT and reversed by the NGF treatment. Both are time-dependent, although their respective time courses cannot yet be precisely defined. One process is a FFT-induced *progressive reduction in size*, shifting large neurons into the midsize subset and midsize neurons into the small cell subset. If all the neurons were to

◀ FIG. 1. Changes in numbers and size distributions of medial septum cholinergic neurons, induced by axotomy and NGF treatment. **Upper panel**, *unilateral fimbria-fornix transection (FFT), no NGF*. **Lower panel**, *FFT plus NGF treatment either delayed (14d + 1, 3, 14 days) or started at lesion time (0–14d)*. NORM, unoperated and untreated control. Neurons were subdivided by cross-sectional size into small ( $<100 \mu\text{m}^2$ , *solid bar*), midsize ( $100$ – $200 \mu\text{m}^2$ , *open bar*) and large ( $>200 \mu\text{m}^2$ , *stippled bar*). Numbers above each bar give the overall average cross-sectional area/neuron ( $\mu\text{m}^2$ ).

retain enough ChAT to remain detectable, differential counts in the three subsets would accurately describe such a size shift. The second process is a *progressive loss of ChAT* (and other markers), which causes cells in all three subsets to lose their detectability, while still remaining available for a delayed NGF action. NGF administration will progressively restore both detectability (ChAT increase) and larger sizes (reverse shift from small to midsize to large cell subsets).

Several implications of NGF-induced neuronal size increases need to be explored. In the aged rat (6), cholinergic neuronal size is considerably smaller than in younger animals, and the NGF effect was to reduce the age-induced hypotrophy but not raise the size above normal. In the apparently uninjured striatum (12), a true hypertrophy resulted from the NGF treatments. In the medial septum after FFT (14; and Table 1, Fig. 1), both consequences of NGF treatment were seen: i) a reversal of the axotomy-induced hypotrophy, and ii) a supranormal contribution to the large neuron subset. An important question, then, is whether the converse phenomenon also occurs, namely that cholinergic hypotrophic states also reflect an NGF regulatory role—in such cases, a reduced availability of endogenous NGF. In the septo-hippocampal model, it is now accepted that FFT affects the medial septum neurons by interrupting a retrograde supply of endogenous NGF from the hippocampal innervation territory. In the aged brain model, it is also reasonable—though with little documentation, as yet—to attribute the age-induced neuronal hypotrophy to a progressive deficit of endogenous trophic support. We are currently exploring a recent, fortuitous observation that CNS cholinergic neurons may display a smaller size in rats born and reared in our own animal facility as compared to those acquired from our standard commercial source (Conner, *unpublished data*). If this observation can be substantiated by future work, it might become possible i) to analyze the external conditions responsible for such an “environment-induced hypotrophy,” and ii) to ascertain a protective linkage between this hypotrophy and a corresponding deficit of endogenous NGF. Ultimately, investigation of these several models would shed some light on whether neuronal cell size is yet another component of adult CNS plasticity, whose control by neuronotrophic factors can lead to modulation of neural functions.

## ACKNOWLEDGMENTS

Work reported here was supported in part by NINCDS grant #NS-16349 and NSF grant BNS-88-08285.

## REFERENCES

1. Bartus RT, Dean RL, Beer B, Lippa AS. The cholinergic hypothesis of geriatric memory dysfunction. *Science* 1982;217:408–417.

2. Cavicchioli L, Flanigan T, Vantini G, Fusco M, Polato P, Toffano G, Walsh FS, Leon A. NGF amplifies the expression of NGF receptor mRNA in mammalian forebrain cholinergic neurons. *Eur. J. Neurosci.* 1989;1:258–262.
3. Chandler CE, Parsons LM, Hosang M, Shooter EM. A monoclonal antibody modulates the interaction of nerve growth factor with PC12 cells. *J. Biol. Chem.* 1984; 259:6882–6889.
4. Chao MV, Bothwell MA, Ross AH, Koprowski H, Lanahan AA, Buck CR, Sehgal A. Gene transfer and molecular cloning of the human NGF receptor. *Science* 1986; 232:518–521.
5. Coyle JT, Price DL, DeLong MR. Alzheimer's disease: a disorder of cortical cholinergic innervation. *Science* 1983;219:1184–1190.
6. Fischer W, Wictorin K, Björklund A, Williams LR, Varon S, Gage FH. Amelioration of cholinergic neuron atrophy and spatial memory impairment in aged rats by nerve growth factor. *Nature* 1987;329:65–68.
7. Fusco M, Oderfeld-Nowak B, Vantini G, Schiavo N, Gradirowska M, Zaremba M, Leon A. Effect of nerve growth factor upon the adult intact and lesioned rat septohippocampal cholinergic system. *Brain Res.* 1989;in press.
8. Gage FH, Wictorin K, Fischer W, Williams LR, Varon S, Björklund A. Retrograde cell changes in medial septum and diagonal band following fimbria-fornix transection: quantitative temporal analysis. *Neuroscience* 1986;19:241–255.
9. Gage FH, Armstrong DM, Williams LR, Varon S. Morphological response of axotomized septal neurons to Nerve Growth Factor. *J. Comp. Neurol.* 1988;269:147–155.
10. Hagg T, Manthorpe M, Vahlsing HL, Varon S. Delayed treatment with nerve growth factor reverses the apparent loss of cholinergic neurons after acute brain damage. *Exp. Neurol.* 1988;101:303–312.
11. Hagg T, Vahlsing HL, Fass R, Manthorpe M, Varon S. Axotomy-induced loss of both ChAT and NGF-receptor positive medial septum neurons is reversed by delayed NGF treatment. *Soc. Neurosci. Abstr.* 1988;14:605.
12. Hagg T, Hagg F, Vahlsing HL, Manthorpe M, Varon S. Nerve growth factor effects on cholinergic neurons of neostriatum and nucleus accumbens in the adult rat. *Neuroscience* 1989;30:95–103.
13. Hagg T, Engvall E, Varon S, Manthorpe M. Laminin-like antigen in rat CNS neurons: distribution and changes upon brain injury and nerve growth factor treatment. *Neuron* 1989;3:721–732.
14. Hagg T, Fass-Holmes B, Vahlsing HL, Manthorpe M, Varon S. Nerve growth factor (NGF) reverses axotomy-induced decreases in choline acetyltransferase, NGF-receptor and size of medial septum cholinergic neurons. *Brain Res.* 1989;505:29–38.
15. Hefti F. Nerve growth factor promotes survival of septal cholinergic neurons after fimbrial transections. *J. Neurosci.* 1986;6:2155–2162.
16. Hefti F, Dravid A, Hartikka J. Chronic intraventricular injections of nerve growth factor elevate hippocampal choline acetyltransferase activity in adult rats with partial septo-hippocampal lesions. *Brain Res.* 1984;293:305–311.
17. Hepler DJ, Wenk GL, Cribbs BL, Olton DS, Coyle JT. Memory impairments following basal forebrain lesions. *Brain Res.* 1985;346:8–14.
18. Kromer LF. Nerve growth factor treatment after brain injury prevents neuronal death. *Science* 1987;235:214–216.
19. Lärkfors L, Ebendal T, Whittemore SR, Persson H, Hoffer B, Olson L. Decreased levels of nerve growth factor (NGF) and its messenger RNA in the aged brain. *Molec. Brain Res.* 1987;3:55–60.

20. Levi-Montalcini R. Developmental neurobiology and the natural history of nerve growth factor. *Annu. Rev. Neurosci.* 1982;5:341–361.
21. Montero CN, Hefti F. Rescue of lesioned septal cholinergic neurons by nerve growth factor: specificity and requirement for chronic treatment. *J. Neurosci.* 1988;8:2986–2999.
22. Varon S. Neural growth and regeneration: a cellular perspective. *Exp. Neurol.* 1977; 54:1–6.
23. Varon S, Manthorpe M, Williams LR, Neuronotrophic and neurite promoting factors and their clinical potentials. *Dev. Neurosci.* 1984;6:73–100.
24. Varon S, Manthorpe M, Davis GE, Williams LR, Skaper SD. Growth factors. In: Waxman SG, ed. *Functional Recovery in Neurological Disease, Advances in Neurology*, Vol. 47. New York: Raven Press, 1988;493–521.
25. Whittemore SR, Seiger A. The expression, localization and functional significance of  $\beta$ -nerve growth factor in the central nervous system. *Brain Res. Reviews* 1987; 12:439–464.
26. Will B, Hefti F. Behavioural and neurochemical effects of chronic intraventricular injections of nerve growth factor in adult rats with fimbria lesions. *Behav. Brain Res.* 1985;17:17–24.
27. Williams LR, Varon S, Peterson G, Wictorin K, Fischer W, Björklund A, Gage FA. Continuous infusion of nerve growth factor prevents basal forebrain neuronal death after fimbria-fornix transection. *Proc. Natl. Acad. Sci. USA* 1986;83:9231–9235.

## **IV. Neurite Outgrowth and Growth Cones**



# Intracellular Calcium and the Control of Neuronal Growth and Form

S. B. Kater, L. R. Mills and P. B. Guthrie

*Department of Anatomy and Neurobiology, Program in Neuronal Growth and Development, Colorado State University, Fort Collins, CO 80523*

The architectural diversity of neurons has been one of the most intriguing areas of investigation from the time early histological methods were first applied to the nervous system. The incredible morphological diversity was marvelled at, especially in light of the seemingly antithetical fact that there was also a high degree of similarity. A century of investigation still leaves us with unanswered fundamental questions about the origins of neuronal form. Certainly we recognize that neuronal architecture, as with any biological phenotype, is the expression of a composite of intrinsic and extrinsic forces. Only recently, however, have we begun to uncover the mechanisms governing the behavior of the primary organelle responsible for the iteration of neuronal form, the neuronal growth cone. Our basic goal has been to understand the mechanisms underlying the actions of growth cones during four very different scenarios: 1) the generation of basic form during embryonic development, and the incredible navigational challenges faced during this period; 2) the maintenance of this (quasi-)stable architecture throughout mature life; 3) the changes in architecture during periods of neuroplasticity in adulthood which may range from repair to reactive remodeling; and 4) the degeneration of specific aspects of neuronal architecture which may become pathological and even result in neuronal death. There is now good reason to believe that understanding the basic regulatory systems in the growth cone is a prerequisite for understanding the diversity of neuronal forms and their developmental histories.

The term neuronal growth cone was introduced by Ramon y Cajal (25), to define the broad, flattened lamellipodia and numerous filopodia that characterize the tip of a growing neurite. The growth cone is a highly dynamic structure; the lamellipodium and filopodia continuously move as the growth cone "explores" its environment. Discriminatory behavior is characteristic of motile growth cones; frequently the growth cone turns, branches, or even stops, apparently in response to invisible cues. This structure is now well recognized as one of the primary organelles responsible for the generation, alteration, and regeneration of neuronal form and connectivity (11).

The neuronal growth cone has been a focus of extensive investigation both *in situ* and in cell culture where it can be more efficiently manipulated. In cell culture, the characteristic long filopodia appear to act as transducers searching out the environment. These same filopodia *in vivo* (e.g., 1) seem to play key roles in neuronal pathfinding. On the other hand, while growing neurites may display broad lamellipodia and large, numerous filopodia, many observations have been made of elongating neurites with rather more circumscribed growth cone structure. For instance, Lopresti et al. (16) have described pioneer growth cones *in situ* which possess the characteristic broad, flattened lamellipodia and multiple filopodia. These pioneer neurons are followed by an additional set of neurons whose growth cones display quite simple morphologies. We suspect that these different forms subserve different functions associated with the outgrowth process. For example, pioneering growth cones, which select appropriate paths and navigate to appropriate targets, might require the broadened lamellipodium and long filopodia as primary agents of pathfinding. Interestingly, we have observed that an individual neuron can display, over different periods of its lifetime, different shaped growth cones (28). In fact, it is now clear that a variety of stimuli, both endogenous to individual neurons and exogenous environmental cues, can play significant roles in the alteration of growth cone form and function.

## REGULATION OF GROWTH CONE BEHAVIOR

Different types of environmental cues have effects which can be regarded as either global or local in nature. Global signals affect all of the growth cones of a given neuron. Alternatively, very local cues can act on individual growth cones. Experimental evidence also provides strong support for the idea that growth cones can behave quite autonomously. In culture, growth cones can survive for several days, perform typical growth cone behaviors, and respond to environmental signals even when severed from the parent neuron (26). Stimuli that would inhibit outgrowth of growth cones connected to the cell body can also inhibit isolated growth cones (10). Observations such as these lend considerable weight to the idea that these organelles can indeed act autonomously in their primary functions of pathfinding *in vivo*.

A wide variety of stimuli, both intrinsic and extrinsic, can alter growth cone form and function. These stimuli have largely appeared as unrelated, but as will be discussed below, we now recognize a common link between them at the level of intracellular second messengers. The longest known of these signals has been the simple surfaces or substrata upon which neurons grow. Neurons growing on, for instance, polylysine as opposed to collagen, display quite different morphologies (2,28).

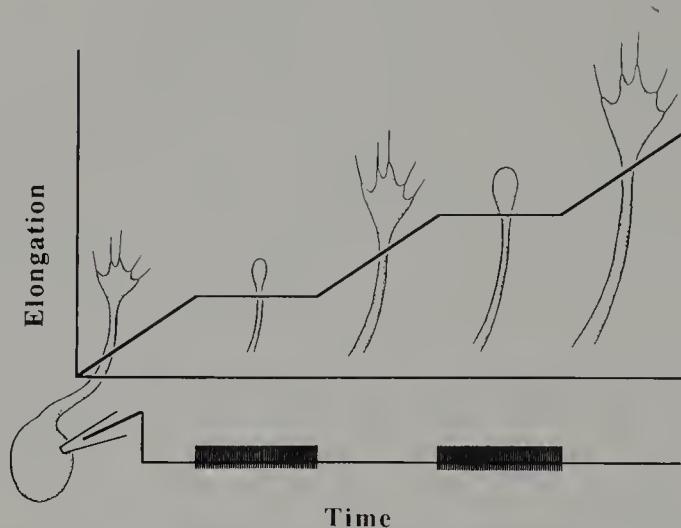
In addition to the substrata on which growth cones exist, simple mechanical stimuli can significantly alter growth cone morphology (e.g., 24). For instance, the application of a brief mechanical stimulus to the growth cone (e.g., a gentle

puff of medium, or a fine touch with a microscopic probe) as well as permanent physical barriers (e.g., a surface scratch in the tissue culture dish) can reliably evoke discrete turning and/or branching behaviors. Thus, both brief mechanical stimuli and mechanical barriers can dramatically alter the outgrowth direction and branching patterns of neurons. Such purely physical navigational aids may well not act alone. More likely they are integrated with other signals (e.g., chemical) to determine the ultimate sequence of neuromorphogenetic events.

Another major set of stimuli that influence growth cone behavior are trophic factors. The best established of these is nerve growth factor (NGF) which is known to have multiple effects ranging from direct effects on growth cone behavior and morphology, to the induction of particular genes (for a review see ref. 27). Interestingly, although NGF has been assumed to act as a diffusible molecule, evidence suggests the likelihood that it may, in fact, also act when substrate bound (5). This raises interesting possibilities for additional roles for diffusible molecules in growth cone navigation.

Our recent work has introduced a whole new class of diffusible molecules that regulate growth cone behaviors and thus could act as guidance cues for neurite outgrowth. Neurotransmitters are now known to regulate a variety of growth cone behaviors in a wide range of nervous systems. For instance, within the snail *Helisoma*, serotonin can inhibit outgrowth and alter the characteristic motile growth cone morphology of selected neurons. Another subset of neurons is totally unaffected by the presence of serotonin (9,10,22). An overlapping, but not identical, subset of neurons, can be inhibited in their outgrowth by dopamine. Similar results have now been obtained with rat hippocampal pyramidal neurons in culture. We have found that glutamate can have inhibitory effects on growth cone elongation. In this system, as well, the action of the neurotransmitter is highly selective for specific growth cones; however, it is not the growth cones of different neurons but rather the different classes of growth cone on the same neurons that show different responsiveness to glutamate. Namely, the dendritic growth cones of hippocampal pyramidal neurons are inhibited in their outgrowth and the dendrites can actually be pruned back by the presence of glutamate whereas, axonal growth cones continue their motile processes (17). Taken together with the now numerous observations in other species (e.g., 13,15), the work on *Helisoma* has demonstrated that an entirely new class of molecules can act as guides to neuronal growth cones. This, then, extends the idea that these basic agents of information transfer in mature nervous systems also play formative roles in neuronal development (14).

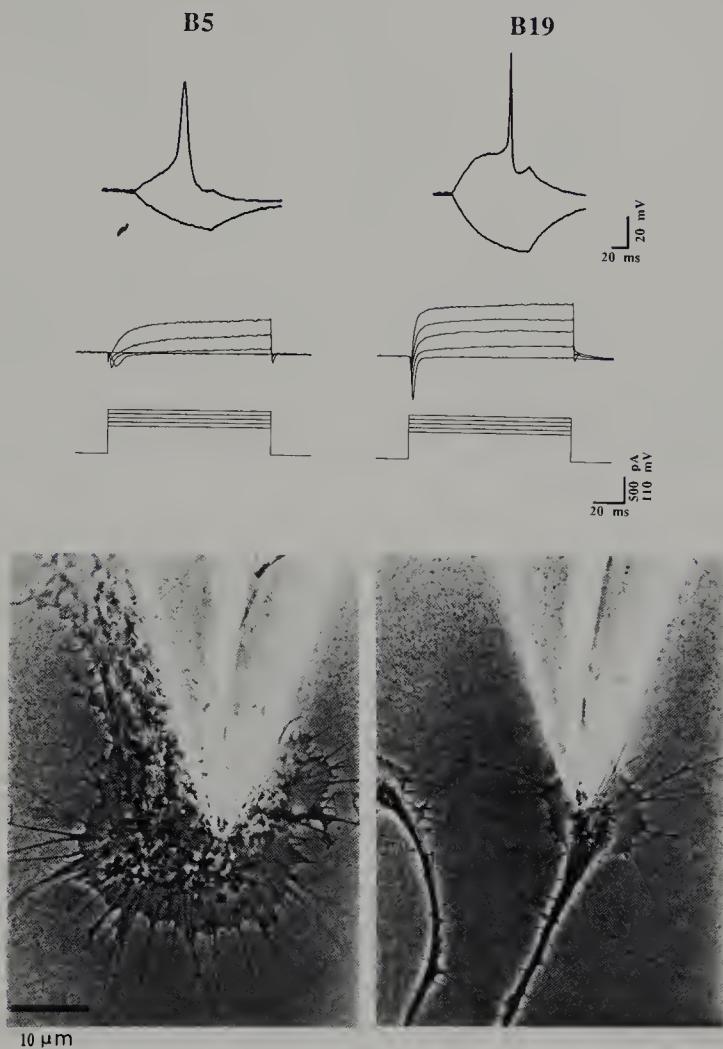
Intrinsic signals can also have major effects upon growth cone behavior. For some time "activity" has been discussed by neurobiologists as a potentially prominent sculptor of neuronal architecture and connectivity. A direct assessment of this idea has come from our work which examined the role of activity upon the growth cones of individual neurons in cell culture. Using an extracellular whole-cell patch electrode, it was possible to generate action potentials experimentally while optically monitoring the behavior of several growth cones of that neuron (Fig. 1). These experiments demonstrated that motile growth cones can be abruptly



**FIG. 1.** A summary of the results of Cohan and Kater (3) of the effect of electrical activity on neurite outgrowth. An extracellular patch electrode, on the cell body, was used to evoke action potentials. Simultaneously, neurite elongation and the status of growth cones were monitored photographically. The results of these studies demonstrated that, with the electrode in place, normal elongation and growth cone behavior were observed. During periods of evoked electrical activity, however, both growth cone behavior and neurite elongation were greatly changed. The firing of action potentials inhibited neurite elongation, filopodia withdrew, and the lamellipodial surface was greatly decreased. Subsequent to cessation of action potentials, general motility resumed. This is a reversible, and highly reproducible, effect.

inhibited in their elongation and pathfinding behaviors by the generation of action potentials. This inhibition was reversible; after cessation of stimulation, motility and growth cone morphology return to normal and the growth cones resume active elongation (3). These results indicate potentially important roles for neuronal circuitry in the generation of neuronal morphology. Quite different neuronal geometries would be expected in neurons participating in actively firing circuits when compared with those belonging to quiescent ensembles of neurons.

It is important to note here that the neuronal growth cone membrane shares many properties in common with other regions of the neuron (7). Perhaps most striking is the fact that growth cones are capable of firing fully regenerative action potentials. In fact, these action potentials are like fingerprints to the identity of the neuron (Fig. 2). A given neuron can be defined on the basis of the voltage-dependent ionic currents all along the nerve cell. Thus, the growth cone possesses an identity which may well be used as either the lock or key in recognition scenarios that occur as the growth cone courses along diverse environments in the processes of synaptogenesis.



**FIG. 2.** Growth cones from different identified neurons are identifiable, and their membranes possess many of the active properties normally associated with mature neuronal membrane. Such growth cones can be surgically isolated from their associated neurite and patch clamped in either a cell-attached or a whole-cell mode. Examples of action potentials and whole-growth cone voltage clamp records from isolated growth cones from two different neurons are shown: buccal neuron B5 (left) and a neuron B19 (right). These records show that growth cones from different neurons possess different, but characteristic, membrane properties; in fact, the membrane properties are characteristic for the neuron that generated the growth cones and can serve to identify growth cones. The phase images at the bottom are typical growth cones for these neurons. These growth cones can be identified on purely morphological criteria (9).

We now have evidence that many growth cone-regulatory stimuli converge at the level of membrane potential. This is most clearly shown for neurotransmitters. Depolarizing agents can dramatically inhibit neurite outgrowth just as was the case for the depolarizations produced by action potentials. For example, serotonin is excitatory (depolarizing) to one subset of neurons, but not another. Neurite outgrowth is blocked in the depolarized subset, but not the other (23). Other transmitters, when they change the membrane potential in the depolarizing direction, also have outgrowth-inhibiting effects on these same neurons. As another example, glutamate is the excitatory transmitter and inhibits the outgrowth of dendrites from cultured hippocampal pyramidal neurons. The most direct demonstration of this effect derives from experiments that negate changes in membrane potential during transmitter application. Using a whole cell patch electrode it is possible to counteract the shifts in membrane potential evoked by a neurotransmitter. Either serotonin or dopamine can block outgrowth of *Helisoma* neuron B19. However, when these neurotransmitters are applied but the membrane potential is held hyperpolarized through current injection via the electrode (Fig. 3), outgrowth continues. Thus, it appears that one major path of growth cone regulation is via regulation of the membrane potential.

## NEURONAL INTEGRATION: THE GROWTH CONE PROCESSES MULTIPLE SIGNALS

While it is clear that individual signals can have profound effects on the behavior of the neuronal growth cone, it is equally clear that many of these signals co-exist both during development and in the mature organism (Fig. 4). Clearly, growth cones must be able to integrate signals provided by unique substrata, specific trophic factors, the presence or absence of certain neurotransmitters, and ongoing electrical activity all at the same time. A good model for this kind of integration of multiple behavioral cues comes from the mature nervous system where individual neurons must integrate multiple convergent inputs. Our best example of the effects of multiple stimuli on growth cone behavior reveals how the presence of a second neurotransmitter can profoundly alter the effect of a first. In *Helisoma*, a primary effect of serotonin is the inhibition of growth cone motility in a specific set of neurons. The presence of acetylcholine, however, can negate the serotonin-induced inhibition of growth cone motility (21). This is likely not an isolated phenomenon, but rather, integration of the effects of multiple transmitters seems a general principle. In rat hippocampal pyramidal cells in cell culture, motility of dendritic growth cones is retarded by glutamate. These growth-inhibiting effects of glutamate can be negated by the inhibitory transmitter GABA (18). In both of these examples, the net effect on growth cone behavior is a composite of the actions of more than one environmental stimulus. It is important to note here that, in both cases, the second neurotransmitter acts via changes in membrane potential.

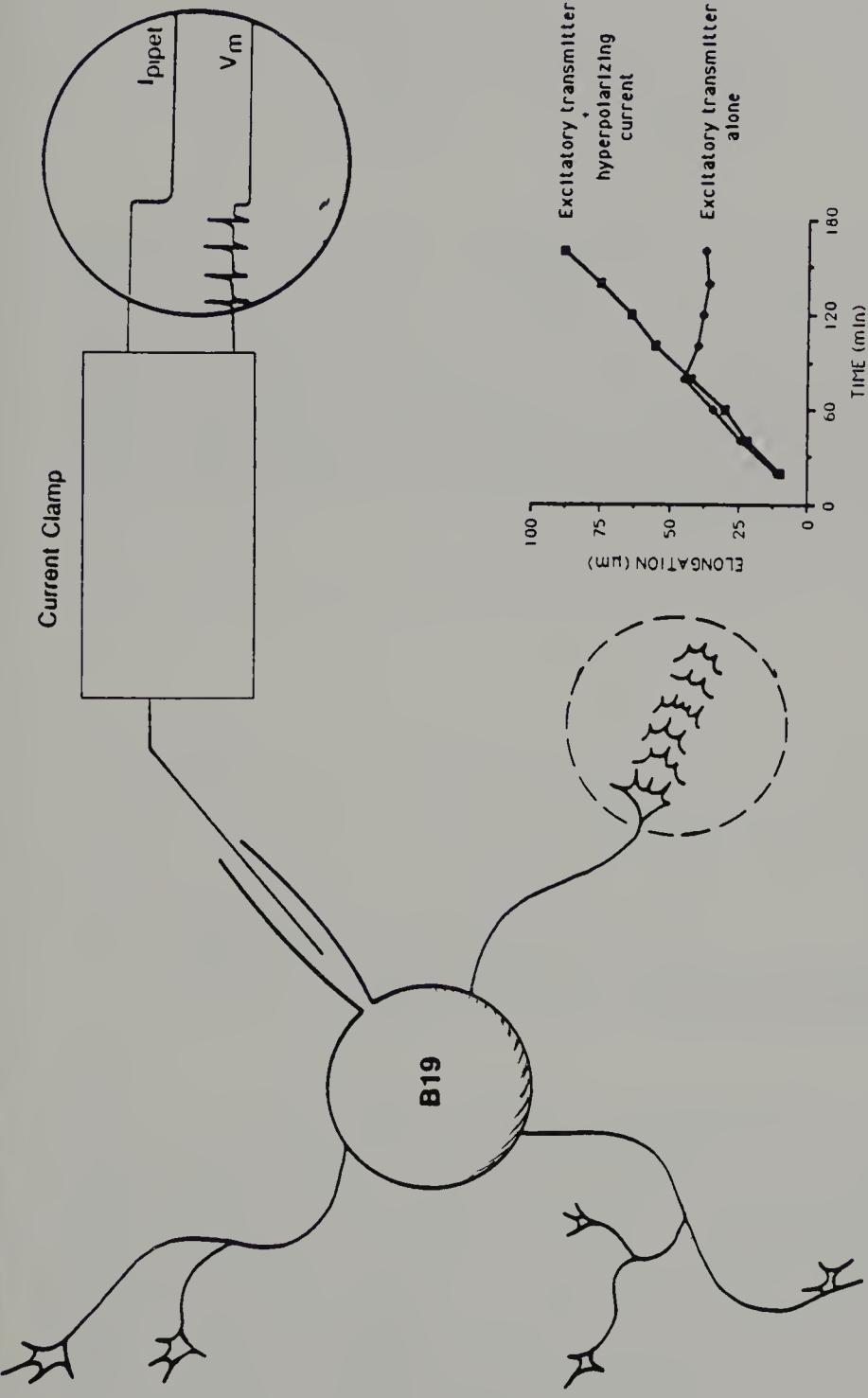
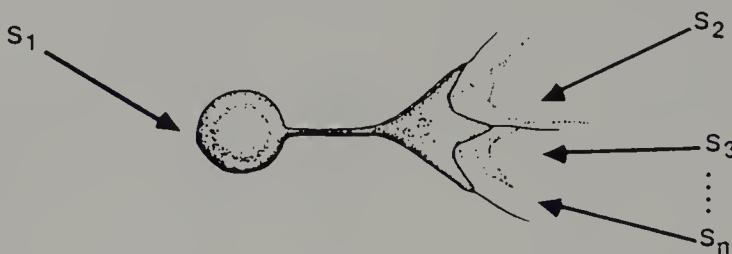


FIG. 3. Neurotransmitters that regulate growth cone behavior do so by acting at the level of membrane potential. As shown in schematic form, it is possible, using patch electrodes or microelectrodes, to record from or stimulate identified *Helisoma* neurons while simultaneously monitoring neurite elongation. Such experiments (23) have demonstrated that those neurotransmitters which inhibit neurite elongation also significantly depolarize the neuron. In addition, the outgrowth inhibitory effects of those neurotransmitters can be blocked by injection of hyperpolarizing current.

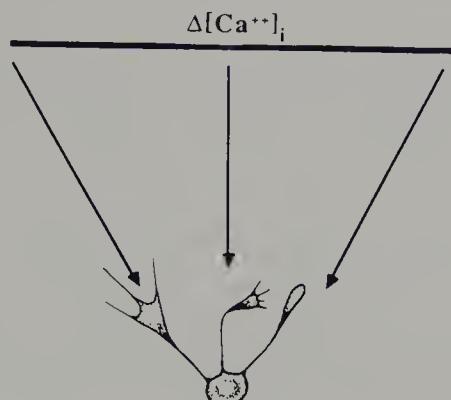


**FIG. 4.** Neuronal growth cones integrate multiple environmental cues with intracellular signals. Such cues include substrate-bound molecules, neurotransmitters, trophic factors, mechanical stimuli, and even the electrical activity generated within the neuron. These signals can act either locally at the level of the growth cone (e.g., neurotransmitters) or have more global effects on the entire neuron (e.g., electrical activity, which would propagate throughout the cell). *In vitro*, it has been possible to show that each of these separate stimuli can affect the behavior of growth cones. *In vivo*, it is clear that many of these stimuli could be present simultaneously and that the neuron must integrate the effects of all stimuli, transforming this net signal into a change in growth cone behavior.

Both acetylcholine and GABA are inhibitory (hyperpolarizing) transmitters to their respective cell types. The mechanisms underlying the integration demonstrated in these examples of outgrowth regulation are precisely the same as those used for neural coding in mature neurons.

## LOCAL CHANGES IN INTRACELLULAR CALCIUM ALTERS GROWTH CONE BEHAVIOR

A neuronal growth cone on a given neuron may exhibit: 1) characteristic long-filopodia with a broadened lamellipodium, 2) a decreased number of filopodia and reduced lamellipodium, or 3) the rounded, phase-bright growth cone with no filopodia that is characteristic of the nonmotile growth cones of *Helisoma* neurons (8). Various stimuli have been found which can transform the growth cone from one of these forms to another. For each of these stimuli, some transduction mechanism must exist to link the stimuli with the intracellular machinery controlling morphology and motility. With the advent of the fluorescent calcium indicator fura-2, it has been possible to directly measure intracellular calcium levels in neurons. The emerging picture is that intracellular calcium concentrations profoundly affect the behavior and morphology of neuronal growth cones (12, Fig. 5).

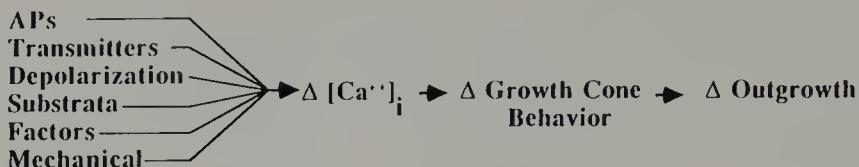


**FIG. 5.** Growth cone behavior changes with local changes in intracellular free calcium concentration. An array of different growth cone morphologies and behaviors is associated with different levels of intracellular calcium. At the usual calcium set point associated with outgrowth, characteristic broadened lamellipodia and long filopodia are observed. With a slight reduction in intracellular calcium concentrations, growth cones become considerably reduced but motility increases. Either significantly increasing or reducing intracellular calcium levels result in a nonmotile form of the growth cone. Preliminary evidence (24) indicates that even directionality may be regulated by intracellular calcium concentrations.

We know, for instance, that the generation of action potentials can greatly increase intracellular calcium in growth cones. Similarly, we know that serotonin greatly increases the concentration of calcium, whereas the presence of acetylcholine can negate the rise in intracellular calcium (21). The equation below shows that the mechanism by which neurons integrate multiple stimuli is by summing the net effects of various stimuli that change intracellular calcium with their own intracellular basal calcium level.

$$[\text{Ca}^{++}]_i = [\text{Ca}^{++}]_{\text{Basal}} + \Delta[\text{Ca}^{++}]_{S1} + \Delta[\text{Ca}^{++}]_{S2} + \dots \Delta[\text{Ca}^{++}]_{Sn}$$

The primary point is that the net intracellular calcium concentration and its spatial distribution is going to be a composite. This composite could be different for different neurons receiving the same stimuli, (e.g., if different neurons have different basal calcium levels, then a given stimulus could have very different effects on those two neurons) and could also vary for an individual neuron when it received different combinations of stimuli. In each case, the summation of various stimuli, some of which raise intracellular calcium and some of which lower intracellular calcium, would produce their net effect at the final common determinant—free intracellular calcium (Fig. 6).



**FIG. 6.** It is now known that various stimuli ranging from action potentials to mechanical deformation result in discrete and predictable changes in intracellular calcium. The form and behavior of the neuronal growth cone are highly sensitive to intracellular calcium concentrations. Thus, these stimuli, acting through changes in intracellular calcium, act to change growth cone behavior. This in turn changes outgrowth in neurons and alters the ultimate architecture and connectivity patterns in which these neurons participate.

At this point it's important to note that increases and decreases in calcium concentration do not *per se* evoke identical behaviors in growth cones of all organisms (cf, 12). The early literature was replete with apparently contradictory effects of treatments intended to alter intracellular calcium. We now recognize two major sources of confusion. On the one hand, we now know that many agents assumed to alter intracellular calcium have effects on multiple second messenger systems. Some agents have only transient effects on calcium concentrations. With the ability to directly measure calcium concentrations, we can now confirm the effects on any pharmacological manipulation. The other major source of confusion is at least partially clarified by the idea that particular neurons have unique calcium set points (6) some of which may be set near the top of the optimum calcium concentration for outgrowth and some set near the bottom. The net result is that an experimental perturbation to raise calcium might take one kind of neuron out of the permissive range of calcium concentration for a given behavior while the same stimulus might optimize calcium for the very same behavior in another neuronal type (12). Our present view, which is largely derived from work on *Helisoma*, is that large deviations in either direction from the calcium set point of a given neuron take that neuron out of the permissive range, and outgrowth ceases.

This model assumes that the effects of many, if not all, regulatory signals summate at the level of intracellular calcium. There is, however a variety of evidence that indicates that other second messengers (e.g., cyclic nucleotides, and protein kinase C, 4,19,20) can regulate growth cone behavior perhaps independently of effects on intracellular calcium. Whether such mechanisms are ancillary or subordinate to the mechanisms proposed here is yet to be determined. Nevertheless, it is clear that intracellular calcium does play a key role in the regulation of neuronal growth cone behavior and ultimately in the genesis of neuronal architecture.

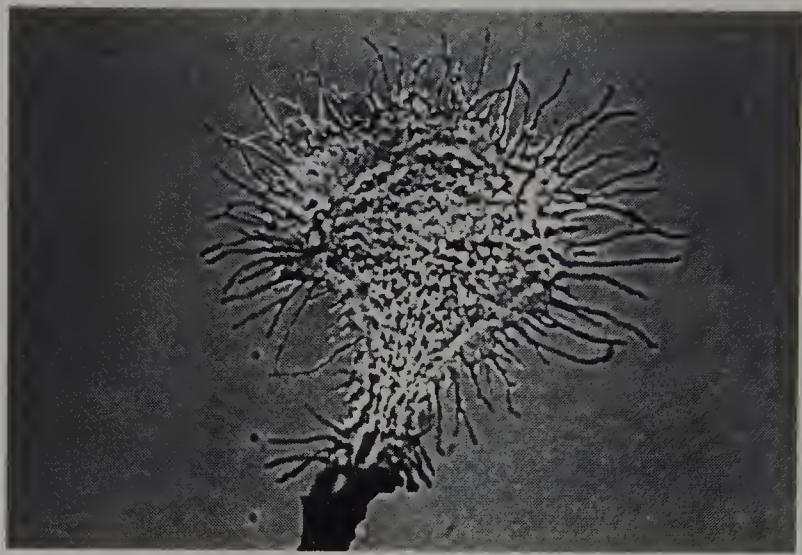
## MULTIPLE COMPONENTS OF CALCIUM HOMEOSTASIS

While a variety of agents can predictably alter intracellular calcium concentration, and these in turn all have effects on growth cone behavior, there is no reason to assume that each acts through the same intracellular regulatory mechanism. Because there are many component processes in the regulation of intracellular calcium, it seems reasonable that different environmental cues act upon different aspects of the overall calcium regulatory system. Free intracellular calcium concentration is a function of calcium influx and release of calcium from intracellular stores, minus the efflux of calcium through pumping systems and sequestration by the buffering capacity of the cytoplasm and organelles such as mitochondria. Different environmental cues might well regulate different aspects of this balance. For instance, some neurotransmitters may well act or alter calcium influx through membrane channels, while other neurotransmitters, perhaps operating through other second messengers, might release calcium from intracellular stores. These are but two examples of an array of possibilities that might extend to the modulation of all of the components of calcium homeostasis.

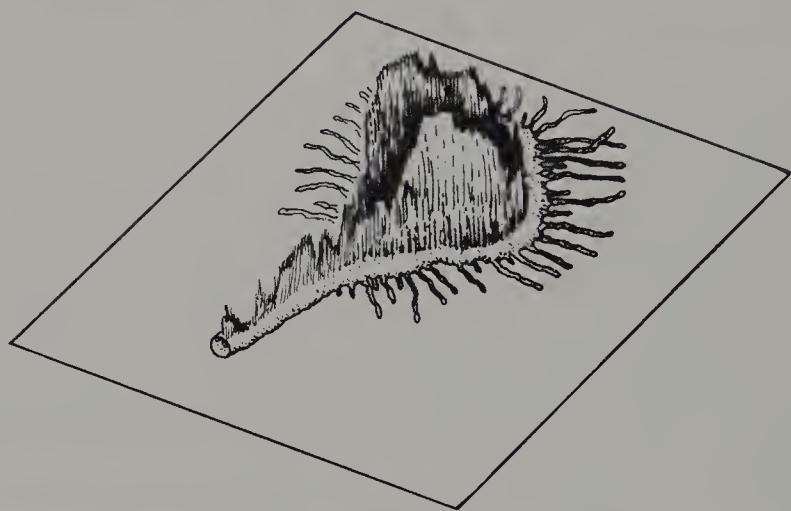
In this review we have only referred to the overall concentration of calcium within a growth cone. At a finer grain, however, we can see that calcium is not likely to be homogeneously distributed even within a single growth cone. For example, we presently have "calcium maps" of individual growth cones that imply the existence of gradients of calcium concentration (Fig. 7). While this observation remains to be understood in terms of its underlying causes, it is clear that it could play a very important role in growth cone behavior. It is well known that there are very different underlying components of growth cone elongation. Processes like actin assemble, tubulin polymerization, and vesicle fusion each have different optimal calcium concentrations. We envision that these processes may each be separately regulated by distinct and different calcium concentrations. The specific variations in calcium concentration that we have observed (the "calcium map") appear to coincide with the distribution of the cytoskeletal elements and the vesicular apparatus used for motility (Fig. 8). This reinforces the idea that precise local concentrations of calcium may indeed regulate the underlying calcium-dependent processes for motility.

## CONCLUSIONS

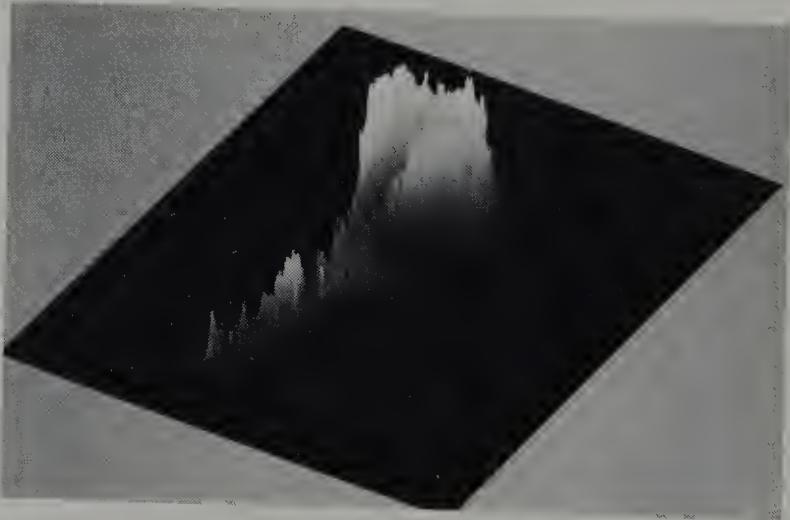
The generation and regeneration of neuronal form and connectivity both undoubtedly rely upon the integration of intrinsic and extrinsic information of many kinds. Our work has demonstrated that the concentration and spatial distribution of intracellular calcium is a key locus for integration of such information. Through a delicate balance of mechanisms that raise and lower free calcium, a steady state



A

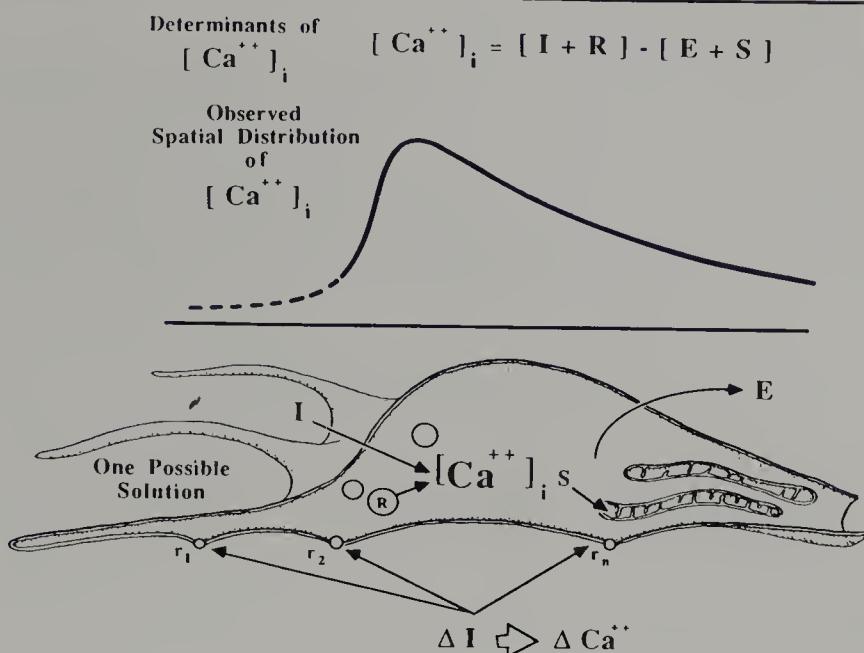


B



C

FIG. 7.



**FIG. 8.** Factors determining calcium concentration within growth cones. The top equation shows the factors determining calcium concentrations within the growth cone. Calcium levels are increased by influx (I) and release (R) from intracellular stores. Levels are decreased by efflux (E) (pumping), and sequestration (S) (both cytoplasmic buffering and intracellular organelles). The middle panel shows the calcium rest levels in a growth cone observed by fura-2 mapping. The bottom panel gives one possible distribution of mechanisms which could explain the observed gradient of distribution of intracellular calcium within the neuronal growth cones. If influx (I) were localized primarily to the leading edge of the main body of the growth cone and/or efflux (E) and sequestration (S) were localized primarily to the growth cone neurite junction, calcium concentrations would be higher at the leading edge than in the neurite. One implication of this gradient is the possibility that anything that perturbs the gradient might have profound effects on the behavior of the growth cone. For example, if neurotransmitter receptors were localized on the leading edge of the growth cone, activation of these receptors would directly increase the influx of calcium at that point, and increase the gradient. Activation of other receptors at the trailing edge of the growth cone (e.g., receptors interacting with extracellular matrix molecules) could reduce the gradient by increasing influx at that point. These ideas, while speculative, offer the opportunity to directly test how particular environmental and intrinsic cues affect the behavior of the neuronal growth cone by specifically affecting components of calcium regulating mechanisms.

**FIG. 7.** Calcium concentrations show local heterogeneity within neuronal growth cones. (A) A phase-contrast image of a buccal neuron B5 growth cone showing the numerous filopodia and lamellipodial membrane. (B) A line drawing showing how the calcium concentrations are mapped onto the growth cone as a three-dimensional projection. Calcium concentrations are mapped vertically with higher concentrations being represented by higher lines. (C) The original three-dimensional calcium map used to generate panel B. Here, both height and brightness are used to show calcium concentration. The important feature of this panel is the gradient in calcium concentration, with the highest concentration at the leading edge of the main body of the growth cone and a significantly lower concentration at the growth cone-neurite junction. Calcium concentrations range from 100 nM for dark gray to 800 nM for white in this panel.

is achieved that appears to have significant regulatory control over neuronal growth cone behavior. A complex regulatory role for the calcium map is important because we now know that environmental signals known to change growth cone behavior also change the calcium map. Cues, both internal and external, alter intracellular calcium levels, and consequently alter growth cone behavior. We have observed such changes with a variety of agents ranging from action potentials (3) through neurotransmitters (9), depolarization of membrane potential (23), changes in substrata (28), growth factors (28), and even mechanical stimuli (24). It is through the alteration of the various components of calcium homeostasis that we envision the complexities of neuronal architecture and connectivity may be fine-tuned throughout the life histories of neuronal ensembles. A change in *any* of the regulatory components of calcium homeostasis would be expected to cause a change in these calcium maps. It now remains to partition and dissect this system, and thereby define how each specific component of the calcium homeostatic system can be regulated to bring about the changes in calcium which ultimately result in changes in neuronal growth cone behavior.

## REFERENCES

1. Bentley D, Toroian-Raymond A. Disoriented pathfinding by pioneer neurone growth cones deprived of filopodia by cytochalasin treatment. *Nature (Lond)* 1986;323:712–715.
2. Bray D. Branching patterns of individual sympathetic neurons in culture. *J. Cell Biol.* 1973;56:702–712.
3. Cohan CS, Kater SB. Suppression of neurite elongation and growth cone motility by electrical activity. *Science* 1986;232:1638–1640.
4. Forscher P, Kaczmarek LK, Buchanan J, Smith SJ. Cyclic AMP induces changes in the distribution and transport of organelles within growth cones of *Aplysia* bag neurons. *J. Neurosci.* 1987;7:3600–3611.
5. Gunderson RW. Sensory neurite growth cone guidance by substrate adsorbed nerve growth factor. *J. Neurosci. Res.* 1985;13:199–212.
6. Guthrie PB, Mattson MP, Mills LR, and Kater SB. Calcium homeostasis in molluscan and mammalian neurons: neuron-selective set-point of calcium rest concentration. *Soc. Neurosci. Abstr.* 1988;14:582.
7. Guthrie PB, Lee RE, Kater SB. A comparison of neuronal growth cone and cell body membrane: electrophysiological and ultrastructural properties. *J. Neurosci.*; in press.
8. Hadley RD, Bodnar DA, Kater SB. Formation of electrical synapses between isolated, cultured *Helisoma* neurons requires mutual neurite elongation. *J. Neurosci.* 1985; 5:3145–3153.
9. Haydon PG, Cohan CS, McCobb DP, Miller HR, Kater SB. Neuron-Specific growth cone properties as seen in identified neurons of *Helisoma*. *J. Neurosci. Res.* 1985; 13:135–147.
10. Haydon PG, McCobb DP, Kater SB. The regulation of neurite outgrowth, growth cone motility, and electrical synaptogenesis by serotonin. *J. Neurobiol.* 1987;18:197–215.

11. Kater SB, Letourneau P, eds. *The Biology of the Neuronal Growth Cone*. New York: Alan R. Liss, 1985.
12. Kater SB, Mattson MP, Cohan, C, Connor J. Calcium regulation of the neuronal growth cone. *Trends in Neurosci.* 1988;11:315–321.
13. Lankford KL, DeMello FG, Klein WL. D1 dopamine receptors inhibit growth cone motility in cultured retinal neurons; evidence that neurotransmitters act as morphogenetic growth regulators in the developing nervous system. *Proc. Natl. Acad. Sci. USA* 1988;85:4567–4571.
14. Lauder JM. Neurotransmitters as morphogenetic signals and trophic factors. In: Ver nadakis A, Privat, A, Lauder JM, Timipas PS, Giacobini E, eds. *Model Systems of Development and Aging of the Nervous System*. Boston: Martinus Nijhoff Publishing, 1987;219–237.
15. Lipton SA, Frosch MP, Phillips MD, Tauck DL, Aizenman E. Nicotinic agonists enhance process outgrowth by retinal ganglion cells in culture. *Science* 1988;239:1293–1296.
16. Lopresti V, Macagno ER, Levinthal C. Structure and development of neuronal connections in isogenic organisms: cellular interactions in the development of the optic lamina of *Daphnia*. *Proc. Natl. Acad. Sci. USA* 1973;70:433–437.
17. Mattson MP, Dou P, Kater SB. Outgrowth-regulating actions of glutamate in isolated hippocampal pyramidal neurons. *J. Neurosci.* 1988;8:2087–2100.
18. Mattson MP, Kater SB. Excitatory and inhibitory neurotransmitters in the generation and degeneration of hippocampal neuroarchitecture. *Brain Res.* 1988;478:337–348.
19. Mattson MP, Taylor-Hunter A, Kater SB. Neurite outgrowth in individual neurons of a neuronal population is differentially regulated by calcium and cyclic AMP. *J. Neurosci.* 1988;8:1704–1711.
20. Mattson MP, Guthrie PB, Kater SB. Intracellular messengers in the generation and degeneration of hippocampal neuroarchitecture. *J. Neurosci. Res.* 1989;21:447–464.
21. McCobb DP, Cohan CS, Connor JA, Kater SB. Interactive effects of serotonin and acetylcholine on neurite elongation. *Neuron.* 1988;1:377–385.
22. McCobb DP, Kater SB. Dopamine and serotonin inhibition of neurite elongation of different identified neurons. *J. Neurosci. Res.* 1988;19:19–26.
23. McCobb DP, Kater SB. Membrane voltage and neurotransmitter regulation of neuronal growth cone motility. *Developmental Biology.* 1988;130:599–609.
24. Mills LR, Murrain M, Guthrie PB, Kater SB. Intracellular calcium concentrations in neuronal growth cones change with induced turning and branching behavior. *Soc. Neurosci. Abstr.* 1988;14:583.
25. Ramon y Cajal S. A quelle époque apparaissent les expansions des cellules nerveuses de la moelle épinière du poulet. *Anat. Anz.* 1890;5:609–613, 631–639.
26. Shaw G, Bray D. Movement and extension of isolated growth cones. *Exp. Cell Res.* 1977;104:55–62.
27. Thoenen H, Barde YA. Physiology of nerve growth factor. *Physiol. Rev.* 1980;60:1284–1335.
28. Wong RG, Hadley RD, Kater SB, Hauser G. Neurite outgrowth in molluscan organ and cell cultures: the role of conditioning factor(s). *J. Neurosci.* 1981;1:1008–1021.



# Trophic Regulation of Myelinogenesis

A. L. Gard, R. Bansal and S. E. Pfeiffer

*Department of Microbiology, University of Connecticut Health Center  
Farmington, CT 06032*

The differentiation of oligodendrocytes (OL) and their elaboration of the myelin sheath is a crucial event of brain development. Saltatory conduction is realized with dramatic savings in energy consumption and space utilization. Accordingly, disruption of the myelinogenic process leads to a variety of serious neurological deficits, many with chronic implications (33). The term myelinogenesis is used here to denote the complete process of OL development from the initial appearance, proliferation, and differentiation of committed progenitor cells, through the synthesis and transport of myelin-specific structural components, to the formation and maintenance of the myelin sheath, including possibilities for remyelination (37).

In brain, OL are derived from neuroectodermal cells of the subventricular germinal zones (39,47). After migrating to the formative white matter and other areas, progenitor cells mature into post-mitotic, functional OL (22,34). In rodents, the rapid progression of myelinogenesis during the first postnatal month has facilitated, through a combination of immunocytochemical studies *in vivo* and in culture, the identification of phenotypic changes which subdivide the OL lineage pathway into sequential, antigenically defined stages (7,18,26,27,41). However, because myelinogenesis progresses along both temporal and spatial gradients within the central nervous system (e.g., 50), the experimental problem of identifying environmental stimuli that regulate the growth control and timing of OL differentiation becomes one of procuring relatively homogenous populations of cells at individual stages within this lineage pathway (summarized in Fig. 1).

Recent progress has demonstrated in optic nerve a bipolar, migratory, bipotential glial progenitor cell (0-2A) that can differentiate into either a Type II astrocyte or an OL, depending on environmental signals (41,43,52,56). The identity and neural origin of two such signals have been identified. Platelet-derived growth factor (PDGF), released by the Type I astrocyte (48), acts as a mitogen to stimulate 0-2A cells to continue proliferating until a subsequent decision is made to differentiate further (40); in the absence of PDGF the cells cease proliferation and rapidly differentiate into OL (35,48). A factor related to ciliary neurotrophic factor (CNTF;3) is also produced by the Type I astrocyte and increases the differentiation probability of 0-2A precursors to Type II astrocytes (21,28).

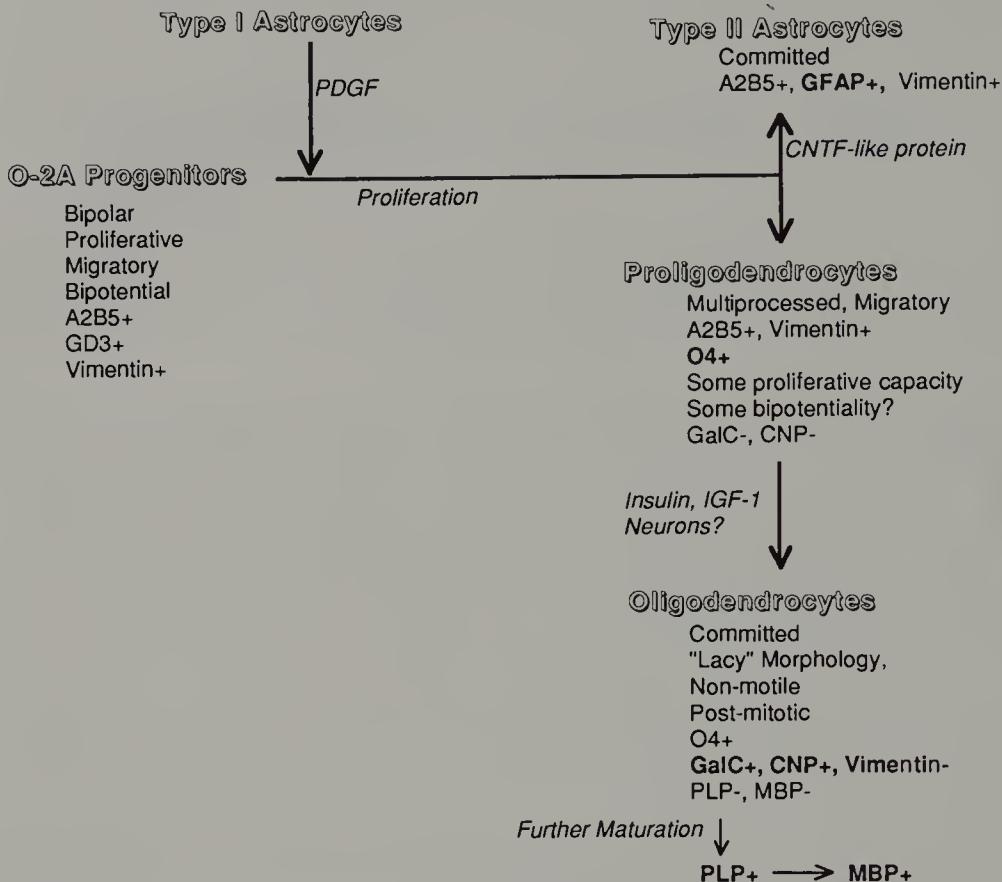


FIG. 1. A current working hypothesis of the developmental lineage of oligodendrocytes in telencephalon. See text for discussion and references.

A decision to enter the OL-specific lineage is characterized by the appearance of the O4 surface antigen in rodents (10,25,53,54), although cultured O4<sup>+</sup> cells may retain some bipotentiality (25,57). These cells are not considered OL *per se* insofar as they do not express the early OL marker galactocerèbroside (GalC) (42). Cells at this stage developing in culture systems are responsive to environmental cues (e.g., 23). For example, the stimulation of OL proliferation and development by insulin and insulin-like growth factor I (31,58) may occur primarily at the O4<sup>+</sup> progenitor stage (30).

*In vivo* the temporal expression of GalC in the OL plasmalemma is followed in succession by an ordered and sequential synthesis of the major myelin-associated proteins during a brief interval preceding myelin sheath formation (47,55). OL developing from precursor cells in culture can differentiate extensively and express myelinogenic parameters over long periods, even in the absence of neurons

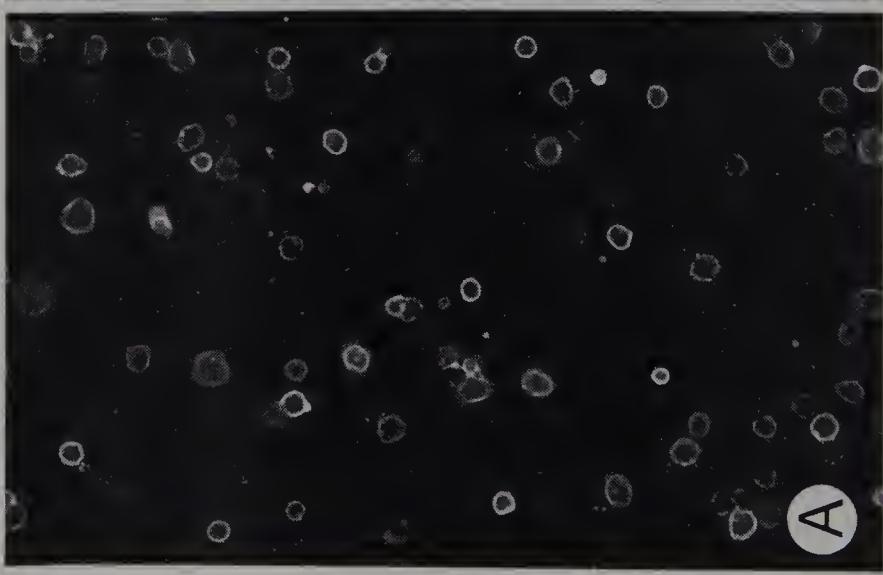
(11,32,37). Nevertheless, a role for other neural cell types in the modulation or differential regulation of specific myelin-associated gene products is suggested by other studies (e.g., 6,29). The developmental state of these cells in experimental situations can be identified with considerable accuracy using a panel of immunological and molecular probes (38).

## OLIGODENDROCYTE PROGENITORS ISOLATED DIRECTLY FROM DEVELOPING TELENCEPHALON AT A SPECIFIC PHENOTYPIC STAGE: MYELINOGENIC POTENTIAL IN A DEFINED ENVIRONMENT

In an immunofluorescence analysis of freshly dissociated tissue (18) we have determined that both the size and relative proportion of the O4<sup>+</sup> subpopulation (O4<sup>+</sup>GalC<sup>-</sup> plus O4<sup>+</sup>GalC<sup>+</sup> cells) increases rapidly during the first postnatal week in the rat telencephalon. O4<sup>+</sup>GalC<sup>-</sup> progenitors first appear at birth ( $10^5$ /telencephalon), two–three days before O4<sup>+</sup>GalC<sup>+</sup> oligodendrocytes. The number of O4<sup>+</sup> cells recovered by a partially selective dissociation procedure reaches a plateau of  $\sim 1.5 \times 10^6$  by P4–P5, when only 20% are GalC<sup>+</sup>.

To study the developmental potential of the O4<sup>+</sup>GalC<sup>-</sup> population, we have used solid-phase immuno-affinity selection ("panning") to isolate  $\sim 20\%$  ( $3.5 \times 10^5$  cells/telencephalon) of the cells in freshly dissociated tissue (18). Complement-mediated immunolysis is used to remove GalC<sup>+</sup> OL in a preliminary step. The remaining O4<sup>+</sup> cells (>97% GalC<sup>-</sup>) provide a developmentally narrow, defined population of oligodendrocyte progenitors (Fig. 2) obtained directly from brain. Despite the reaction of O4 antibody with purified sulfatide, O4<sup>+</sup>GalC<sup>-</sup> cells metabolically labeled with  $^{35}\text{SO}_4$  *in situ* also do not synthesize detectable levels of sulfogalactolipids, indicating the presence of an unidentified antigen recognized by this antibody (3,19,51).

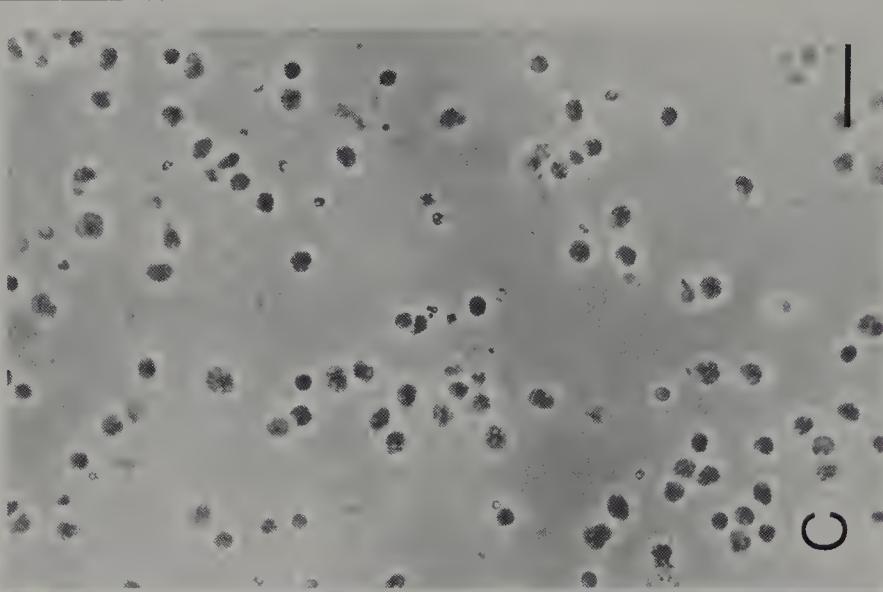
When isolated progenitors are cultured in a defined medium, more than 90% of the seeded cells rapidly attach to a polyornithine substratum. Although their initial viability exceeds 98%, the plating efficiency decreases to  $\sim 25\%$  over the first three days in culture (DIC), and then remains relatively constant through 10 DIC before abruptly declining. The requirement in culture for survival factors present in brain is thereby suggested. Within 1–2 DIC most cells extend two–five processes 10–40  $\mu\text{m}$  in length with terminal growth cone-like expansions (Fig. 3). Numerous filopodia and lamellipodia are observed to extend from these processes when observed after labeling the cell surface with O4 by immunofluorescence; by 3–4 DIC elongated processes with extensive secondary branching appear; by 7 DIC the processes of most cells are 50–100  $\mu\text{m}$  long and highly reticulate with expanded membranous sheets and leaflets from which filopodia extended distally (Fig. 4). These changes parallel the morphogenesis of OL *in situ* through a "lacy" stage, during which the synthesis of myelin proteins commences and the contact of ap-



A



B



C

FIG. 2. Freshly isolated progenitors after immunoselection from 4–5 day-old postnatal rat telencephalon labeled for O4 (A) and GalC (B). (C), corresponding field under phase contrast. Scale bar, 50  $\mu$ m.

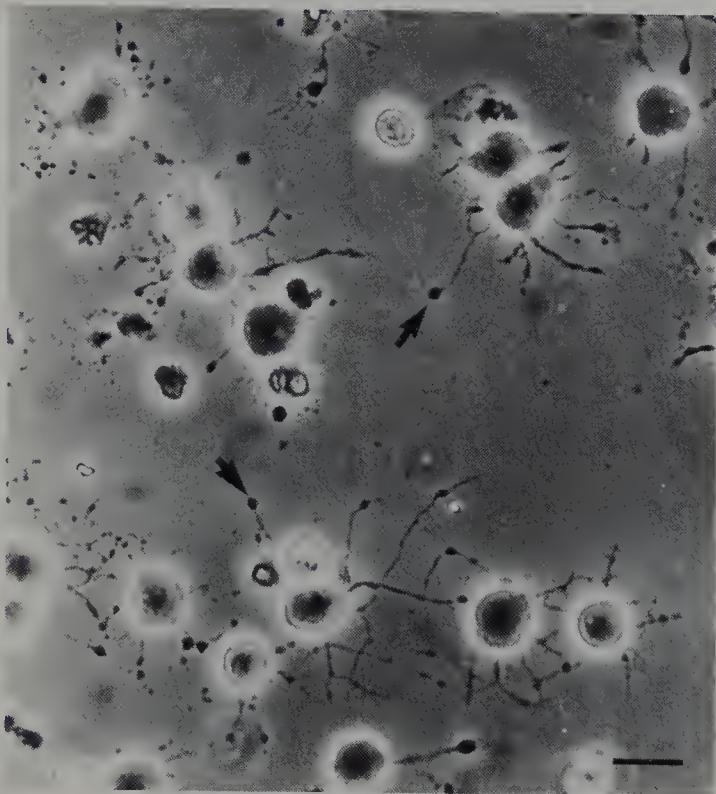


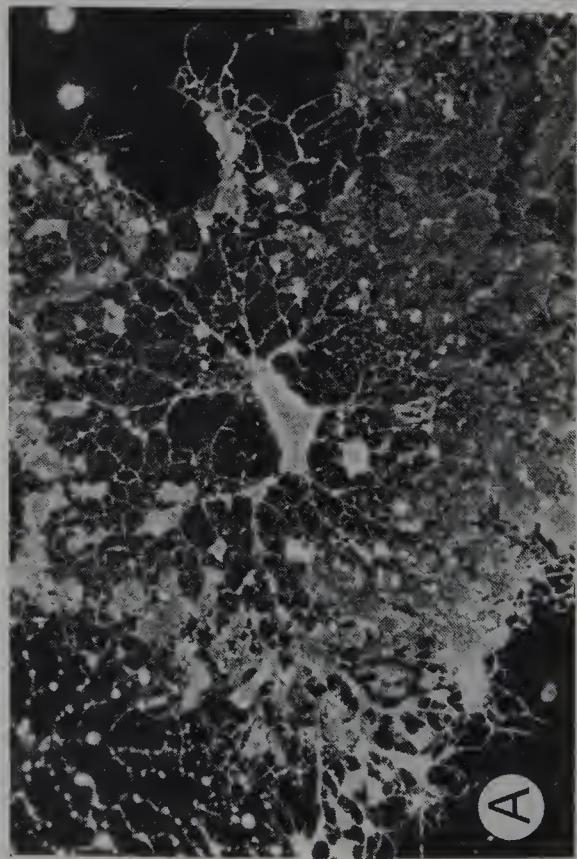
FIG. 3. Morphology of  $O4^+$ GalC $^-$  progenitors after 1 DIC on polyornithine substratum in modified N2 medium (see 18 for composition). Terminal end bulb structures are indicated (arrows). Scale bar, 12  $\mu m$ .

propriate axons is predicted, shortly before myelination *per se* (16,55). It is noteworthy that throughout this time the cultures remain essentially devoid of immunocytochemically recognizable neurons and astrocytes.

At least 80% of the isolated  $O4^+$ GalC $^-$  progenitors undergo extensive biochemical differentiation in culture. A relatively small, maximal set of nutritional supplements is sufficient for oligodendrocytes to carry out a myelinogenic cascade of differentiated gene expression in a temporally normal manner, in quantitatively significant amounts, in normal ratios of myelin protein isoforms, and in a regulated relationship to the inclusion of myelin-specific products into myelin-like membrane sheets (Fig. 4). This sequence mimics the highly conserved order and relative timing of antigen expression detected immunohistochemically among OL differentiating in regions of the developing rat brain (47). GalC appears after 1–3 DIC, as cells display a distinctly more complex process outgrowth, almost simultaneously with weak 2',3'-cyclic nucleotide 3'-phosphohydrolase (CNP) immunoreactivity. Proteolipid protein (PLP) and myelin basic protein (MBP), respectively, appear two-



B



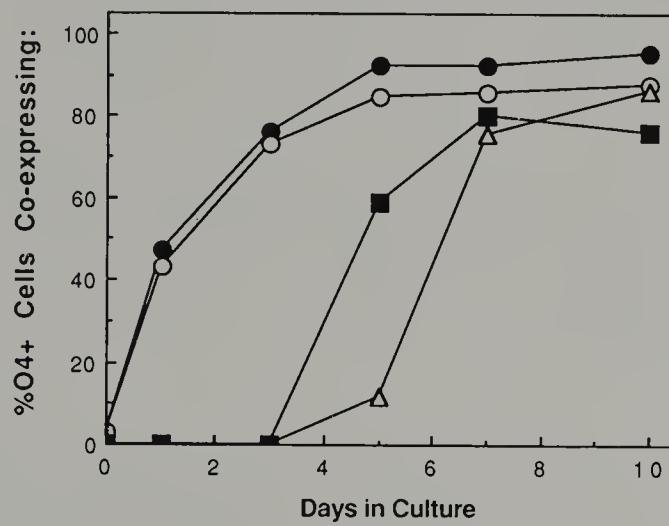
A

FIG. 4. Isolated O<sup>4+</sup>GalC<sup>-</sup> cells produce expanses of GalC<sup>+</sup> myelin-like membrane by 7 DIC (A) which also contain CNP and MBP (18). In contrast, PLP immunofluorescence (B, same field) is restricted largely to the cell body 7 DIC. Scale bar, 10  $\mu$ m.

three and four–five days after GalC expression (Fig. 5). We conclude that a specific (“committed”) regulatory program of terminal differentiation is in place in O4<sup>+</sup>GalC<sup>-</sup> cells *in vivo*, cells which we have termed “proligodendrocytes.” An important corollary of this finding is that contact with other cell types, in particular neurons and astrocytes, or unknown growth factors unique to these cell types, is not required for subsequent gene expression.

A smaller population (~15–20%) of O4<sup>+</sup>GalC<sup>-</sup> cells fails to progress in their morphological and biochemical differentiation during the 10-day course of study. Although many acquire a sparse punctate labeling pattern on their cell bodies with an anti-glycolipid antibody reactive for GalC and sulfatide (3,46), the myelin proteins are not expressed at detectable levels. These cells may have significance for understanding progenitors described in adult brain (15,59) that may be recruited during limited remyelination under pathological conditions.

To conclude, this study demonstrates that OL progenitors which have attained the O4<sup>+</sup>GalC<sup>-</sup> stage of differentiation in a normal germinal environment are comprised of two subpopulations with markedly different states of myelinogenic potential once placed into culture. The existence of both may simply relate to a further subdivision of the O4<sup>+</sup>GalC<sup>-</sup> stage within a single lineage pathway by a critical regulatory signal that triggers a subsequent program of ordered myelinogenic expression. Alternatively, the nonresponding cells may reflect a secondary, di-



**FIG. 5.** Developmental time course of myelin-associated antigen expression by isolated progenitors differentiating in culture analyzed by immunofluorescence microscopy. The proportion of O4<sup>+</sup> cells doubly labeled for GalC (●); CNP (○); PLP (■); and MBP (△).

vergent mechanism requiring alternative signals for terminal OL differentiation. Future studies will focus on the identification of factors which promote cellular differentiation in this highly simplified culture system.

## REVERSIBLE INHIBITION OF OLIGODENDROCYTE PROGENITOR DIFFERENTIATION BY A MONOCLONAL ANTIBODY AGAINST SURFACE GALACTOLIPIDS

The expressions of GalC and sulfatide on the surface of OL occur at a critical point in the developmental lineage (Fig. 1), and have been accepted as important diagnostic markers of OL maturation (32,42). We have proposed that these galactolipids have functional roles in the regulation of OL differentiation and myelination (1). Several studies reinforce the plausibility of this hypothesis.

When anti-GalC sera were added without complement to explant cultures of cerebellum or spinal cord prior to myelin formation, normal myelination was inhibited (8,9). Many OL differentiated, but the extensive myelin-like membrane was only rarely associated with axons (44). Removal of the antiserum led to fairly prompt, albeit often reduced, myelination. Ranscht et al. (45) used an anti-GalC to reversibly block Schwann cell myelination of sensory axons in culture (2). Although ensheathment of small axons and basal lamina formation were unaffected, membrane elongation and P0 and MBP expression were inhibited. Dyer and Benjamins (12,13) demonstrated that mature OL in culture respond to anti-GalC treatment by rapidly internalizing the membrane-bound antibodies and reorganizing surface GalC into patches. Long-term exposure of OL to anti-GalC caused extensive contraction of membrane sheets with accompanying reorganization of the underlying cytoskeleton.

We have studied the effects of an anti-glycolipid (R-mAb) described by Ranscht et al. (46) on the development of oligodendrocyte progenitors growing in culture. R-mAb was originally described as having a primary specificity for GalC, with a 16-fold lower crossreactivity with sulfatide. While it does react with GalC and the structurally related galactosyldiacylglycerol, recent experiments in our laboratory have demonstrated that under a variety of conditions the crossreaction with sulfatide is much stronger than previously reported, generally equalling the reaction with GalC (3).

In the presence of R-mAb only a basal level of weakly stained R-mAb<sup>+</sup> cells present in the starting population is maintained and MBP<sup>+</sup> cells do not appear (Fig. 6 A, B). A significant reduction in the appearance of R-mAb<sup>+</sup> and MBP<sup>+</sup> cells occurs at antibody dilutions as low as ~2 µg/ml total IgG<sub>3</sub>. In contrast, the number of O4<sup>+</sup> cells is not affected by R-mAb treatment, indicating that differentiation and entry of O-2A precursors into the oligodendrocyte pathway, and O4<sup>+</sup> cell viability, are not inhibited.

Biochemical studies demonstrate that R-mAb treatment inhibits [<sup>3</sup>H]galactose incorporation into GalC and sulfatide, and the development of CNP activity, MBP

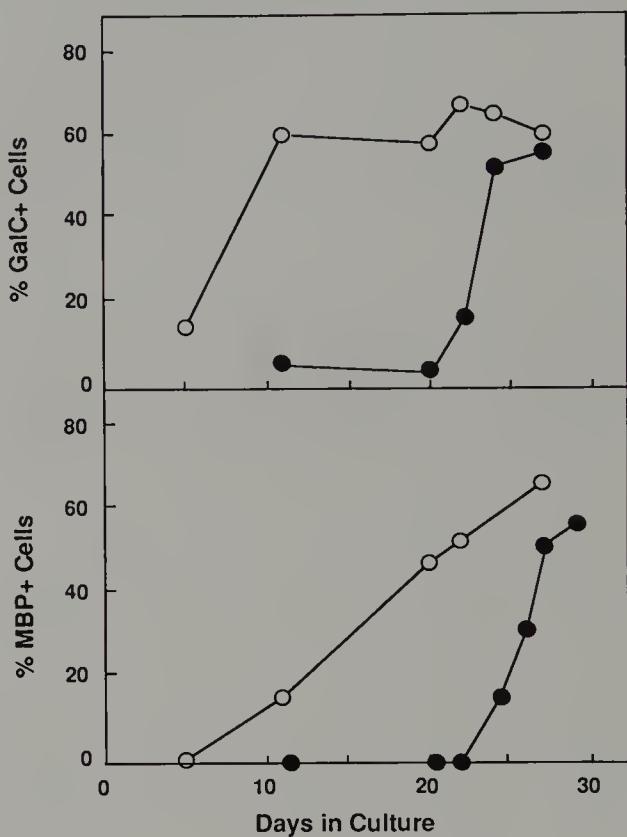
RNA levels, and MBP protein content compared with untreated control cultures (Table 1). Therefore, the block in MBP expression appears to affect either transcription or message stability. In contrast, the levels of total protein and astrocytic glial fibrillary acidic protein in antibody-treated cultures are nearly normal. The point in the OL lineage at which the inhibition occurs must be restricted to the period after the appearance of the O4<sup>+</sup>GalC<sup>-</sup> progenitor through the onset of GalC expression (Fig. 1). Antibody-blocked cells have a distinctive morphology with sparsely branched processes instead of the typically branched process network of mature OL (Fig. 7). Approximately 90% of the blocked cells express vimentin-containing intermediate filaments. Thus, the R-mAb-blocked cells retain these characteristics of the O4<sup>+</sup>GalC<sup>-</sup> progenitor cells in brain (18,19).

Upon removal of the antibody, the cells rapidly resume the normal, temporal sequence of differentiation, reaching control levels of differentiated expression (Fig. 6 A, B). That is, the expression of GalC occurred first, followed only then by the expression of MBP; that is, the normal, characteristic lag between the temporal development of R-mAb and MBP expression is maintained. Therefore, the block must either occur before or prevent the activation of an important regulatory event leading to the terminal differentiation sequence. Upon release, previously blocked cells differentiate into OL more synchronously than control cultures. The asynchrony of OL development is maintained in primary cultures of dissociated fetal brain regions. Since the number of O4<sup>+</sup> cells increases normally during a prolonged treatment of mixed primary cultures with the blocking antibody, cells developmentally younger than those at the block point must continue to progress and accumulate at the point of inhibition, apparently at or just after the GalC<sup>-</sup>/GalC<sup>+</sup> interface. The reversion must be due to recovery of the blocked cells themselves, rather than repopulation by more immature progenitors still flowing into the developing population, since similar results were obtained in experiments (see ref.

TABLE 1. Biochemical analyses of mixed primary cultures treated with R-mAb

Addition	% of Control		
	GalC	MBP	GFAP
None	94	75	90
R-mAb 5–20 DIC	3	1	94
R-mAb 5–20 DIC, then removed 20–29 DIC	97	91	79

Cultures initiated from 19–21 day fetal rat telencephalon were grown in normal medium, or in medium supplemented with R-mAb at 1/40 dilution from 5–20 DIC, and analyzed at 20 DIC, or grown for an additional 9 days in fresh medium lacking R-mAb to study the reversibility of the antibody-induced block. Cultures were then harvested and analyzed for various markers of myelinogenesis and compared with the levels in untreated, 20 DIC control cultures, set at 100%.



**FIG. 6.** Reversible inhibition of myelinogenesis in culture by R-mAb. Mixed primary cultures were initiated from 19–21 day fetal rat telencephala. Antibody was added to one set of cultures 5 days after plating and removed at 20 days. Cultures were sampled at the times shown and assayed for developmental expression of myelinogenic markers. The data are presented as the percent of O4<sup>+</sup> cells that were also stained in double-label immunofluorescence microscopy with an antibody against the indicated differentiation antigen. ○, untreated control cultures; ●, cultures treated with R-mAb-treated cultures from 5–20 DiC, and then grown further in antibody-free medium until 28 DIC.

2) in which earlier stages of the OL lineage were removed by using enriched O4<sup>+</sup>GalC<sup>-</sup> progenitors (above).

Several other antibodies that bind to surface antigens of OL and/or their progenitors, including anti-N-CAM (5), HNK-1 (see below), O1 (53), 1A9 (17), and anti-cholesterol (49) are all ineffective at blocking differentiation, as are non-specific purified mouse myeloma control IgG<sub>3</sub>, and proteins concentrated from culture medium used for growing R-mAb-producing hybridoma cells. Since O1 mAb, which reacts specifically with GalC (3,51), did not inhibit OL differentia-

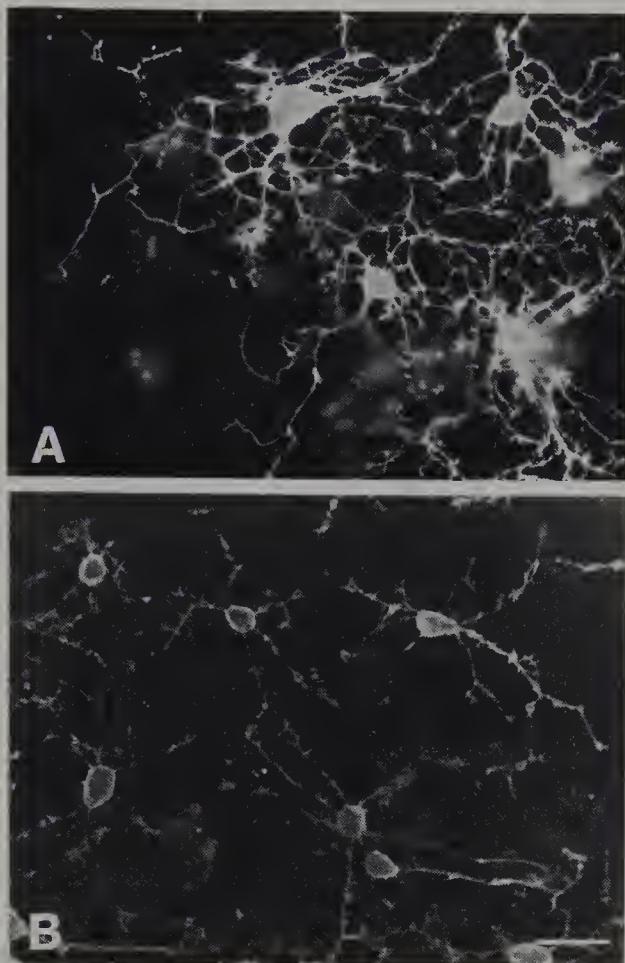


FIG. 7. Immunofluorescence photomicrograph of  $O4^+$  cells in mixed primary cultures after 12 DIC in (A) untreated control cultures, or (B) cultures treated with R-mAb from 3–12 DIC. Bar, 25  $\mu\text{m}$ .

tion, the target molecule could be sulfatide. The absence of inhibition by antibody HNK-1 makes it unlikely that molecules with the HNK-1 epitope, such as myelin associated glycoprotein, fibronectin receptor, cytотactин, or sulfoglucuronylglycolipid (20,24,36) are the target antigens.

The reversible antibody-induced inhibition of myelinogenesis at a specific progenitor stage of OL development offers interesting experimental possibilities for studying the environmental regulation of these cells. In addition, it has important implications for demyelinating diseases such as multiple sclerosis (14), in which a similar inhibition would impair remyelination by populations of developmentally quiescent “adult” progenitor cells (59).

## ACKNOWLEDGMENTS

This work was supported by fellowship NS07720 (A.L.G.) and grant NS10861 (S.E.P.) from NINCDS.

## SUMMARY

Myelinogenesis is the sum of developmental events leading to the formation of the myelin sheath. It includes the commitment, proliferation, and differentiation of myelinogenic precursor cells; the synthesis, transport, and assembly of myelin membrane structural components; the interaction of oligodendrocyte processes with neurons; the formation of compacted, multilamellar myelin sheaths, and the maintenance of the finished product. At each of these steps there is evidence for environmental regulation. In this chapter, some of the evidence for extrinsic control of myelinogenesis is reviewed, followed by a description of some of our recent observations regarding the development of isolated oligodendrocyte progenitors and the reversible, stage-specific inhibition of this development by antibody perturbation.

## REFERENCES

1. Bansal R, Gard AL, Pfeiffer SE. Stimulation of oligodendrocyte differentiation in culture by growth in the presence of a monoclonal antibody to sulfated glycolipid. *J. Neurosci. Res.* 1988;21:260–267.
2. Bansal R, Pfeiffer SE. Reversible inhibition of oligodendrocyte progenitor differentiation by a monoclonal antibody against surface galactolipids. *Proc. Natl. Acad. Sci. USA* 1989;86:6181–6185.
3. Bansal R, Warrington A, Gard AL, Ranscht B, Pfeiffer SE. Multiple and novel specificities of monoclonal antibodies O1, O4 and R-mAb used in the analysis of oligodendrocyte development. *J. Neurosci. Res.* 1989;24:548–557.
4. Barbin G, Manthrope M, Varon S. Purification of the chick eye ciliary neuronotrophic factor. *J. Neurochem.* 1984;43:1468–1478.
5. Bhat S, Silberberg D. Rat oligodendrocytes have cell adhesion molecules. *Dev. Brain Res.* 1985;19:139–145.
6. Bologa L, Aizenman Y, Chiappelli F, deVellis J. Regulation of myelin basic protein in oligodendrocytes by a soluble neuronal factor. *J. Neurosci. Res.* 1986;15:521–528.
7. Curtis R, Cohen J, Fok-Seang J, Hanley MR, Gregson NA, Reynolds R, Wilkin GP. Development of macroglial cells in rat cerebellum. I. Use of antibodies to follow early *in vivo* development and migration of oligodendrocytes. *J. Neurocytol.* 1988;17:43–54.
8. Diaz M, Bornstein MB, Raine CS. Disorganization of myelinogenesis in tissue culture by anti-CNS antiserum. *Brain Res.* 1978;154:231–239.

9. Dorfman SH, Fry JM, Silberberg DH. Antiserum induced myelination inhibition *in vitro* without complement. *Brain Res.* 1979;177:105–114.
10. Dubois-Dalcq M. Characterization of a slowly proliferative cell along the oligodendrocyte differentiation pathway. *EMBO J.* 1987;6:2587–2595.
11. Dubois-Dalcq M, Behar T, Hudson L, Lazzarini RA. Emergence of three myelin proteins in oligodendrocyte cultures without neurons. *J. Cell Biol.* 1986;102:384–392.
12. Dyer CA, Benjamins JA. Redistribution and internalization of antibodies to galactocerebroside by oligodendroglia. *J. Neurosci.* 1988a;8:883–891.
13. Dyer CA, Benjamins JA. Antibody to galactocerebroside alters organization of oligodendroglial membrane sheets in culture. *J. Neurosci.* 1988b;8:4307–4318.
14. Elias SB. Oligodendrocyte development and the natural history of multiple sclerosis. *Arch. Neurol.* 1987;44:1294–1299.
15. French-Constant C, Raff MC. Proliferating bipotential glial progenitor cells in adult rat optic nerve. *Nature* 1986;319:499–502.
16. Friedrich, Jr. VL, Sternberger NH. The “lacy” oligodendrocyte: an immature form revealed by immunocytochemical staining. *Anat. Rec.* 1983;205:58A-59A.
17. Gard AL, Dutton GR. Myelin-specific domain on the plasmalemma of oligodendroglia: differential expression in the rat and hypomyelinating mouse mutants *jimpy* and *quaking*. *J. Neurosci. Res.* 1987;17:329–343.
18. Gard AL, Pfeiffer SE. Oligodendrocyte progenitors isolated directly from developing telencephalon at a specific phenotypic stage: myelinogenic potential in a defined environment. *Development* 1989;106:119–132.
19. Gard AL, Bansal R, Pfeiffer SE. Developmental phenotype of late-stage oligodendrocyte progenitors *in vivo*. *Trans. Amer. Soc. Neurochem.* 1989;20:248 (abstr.).
20. Grumet M, Hoffman S, Crossin KL, Edelman GM. Cytotactin, an extracellular matrix protein of neural and non-neuronal tissues that mediates glia-neuron interaction. *Proc. Natl. Acad. Sci. USA* 1985;82:8075–8079.
21. Hughes SM, Lillien LE, Raff MC, Rohrer H, Sendtner M. Ciliary neurotrophic factor induces type-2 astrocyte differentiation in culture. *Nature* 1988;335:70–73.
22. Imamoto K, Paterson JA, Leblond CP. Radioautographic investigation of gliogenesis in the corpus callosum of young rats. I. Sequential changes in oligodendrocytes. *J. Comp. Neurol.* 1978;180:115–138.
23. Keilhauer G, Meier DH, Kuhlmann-Krieg S, Nieke J, Schachner M. Astrocytes support incomplete differentiation of an oligodendrocyte precursor cell. *EMBO J.* 1985;4:2499–2504.
24. Künemund V, Jungalwala FB, Fisher G, Chou DKH, Keilhauer G, Schachner M. The L2/HNK-1 carbohydrate of neural cell adhesion molecules is involved in cell interactions. *J. Cell Biol.* 1988;106:213–223.
25. Levi G, Aliosi F, Wilkin GP. Differentiation of cerebellar bipotential glial precursors into oligodendrocytes in primary culture: developmental profile of surface antigens and mitotic activity. *J. Neurosci. Res.* 1987;18:407–417.
26. LeVine SM, Goldman JE. Embryonic divergence of oligodendrocyte and astrocyte lineages in developing rat cerebrum. *J. Neurosci.* 1988;8:3992–4006.
27. LeVine SM, Goldman JE. Ultrastructural characteristics of G<sub>D3</sub> ganglioside-positive immature glia in rat forebrain white matter. *J. Comp. Neurol.* 1988;277:456–464.
28. Lillien LE, Sendtner M, Rohrer H, Hughes SM, Raff MC. Type-2 astrocyte development in rat brain culture is initiated by a CNTF-like protein produced by Type-1 astrocytes. *Neuron*. 1988;1:485–494.

29. Macklin WB, Braun PE, Lees MB. Electroblot analysis of the myelin proteolipid protein. *J. Neurosci. Res.* 1982;7:1–10.
30. McMorris FA, Dubois-Dalcq M. Insulin-like growth factor I promotes cell proliferation and oligodendroglial commitment in rat glial progenitor cells developing *in vitro*. *J. Neurosci. Res.* 1988;21:199–209.
31. McMorris FA, Smith TM, DeSalvo S, Furlanetto R. Insulin-like growth factor I/somatomedin C: a potent inducer of oligodendrocyte development. *Proc. Natl. Acad. Sci. USA* 1986;83:822–826.
32. Mirsky R, Winter J, Abney ER, Pruss RM, Gavrilovic J, Raff MC. Myelin-specific proteins and galactolipids in rat Schwann cells and oligodendrocytes in culture. *J. Cell Biol.* 1980;84:483–494.
33. Morell, P., ed. *Myelin*. New York: Plenum Press, 1984.
34. Mori S, Leblond CP. Electron microscopic identification of three classes of oligodendrocytes and a preliminary study of their proliferative activity in the corpus callosum of young rats. *J. Comp. Neurol.* 1970;139:1–30.
35. Noble M, Murray K, Stroobant P, Waterfield MD, Riddle P. Platelet-derived growth factor promotes division and motility and inhibits premature differentiation of the oligodendrocyte/type-2 astrocyte progenitor cell. *Nature* 1988;333:560–562.
36. Pesheva P, Juliano RL, Schachner M. Expression and localization of the fibronectin receptor in the mouse nervous system. *J. Neurosci. Res.* 1988;20:420–430.
37. Pfeiffer SE. Oligodendrocyte development in culture systems. In: Norton WT, ed. *Oligodendroglia, Advances in Neurochemistry*, vol. 5, New York/London: Plenum Press, 1984;233–298.
38. Pfeiffer SE, Gard AL. Biochemical, immunological, and molecular cell-type specific markers of the central nervous system. In: Kornblith PL, Walker MD, eds. *Advances in Neurooncology* Mount Kisco, NY: Futura, 1988;3–40.
39. Privat A. Postnatal gliogenesis in the mammalian brain. *Int. Rev. Cytol.* 1975;40:281–323.
40. Raff MC, Lillien LE, Richardson WD, Burne JF, Noble M. Platelet-derived growth factor from astrocytes drives the clock that times oligodendrocyte development in culture. *Nature* 1988;333:562–565.
41. Raff MC, Miller RH, Noble MD. A glial progenitor cell that develops *in vitro* into an astrocyte or an oligodendrocyte depending on the culture medium. *Nature* 1983;303:390–396.
42. Raff MC, Mirsky R, Fields KL, Lisak RP, Dorfman SH, Silberberg DH, Gregson NA, Liebowitz S, Kennedy MC. Galactocerebroside is a specific cell surface antigenic marker for oligodendrocytes in culture. *Nature* 1978;274:813–816.
43. Raff MC, Williams BP, Miller RH. The *in vitro* differentiation potential of a bipotential progenitor cell. *EMBO J.* 1984;3:1857–1864.
44. Raine CS, Diaz M, Pakizingan M, Bornstein MB. Antiserum-induced dissociation of myelinogenesis *in vitro*. An ultrastructural study. *Lab. Invest.* 1978;38:397–403.
45. Ranscht B, Wood PM, Bunge RP. Inhibition of *in vitro* peripheral myelin formation by monoclonal anti-galactocerebroside. *J. Neurosci.* 1987;7:2936–2947.
46. Ranscht B, Clapshaw PA, Noble M, Seifert W. Development of oligodendrocytes and Schwann cells studied with a monoclonal antibody against galactocerebroside. *Proc. Natl. Acad. Sci. USA* 1982;79:2709–2713.
47. Reynolds R, Wilkin GP. Development of macroglial cells in rat cerebellum II. An *in*

- situ* immunohistochemical study of oligodendroglial lineage from precursor to mature myelinating cell. *Development* 1988;102:409–425.
48. Richardson WD, Pringle N, Mosley MJ, Westermark B, Dubois-Dalcq M. A role for platelet-derived growth factor in normal gliogenesis in the central nervous system. *Cell* 1988;53:309–319.
49. Swartz Jr. GM, Gentry MK, Amendel LM, Blanchette-Makie EJ, Alving CR. Antibodies to cholesterol. *Proc. Natl. Acad. Sci. USA* 1988;85:1902–1906.
50. Skoff RP, Price DL, Stocks A. Electron microscopic autoradiographic studies of gliogenesis in rat optic nerve. II. Time of origin. *J. Comp. Neurol.* 1976;169:313–334.
51. Singh H, Pfeiffer SE. Myelin-associated galactolipids in primary cultures from dissociated fetal rat brain: biosynthesis, accumulation and cell surface expression. *J. Neurochem.* 1985;45:1371–1381.
52. Small RK, Riddle P, Noble M. Evidence for migration of oligodendrocyte-type-2 astrocyte progenitor cells into the developing rat optic nerve. *Nature* 1987;328:155–157.
53. Sommer I, Schachner M. Monoclonal antibodies (O1 to O4) to oligodendrocyte cell surfaces: an immunocytochemical study in the central nervous system. *Dev. Biol.* 1981;83:311–327.
54. Sommer I, Schachner M. Cells that are O4 antigen-positive and O1 antigen-negative differentiate into O1 antigen-positive oligodendrocytes. *Neurosci. Lett.* 1982;29:183–188.
55. Sternberger NH, Itoyama Y, Kies MW, Webster HdeF. Myelin basic protein demonstrated immunocytochemically in oligodendroglia prior to myelin sheath formation. *Proc. Natl. Acad. Sci. USA* 1978;75:2521–2524.
56. Temple S, Raff MC. Clonal analysis of oligodendrocyte development in culture: evidence for a developmental clock that counts cell divisions. *Cell* 1986;44:773–779.
57. Trotter J, Schachner M. Cells positive for the O4 surface antigen isolated by cell sorting are able to differentiate into astrocytes or oligodendrocytes. *Devel. Brain Res.* 1989;46:115–122.
58. van der Pal RHM, Koper JW, van Golde LMG, Lopes-Cardozo M. Effects of insulin and insulin-like growth factor (IGF-I) on oligodendrocyte-enriched glial cultures. *J. Neurosci. Res.* 1988;19:483–490.
59. Wolswijk G, Nobel M. Identification of an adult-specific glial progenitor cell. *Development* 1989;105:387–400.



# Synaptic Plasticity in the Adult Sacral Spinal Cord: Effects of Lesions and Hormones

Michael S. Beattie<sup>1,2,3</sup>, Jacqueline C. Bresnahan<sup>2,3</sup>  
and M. Gail Leedy<sup>2</sup>

<sup>1</sup> Division of Neurosurgery, <sup>2</sup> Department of Anatomy and <sup>3</sup> Neuroscience Program  
The Ohio State University College of Medicine  
Columbus, OH 43210

**W**ork from a number of laboratories over the past 20 years has demonstrated that, contrary to earlier opinion, synaptic plasticity can occur in the adult central nervous system (CNS) in response to lesions (6). "Synaptic plasticity" here refers to changes in the organization or number, or replacement of lost synapses, as defined by morphological studies. Synaptic replacement after destruction of afferent pathways has been particularly well-documented in rostral CNS systems, for example, the hippocampus (6) and septal region (32); and Cotman (6) and others (e.g., 11) have suggested that indeed, synaptic turnover may be a normal, ongoing process that reflects the continuation into adulthood of some of the developmental capacity for nervous system growth. This concept is strengthened by recent findings which suggest that synaptic turnover and growth may be affected in hypothalamic nuclei responsive to circulating hormones or osmotic stress (11,15). The role of neuronal, glial, and hormonal trophic factors in synaptic plasticity is the subject of recent discussions (e.g., 28).

The occurrence of synaptic reorganization in the spinal cord after lesions, however, has been a matter of some controversy (see, e.g., 31), and the potential role of synaptic growth and replacement in mediating functional changes after spinal cord damage has been questioned (but see 10). There has been some evidence that "humoral" factors may mediate rapid changes in spinal motoneuron excitability after spinal cord transection (see 26).

Hormonal alterations have been shown to affect sacral spinal reflexes in the adult, as well as neonatal rat (3,4). In the experiments described here, we have used quantitative electron microscopic techniques to examine the possibility that changes in synaptic inputs to identified sacral spinal cord efferent neurons might occur after spinal cord transection in the cat, and after testosterone deprivation and replacement in the rat. The results suggest that synaptic reorganization does occur in some, but perhaps not all, sacral spinal systems after lesions, and that

hormone levels can affect the number of synapses contributing to activation of sacral motoneurons innervating muscles involved in sexual reflexes.

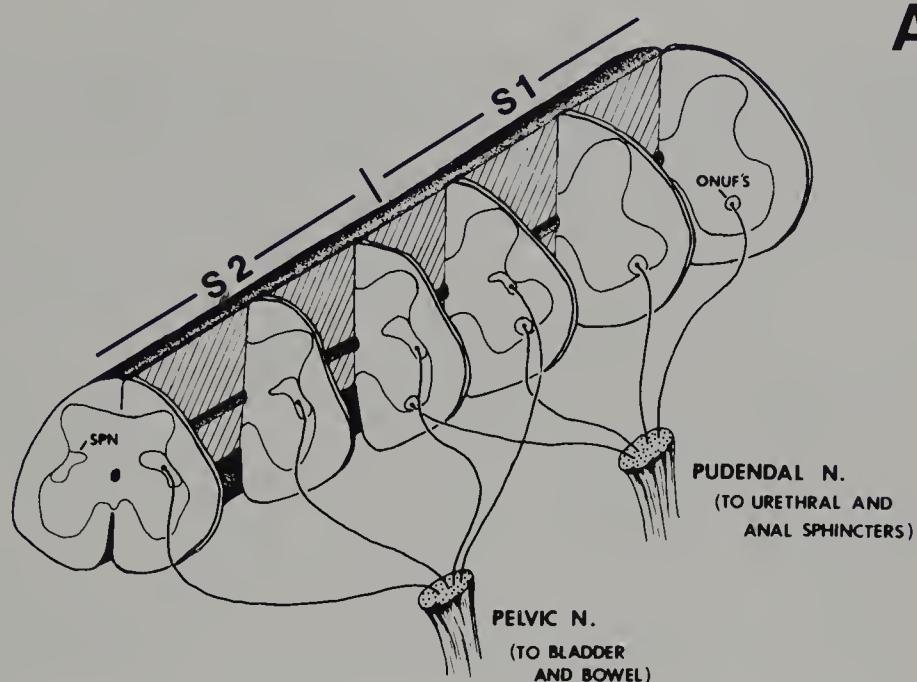
## **ORGANIZATION OF SACRAL MICTURITION AND DEFECATION REFLEXES IN THE CAT**

The efferent neurons controlling micturition and defecation are located within spinal segments S1 and S2 in the cat (Fig. 1A). Parasympathetic preganglionic neurons innervating the postganglionic neurons of the bladder and colon are located in the sacral parasympathetic nucleus (SPN), which can be divided, in the cat, into a dorsal band subdivision innervating the colon, and a lateral band innervating the bladder (20,27). The motoneurons innervating the external striated anal and urethral sphincters are located in the homologue to Onuf's (29) nucleus (33). The rostral-caudal extents of these two groups of neurons overlap somewhat, and the dendritic arbors of Onuf's nucleus motoneurons extend into regions occupied by the preganglionic efferent neurons (21,24). Normal elimination reflexes depend on the integration of somatic and visceral outflow. Effective micturition and defecation require the coordination of these two efferent populations; e.g., micturition depends upon the simultaneous activation of bladder contraction and relaxation of the external (and internal) urethral sphincters. The organization of elimination reflexes is quite complex, and involves rostral control and sympathetic outflow (via the hypogastric nerve) as well as parasympathetic and somatic components. For additional information, the reader is referred to the reviews provided by de Groat and his colleagues (7,8). Our focus will be on the efferent elements supplying peripheral innervation via the pudendal and pelvic nerves.

## **EFFECTS OF SPINAL CORD TRANSECTION ON ELIMINATION REFLEXES AND SYNAPTIC INPUTS TO EFFERENT NEURONS**

One of the common consequences of spinal cord lesions is the development of spastic bladder and/or bladder-sphincter dys-synergy (30). It might be noted here that the first report of putative axonal sprouting in the mammalian spinal cord suggested that such sprouting might be the cause of limb spasticity after spinal cord damage (22). Spinal cord transection also results in the development of a "somato-vesicle" reflex not present in the normal adult (see 36), although extant in the neonatal cat. de Groat and co-workers (35) have postulated that this new (or re-emergent) reflex may be due to the sprouting of dorsal root afferents after transection.

Our strategy for gathering morphological evidence for or against synaptic plasticity in the circuits mediating eliminative reflexes has been to focus our analysis on the elements that constitute the reflex final common pathways, i.e., the somatic motoneurons innervating the sphincters, and the preganglionic neurons innervating the bladder and colon. This approach has distinct advantages in terms of



NORMAL (n=6)

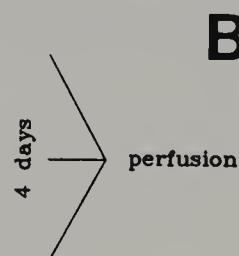
- pudendal n.  
label

ACUTE TX (n=6)

- cord transection  
and pudendal n.  
label

CHRONIC TX (n=6)

- pudendal n.  
label



NORMAL (n=6)

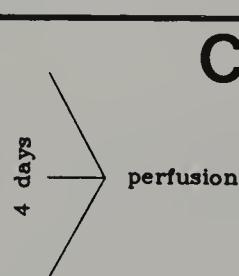
- pelvic n.  
label

ACUTE TX (n=6)

- cord transection  
and pelvic n.  
label

CHRONIC TX (n=6)

- pelvic n.  
label



**FIG. 1.** **A:** A schematic diagram of the sacral spinal cord segments containing the parasympathetic preganglionic neurons in the sacral parasympathetic nucleus (SPN) innervating the bladder and bowel, and the motoneurons in Onuf's nucleus innervating the external urethral and anal sphincters. **B, C:** The experimental design for groups used to evaluate the effect of spinal cord transection (TX) on synaptic input to Onuf's nucleus motoneurons (**B**) and sacral parasympathetic preganglionic neurons (**C**).

limiting the sampling problems associated with quantitative electron microscopic (EM) studies, that is, we look only at synapses onto neurons known to be relevant to the reflexes in question. There are some disadvantages to this approach, however. First, synaptic reorganization of inputs to interneurons involved in the reflexes are not measured. Second, we can only unequivocally identify the somata and proximal dendrites of projection neurons; plasticity of inputs to the distal dendrites will not be detected. Third, we have, to date, utilized only EM morphological criteria to determine synaptic types; we do not yet know the source of the synapses we are measuring. Nevertheless, we can address a very specific hypothesis: spinal cord transection results in denervation of reflex final common pathway neurons, and reinnervation by segmental afferents.

Figure 1 (B and C) shows the experimental design for our studies on cat sacral reflex circuits. Horseradish peroxidase was applied to the pudendal (or pelvic) nerves of normal cats, cats with an acute (four days) spinal cord transection, or cats with chronic (10–11 weeks) lesions. Using techniques for sequential light microscopy (LM) and EM developed in our laboratory (1,20,24), we can identify labeled projection neurons in the Onuf's nucleus and SPN, measure and characterize the neurons at the LM level, then take thin sections through identified neurons and quantitate the synaptic inputs. Large electron micrographic photomontages are constructed, and all elements in apposition to the membranes of somata and attached proximal dendrites are classified and measured at 22,500X magnification (see Fig. 2A). Classifications include other somata and dendritic profiles, axons, glia, and synaptic contacts containing either round, pleomorphic, or flat synaptic vesicles (see 17,20,24). The presence and number of dense-cored vesicles are also noted, and the presence and length of morphologically defined active zones are also determined.

Cats with chronic spinal cord transection displayed the typical course of reflex changes. Immediately after transection, eliminative reflexes were absent. Over the course of the first two weeks, tactile stimulation of the perineal region came to elicit defecation and weak micturition in most animals. By 10 weeks all animals released feces and at least some urine in response to perineal stimulation, and most exhibited effective voiding. All animals also exhibited varying degrees of apparent sphincter-bladder dys-synergia.

Evidence for acute denervation of SPN and Onuf's neurons was found at four days after spinal cord transection. Figure 2B shows a photomicrograph and Fig. 3A shows a plot of dark, osmophilic fibers and swellings seen in the SPN in a thick plastic section processed for EM. Similar putative degeneration products were seen at four days in Onuf's nucleus. Using EM, we also observed several examples of degenerating terminals in the neuropil of these nuclei, some of which were apposed to identified SPN and Onuf's nucleus neurons (5,19). Although it is difficult to extrapolate from the degeneration seen at one time point to total amount of synaptic degeneration caused by the lesion over time, these observations at least support the proposition that spinal transection produces synaptic removal of some elements from the somata and proximal dendrites of the neurons analyzed for synaptic reorganization.

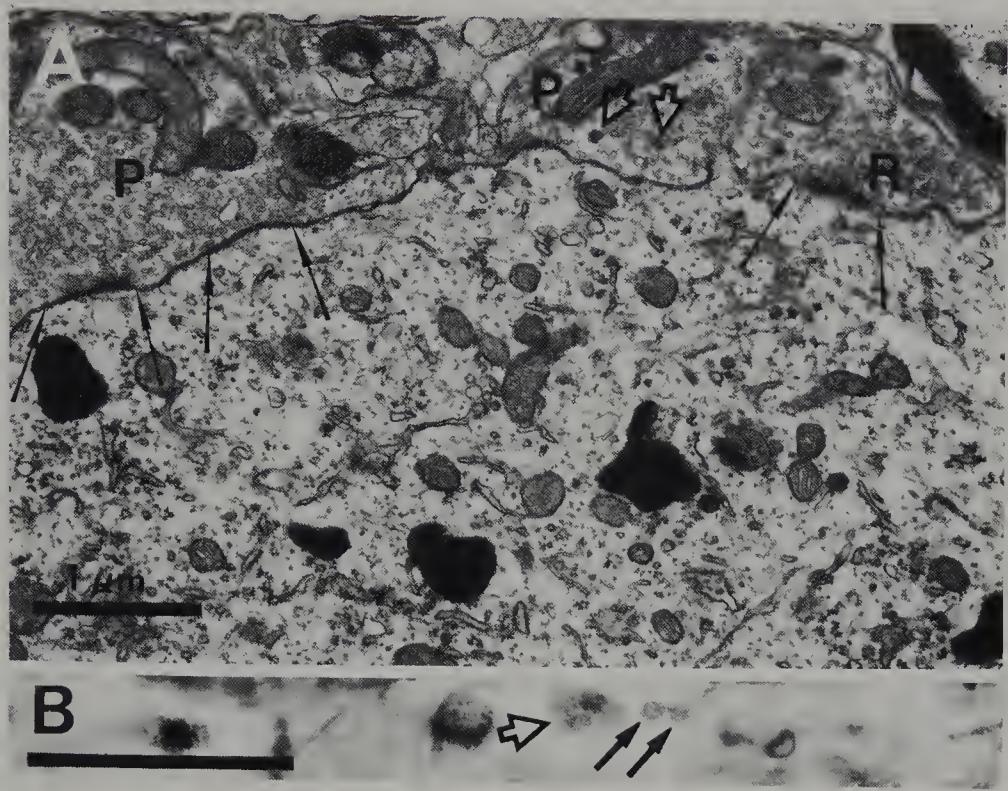
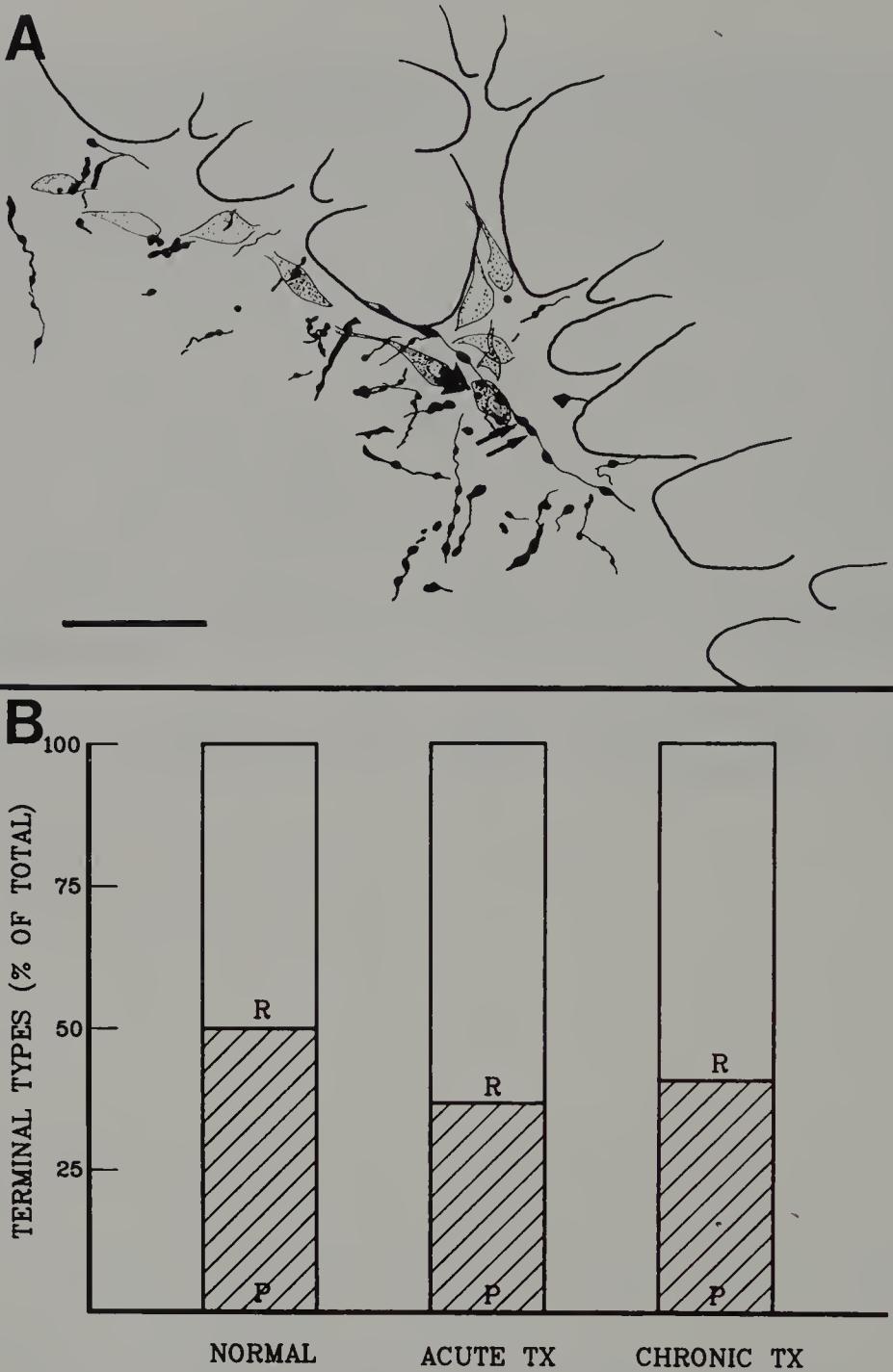


FIG. 2. A: Electron micrograph showing synaptic inputs onto an identified Onuf's nucleus motoneuron. Synaptic active sites are indicated by arrows; terminals containing pleiomorphic (P), and round (R) synaptic vesicles are marked as are dense cored vesicles (*block arrows*). B: A dark osmiophilic fiber with swellings is shown (*arrows*); an adjacent swelling overlies a labeled cell body (*block arrow*). The same field is illustrated in Fig. 3A. Bar, 50  $\mu$ m; plastic section.

Evidence for synaptic reorganization of inputs to Onuf's nucleus motoneurons comes from the following observations: 1) Despite the qualitative evidence for acute denervation, there was no difference in the number of synapses counted on somatic or dendritic membranes, or the proportional membrane length occupied by synapses across the three groups (Table 1); 2) There was a rapid decrease in the mean size of synaptic profiles in apposition to identified neurons, and this effect was more apparent on proximal dendrites (Table 1); 3) Active site length and proportion of membrane in apposition to active sites decreased at four days, with some evidence for recovery on proximal dendrites in the chronic animals (Table 1); 4) The proportion of the synaptic terminal population classified as containing "round" synaptic vesicles increased in both the acute and chronic transection groups (Fig. 3B).

The lack of change in synaptic coverage and number could mean that no sig-



**FIG. 3. A:** A plot of putative degenerating fibers and terminals in the SPN. The swellings indicated in Fig. 2B are comparably indicated in this figure. **B:** Bar graphs showing a significant increase in the proportion of terminals containing round (R) vs pleomorphic (P) synaptic vesicles which contact Onuf's nucleus motoneurons after acute or chronic spinal transection (TX) ( $\chi^2 = 16.62$ ,  $p < 0.01$ ).

TABLE 1. *Onuf's nucleus motoneurons*

	Normal	Acute	Chronic
Synapses/100 $\mu\text{m}$			
	(X + s.e.m.)		
Somata	12.6 + 1.2	13.1 + 1.5	13.5 + 1.2
Dendrites	26.1 + 1.1	27.5 + 4.1	24.7 + 3.8
% Membrane with terminals			
Somata	20.5 + 2.6	17.6 + 1.6	19.3 + 1.8
Dendrites	44.8 + 5.4	35.4 + 5.0	35.9 + 4.4
Terminal area ( $\mu\text{m}^2$ )			
Somata	1.04 + 0.11*	0.88 + 0.05	0.94 + 0.11
Dendrites	1.38 + 0.20*	0.95 + 0.07	0.97 + 0.06
Active site length ( $\mu\text{m}$ )			
Somata	0.39 + 0.04§	0.30 + 0.02	0.31 + 0.03
Dendrites	0.40 + 0.05§	0.20 + 0.04	0.28 + 0.05
% Membrane with active sites			
Somata	1.36 + 0.22†	0.97 + 0.16	1.18 + 0.22
Dendrites	3.74 + 0.73†	1.84 + 0.40	2.92 + 0.68

\* Overall ANOVA (somata + dendrites),  $F_{2,92} = 3.60$ ,  $p < 0.03$ , post-hoc Scheffe: Normal > acute = chronic.

† Overall ANOVA (somata + dendrites,  $F_{2,92} = 4.40$ ,  $p < 0.02$ , post-hoc Scheffe: Normal > acute = chronic.

§ Overall ANOVA (somata + dendrites),  $F_{2,92} = 8.45$ ,  $p < 0.0005$ , post-hoc Scheffe: Normal > acute = chronic.

(Normal = no transection, Acute = four days post-transection, Chronic = 10–11 weeks post-transection.)

nificant denervation, and no reorganization, had occurred. However, in the face of evidence for changes in synaptic morphology, particularly vesicle type, it is more likely that denervation did occur, and that lost synapses were replaced, within four days, by a different population of inputs.

In contrast, the same measures taken on SPN preganglionic neurons showed a stepwise decline in the synaptic coverage and in the number of synaptic terminals per unit membrane (Table 2) (18). Further, no differences in the proportions of different synaptic terminal types were found. Glial coverage of neuronal membrane increased (18). While these data do not preclude the presence of synaptic reorganization of the reduced synaptic inputs, they do not provide evidence for sprouting at this level of analysis, and rather, suggest that spinal cord transection produces a chronic relative denervation of the neurons innervating the bladder.

## SYNAPTIC PLASTICITY IN RESPONSE TO HORMONAL CHANGES IN THE RAT

The spinal nucleus of the bulbocavernosus (SNB) is a sexually dimorphic group of motoneurons in the lumbar spinal cord of the rat that innervates the bulbocavernosus (BC), levator ani, and external anal sphincter muscles (25,34). The

TABLE 2. SPN preganglionic neurons

	Normal	Acute	Chronic
Synapses/100 $\mu\text{m}^*$	(Mean + s.e.m.)		
Somata	10.64 + 1.18	11.93 + 1.12	9.26 + 2.00
Dendrites	18.87 + 4.38	19.30 + 3.25	11.22 + 1.94
% membrane with terminals†			
Somata	16.37 + 1.93	17.31 + 1.81	11.66 + 2.79
Dendrites	28.03 + 4.79	28.89 + 4.62	15.46 + 2.66

\* Overall ANOVA (somata + dendrites),  $F_{2,105} = 2.91$ ,  $p < 0.059$ .

† Overall ANOVA (somata + dendrites),  $F_{2,105} = 5.70$ ,  $p < 0.005$ , post-hoc Scheffe: Normal = Acute > Chronic,  $p < 0.005$ .

(Normal = no transection, Acute = four days post-transection, Chronic = 10–11 weeks post-transection.)

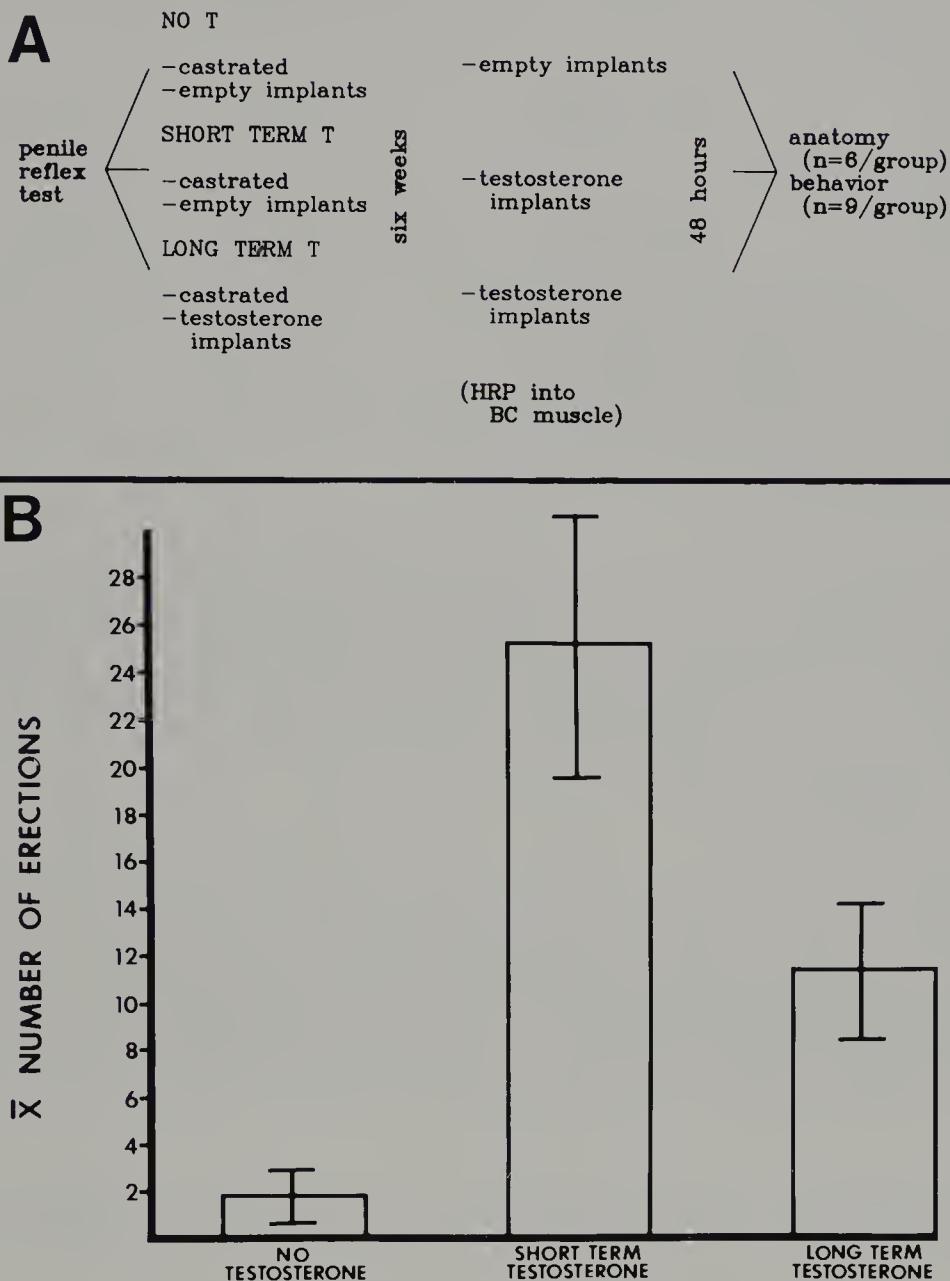
BC is involved in penile erection (13). These motoneurons are homologous to those in Onuf's nucleus in the cat in that they innervate perineal musculature and share a number of organization features, including dendritic bundling and cell-cell junctional specializations (see 19,23). The SNB has received considerable attention since the discovery that its development is dependent upon androgens (3). Testosterone deprivation (by castration) in adult males also affects SNB motoneurons: Chronic deprivation results in reduced cell size and dendritic arbor shrinkage (16). Seasonal variations in testosterone also affect SNB motoneuron dendrites (9).

Castration and testosterone deprivation also result in the virtual elimination of normal penile reflexes (12,14). These reflexes can be reinstated, however, by exogenous testosterone, and the reflex return is rapid (within 48 hr) (14).

Sequential light and electron microscopic quantitative analysis was used to determine whether testosterone deprivation affects synaptic inputs to SNB motoneurons as well as cell size, and to ask whether rapid reflex reinstatement with exogenous testosterone is accompanied by changes in synaptic coverage, number, or shifts in terminal types. In these experiments, HRP was injected into the BC muscle in order to unequivocally identify motoneurons projecting to it, and therefore involved in reflex production. The experimental design is shown in Fig. 4A.

As reported by others (2,3), chronic testosterone deprivation resulted in a decrease in SNB cell size (17). Forty-eight hours of replacement therapy reinstated penile reflexes (Fig. 4B), but did not reinstate normal cell size. In addition, we measured nuclear size and nucleolar size using the LM. Nuclear size paralleled cell size, i.e., short-term replacement was not different from chronic deprivation, and continuous replacement maintained nuclear size. Nucleolar size after 48 hr of replacement therapy was significantly reduced in comparison to no testosterone and long-term testosterone groups (19).

Table 3 summarizes the synaptic morphometric measurements for each group. Chronic testosterone deprivation (NO T) substantially reduced both synaptic num-



**FIG. 4.** A: Experimental design for the experiment testing the effects of testosterone (T) on synaptic input to identified bulbocavernosus (BC) motoneurons. B: Behavioral results showing significant group differences ( $F_{2,24} = 10.95$ ,  $p < 0.0005$ ) in reflex activity after the experimental manipulations described in A.

TABLE 3. Rat SNB motoneurons

	No T	Short term T	Long term T
Synapses/100 $\mu\text{m}^*$			
	(Mean + s.e.m.)		
Somata	19.7 + 1.6	22.6 + 2.3	21.9 + 1.9
Dendrites	15.3 + 1.7	20.9 + 1.9	24.5 + 2.4
% membrane with terminals†			
Somata	27.7 + 2.9	31.8 + 3.5	35.3 + 2.7
Dendrites	22.8 + 3.6	35.8 + 4.0	42.0 + 4.3

\* Overall ANOVA (somata + dendrites),  $F_{2,110} = 3.77$ ,  $p < 0.05$ , orthogonal comparisons: LONG TERM T > SHORT TERM T > NO T

† Overall ANOVA (somata + dendrites),  $F_{2,110} = 6.61$ ,  $p < 0.005$ , orthogonal comparisons: LONG TERM T > SHORT TERM T > NO T

(No T, castration for six weeks without testosterone replacement, Short term T, castration for six weeks followed by 48 hr testosterone replacement, Long term T, castration for six weeks with chronic testosterone replacement.)

bers and coverage when compared with LONG TERM T controls. SHORT TERM T replacement was sufficient to increase both measures significantly. Changes in synaptic coverage and numbers were mirrored by glial coverage; i.e., membrane apposed to glial elements was greatest in the NO T group, least in the LONG TERM T group, and intermediate in the SHORT TERM T animals (17). There were no significant differences in the distribution of terminal types (i.e., round, pleomorphic, flat).

The results of this study provide evidence that testosterone levels can mediate both penile reflex activity and the amount of synaptic inputs to the motoneurons providing the final common pathway for that reflex in the adult rat. Further, changes in synaptic input can occur rapidly, before more gross changes in cell and nuclear size. The glial ensheathment of SNB cells is implicated in this response, because when synapses are removed, they are replaced by glial appositions, and glial appositions decrease when apparently new contacts are established.

## SYNAPTIC PLASTICITY, REFLEX PLASTICITY AND RECOVERY OF FUNCTION

The studies reviewed here show that changes in synaptic connections in the adult mammalian spinal cord can occur under conditions in which reflex alterations also occur. They thus provide support for the idea that synaptic changes may mediate, at least in part, reflex alterations in adult mammals. The simple association of morphologic changes with behavior does not, of course, establish a cause-effect relationship. Indeed, without knowledge of the functional classes of synapses involved in the quantitative changes reported here, it is difficult to predict what

behavioral outcome "should" occur. Further, more detailed studies of the reflexes themselves may be necessary to provide correlations between various reflex component changes and changes in synaptic inputs to different elements of the relevant spinal circuits. Nevertheless, the changes reported occur on the somata and proximal dendrites of the efferent neurons directly mediating reflex action. The role of synaptic reorganization may therefore be relatively easy to interpret in behavioral terms when more specific data on synaptic types become available.

Previous studies of synaptic plasticity in the mammalian CNS have distinguished between "heterotypic" and "homotypic" sprouting (e.g., 10). In our studies of lesion-induced reorganization in Onuf's nucleus, it appears that one class of morphologically defined synapses has been replaced by another, suggesting that replacement is not restricted to similar synaptic types, i.e., heterotypic sprouting has occurred. In contrast, in the SPN, removal of synaptic inputs by transection seemed not to be followed by replacement of like or unlike synapses. In the case of hormone-regulated synaptogenesis, our results suggest a fairly uniform process of synaptic removal and replacement (i.e., homotypic sprouting). Together, these results point to the possible presence of both types of sprouting in the mature spinal cord; their expression may be mediated by different mechanisms. The hormonal trophic effects might represent a more generalized effect on regulation of synaptic contacts, whereas lesion-induced plasticity may call into play the effects of degeneration products and grossly altered neural circuitry which in some cases leads to synaptic replacement but in others to chronic loss of synaptic input.

The quantitative techniques used in these studies allow for at least preliminary comparisons between the effects of spinal cord transection on different circuit elements, and between the effects of lesions and hormonal manipulations on similar motoneuron pools. More fine-grained analysis of specific afferent systems contributing to this plasticity should yield insights into the rules and mechanisms controlling reflex alterations and behavioral modification in the adult mammalian spinal cord. Such studies of synaptic plasticity and the role that normally occurring and lesion-induced trophic factors may play in its mediation are also likely to yield strategies for promoting growth which may facilitate recovery of function after CNS lesions.

## SUMMARY

Quantitative electron microscopy (EM) was used to study potential alterations in synaptic inputs to identified efferent neurons in the cat and rat sacral spinal cord in response to lesions and hormone manipulations which alter spinal reflexes. Spinal cord transection in the cat resulted in an apparent reorganization and synaptic replacement on Onuf's nucleus motoneurons. In contrast, preganglionic neurons showed evidence for chronic denervation of proximal dendrites and somata. Deprivation of testosterone in the rat resulted in decreased synaptic inputs onto

motoneurons innervating the bulbocavernosus muscle. Replacement of testosterone resulted in a significant increase in synaptic input within 48 hr. These results suggest that alterations in synaptic inputs to motoneurons may play a role in both humoral and injury-induced changes in spinal reflexes in adult mammals.

## ACKNOWLEDGMENTS

Supported by NS-10165 to M.S.B. and J.C.B. and NS-07747 (NRSA) to M.G.L. The authors thank T. Van Meter and J. Koman for excellent technical assistance and for illustrations.

## REFERENCES

1. Beattie MS, Bresnahan JC, King JS. Ultrastructural identification of dorsal root primary afferent terminals after anterograde injury filling with horseradish peroxidase. *Brain Res.* 1978;153:127-134.
2. Breedlove SM. Hormonal control of the anatomical specificity of motoneuron to muscle innervation in rats. *Science* 1985;227:1357-1359.
3. Breedlove SM, Arnold AP. Sexually dimorphic motor nucleus in the rat lumbar spinal cord: response to adult hormone manipulation, absence in androgen-insensitive rats. *Brain Res.* 1981;225:297-307.
4. Breedlove SM, Arnold AP. Hormonal control of a developing neuromuscular system. II. Sensitive periods for the androgen-induced masculinization of the rat spinal nucleus of the bulbocavernosus. *J. Neurosci.* 1983;3:424-432.
5. Bresnahan JC, Leedy MG, Beattie MS. Effects of spinal cord transection on Onuf's nucleus motoneurons of cats. *Soc. Neurosci. Absts.* 1988;13:165.
6. Cotman CW. Overview. In: Cotman CW, ed. *Synaptic Plasticity*, New York: Guilford Press, 1985;1-12.
7. de Groat WC, Kawatani M, Hisamitsu T, Lowe I, Morgan C, Roppolo J, Booth AM, Nadelhaft I, Kuo D, Thor K. The role of neuropeptides in the sacral autonomic reflex pathways of the cat. *J. Auton. Nerv. Sys.* 1983;7:339-350.
8. de Groat WC, Nadelhaft I, Milne RJ, Booth AM, Morgan C, Thor K. Organization of the sacral parasympathetic reflex pathways of the cat. *J. Auton. Nerv. Sys.* 1981;3:135-160.
9. Forger NG, Breedlove SM. Seasonal variation in mammalian striated muscle mass and motoneuron morphology. *J. Neurobiol.* 1987;18:155-165.
10. Goldberger ME, Murray M. Recovery of function and anatomical plasticity after damage to the adult and neonatal spinal cord. In: Cotman CW, ed. *Synaptic Plasticity*, New York: Guilford Press, 1985;77-110.
11. Gorski RA. Gonadal hormones as putative neurotrophic substances. In: Cotman CW, ed. *Synaptic Plasticity*, New York: Guilford Press, 1985;287-310.
12. Gray GD, Smith ER, Davidson JM. Hormonal regulation of penile erection in castrated male rats. *Physiol. Behav.* 1980;24:463-468.

13. Hart BL, Melese-d'Hospital PY. Penile mechanisms and the role of striated penile muscles in penile reflexes. *Physiol. Behav.* 1983;31:807–813.
14. Hart BL, Wallach SJR, Melese-d'Hospital PY. Differences in responsiveness to testosterone of penile reflexes and copulatory behavior of male rats. *Horm. Behav.* 1983; 17:274–283.
15. Hatton GI. Reversible synapse formation and modulation of cellular relationships in the adult hypothalamus under physiological conditions. In: Cotman CW, ed. *Synaptic Plasticity*, New York: Guilford Press, 1985;373–406.
16. Kurz EM, Sengelaub DR, Arnold AP. Androgens regulate the dendritic length of mammalian motoneurons in adulthood. *Science* 1986;232:395–398.
17. Leedy MG, Beattie MS, Bresnahan JC. Testosterone-induced plasticity of synaptic inputs to adult mammalian motoneurons. *Brain Res.* 1987;424:386–390.
18. Leedy MG, Beattie MS, Bresnahan JC. Effects of spinal cord transection on synaptology of preganglionic neurons in the sacral parasympathetic nucleus of cats. *Soc. Neurosci. Absts.* 1988;14:698.
19. Leedy MG, Bresnahan JC, Beattie MS. Testosterone-dependent ultrastructural alterations in the spinal nucleus of the bulbocavernosus (SNB) in male rats. *Soc. Neurosci. Absts.* 1987;13:166.
20. Leedy MG, Bresnahan JC, Mawe GM, Beattie MS. Differences in synaptic inputs to preganglionic neurons in the dorsal and lateral band subdivisions of the cat sacral parasympathetic nucleus. *J. Comp. Neurol.* 1988;268:84–90.
21. Li Q, Leedy MG, Beattie MS, Bresnahan JC. Cat motoneurons innervating the urethral and anal sphincters have different dendritic arbors. *Soc. Neurosci. Absts.* 1988; 14:337.
22. Liu CM, Chambers WW. Intraspinal sprouting of dorsal root axons. *Arch. Neurol.* 1958;79:46–61.
23. Matsumoto A, Micevych PE, Arnold AP. Androgen regulates synaptic input to motoneurons of adult rat spinal cord. *J. Neurosci.* 1988;8:4168–4176.
24. Mawe GM, Bresnahan JC, Beattie MS. A light and electron microscopic analysis of the sacral parasympathetic nucleus after labelling primary afferent and efferent elements with HRP. *J. Comp. Neurol.* 1986;250:33–57.
25. McKenna KE, Nadelhaft I. The organization of the pudendal nerve in the male and female rat. *J. Comp. Neurol.* 1986;248:532–549.
26. Mendell LM. Modifiability of spinal synapses. *Physiol. Rev.* 1984;64:260–324.
27. Nadelhaft I, de Groat WC, Morgan C. Location and morphology of parasympathetic neurons in the sacral spinal cord of the cat revealed by retrograde axonal transport of horseradish peroxidase. *J. Comp. Neurol.* 1980;193:265–281.
28. Nieto-Sampedro M. Growth factor induction and order of events in CNS repair. In: Stein DG, ed. *Pharmacological Approaches to the Treatment of Brain and Spinal Cord Injury*, New York: Plenum Press, 1988;310–338.
29. Onuf B. On the arrangement and function of the cell groups of the sacral region of the spinal cord in man. *Arch. Neurol. Psychiat.* 1900;3:387–412.
30. Pedersen E. Regulation of the bladder and colon-rectum in patients with spinal lesions. *J. Auton. Nerv. Sys.* 1983;7:329–338.
31. Pubols LM, Sessle BJ, eds. *Effects of Injury on Trigeminal and Spinal Somatosensory Systems*. New York: A. R. Liss, 1987.
32. Raisman G. Synapse formation in the septal nuclei of adult rats. In: Cotman CW, ed. *Synaptic Plasticity*, New York: Guilford Press, 1985;13–38.

33. Sato M, Mizuno N, Konishi A. Localization of motoneurons innervating the perineal muscles: a HRP study in cat. *Brain Res.* 1978;140:149–154.
34. Schroder HD. Organization of the motoneurons innervating the pelvic muscles of the male rat. *J. Comp. Neurol.* 1980;192:567–587.
35. Thor K, Kawatani M, de Groat WC. Plasticity in the reflex pathways to the lower urinary tract of the cat during postnatal development and following spinal cord injury. In: Goldberger ME, Gorio A and Murray A, eds. *Development and Plasticity of the Spinal Cord*, Padova, Italy: Liviana Press, 1986;65–80.
36. Thor KB, Blais DP, de Groat WC. Behavioral analysis of the postnatal development of micturition in kittens. *Dev. Brain Res.* 1989;46:137–144.

## **V. Neuronal Regeneration and Recovery of Function**



# Energy Depletion, Calcium and the Cytoskeleton: A Model for Trophic Intervention

B. T. Stokes, Q. Li, R. A. Altschuld, B. E. Batten and  
D. K. Anderson\*

The Ohio State University College of Medicine, Columbus, OH 43210;

\*University of Cincinnati College of Medicine, Cincinnati, OH 45267

**T**he use of trophic or growth factors to promote regeneration or repair in the injured central nervous system (CNS) presupposes a thorough understanding of the mechanisms by which neurons become injured. The time course and magnitude of this injury process is commonly divided into two principle epochs: acute and secondary. Trophic factors are more likely to provide a protective or nutritive action later in the acute phase simply because plastic reactions of the CNS take time to occur. The trophic effects on secondary degradation on the other hand, are also likely to involve synaptic plasticity and/or frank regeneration of available neural elements. Distinguishing between these possibilities is difficult and will probably require the use of appropriate *in vitro* model systems that can be easily compared with the *in vivo* injury process. It is clear that we have made only limited progress in the past decade in advancing such models and mechanistic explanations of these trophic actions at the cellular level.

This chapter outlines one such approach to a model system in which metabolic injury, calcium homeostasis, and protein metabolism in deenergized cultured spinal neurons are studied in the same population of cells. As such, it provides a way to examine important hypotheses that attempt to relate the process of energy depletion to structural alterations after the ischemic insult when the potential for reversal is likely to be the greatest. In addition, because the mechanisms of action of such factors as gangliosides (see Ledeen, *this symposium*) are likely to be exerted via alterations in calcium metabolism, it also provides a relevant cellular model in which to address these pharmacological issues.

## ENERGY METABOLISM AND CELL INJURY

Injury to neuronal cells is associated with a decline in high energy phosphates, a loss of cation homeostasis, and possibly, an increase in reactive oxygen radicals.

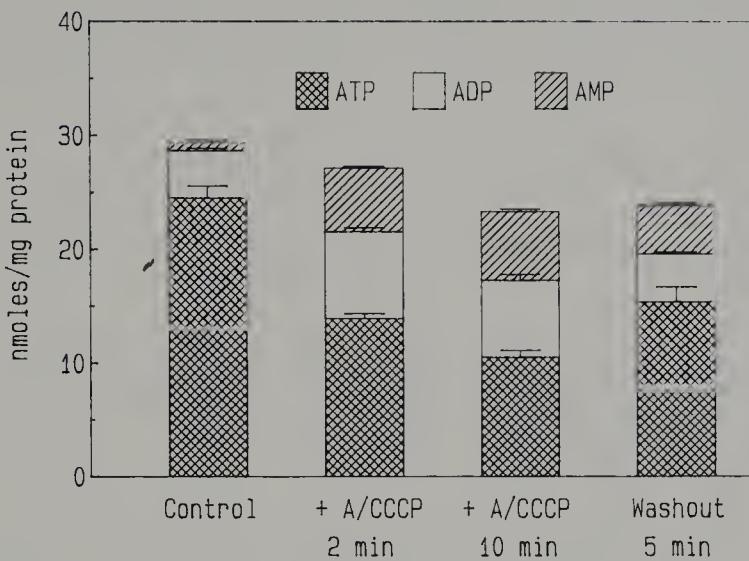
In ischemia, but possibly not hypoglycemia, there is also a decline in cellular pH. The exact linkage between these events and the physiologic and pathologic sequelae of neuronal injury at the cellular level, particularly in the spinal cord, has not been clearly defined (recent reviews of these issues include 4,32,39,40). The pH changes are of some importance because the relative sodium load precipitated by energy depletion will affect membrane  $\text{Na}^+/\text{H}^+$  exchangers and thus indirectly affect operation of  $\text{Na}^+/\text{Ca}^{+2}$  exchanger on the cell membrane. Relative alterations in pH may also affect sodium loading during deenergization.

It is now clear that a variable rate of energy depletion is characteristic of ischemic or traumatically induced spinal cord injury. Within 5 min after cord contusion injury, ATP levels and energy charge have fallen to a fraction of their control values (19,36,37), particularly in the grey matter (38). During spinal ischemia a more gradual decline in these reserves takes place and energy metabolites respond quickly to the restoration of control conditions (2,12). Corresponding alterations in other of the adenine nucleotide metabolites have been examined (32,33) that seem to mimic the known chemistry of these pathways in the cerebrum (31). Interestingly, after electrical shock, adenine nucleotide degradation in the rat brain seems to occur preferentially via adenylate deaminase; but during ischemia, dephosphorylation of AMP appears to be the dominant pathway. In both cases, however, there is a net decline in the size of the adenylate pool and an increase in adenosine, inosine, and hypoxanthine.

The rapidity with which ATP loss occurs in the traumatized spinal cord can also be mimicked in culture preparations (Fig. 1). Here we have shown that cultured spinal neuronal cells lose ATP and total adenine nucleotides when challenged with amytal (A) and carbonyl cyanide m-chlorophenyl hydrazone (CCP); these are a NADH dehydrogenase inhibitor and a proton ionophore, respectively. This is associated with an increase in ADP and AMP as the total nucleotide pool falls. Upon removal of these compounds, there is a rebound in ATP toward control values with a concomitant decrease in the ADP pool; the size of the total adenine nucleotide pool, however, remains depressed. The loss of total adenylates in these protocols most likely involves the production and cellular efflux of adenosine, inosine, and hypoxanthine. However, cell death in a fraction of the population could also cause a decline in measured adenine nucleotides. In addition, the incomplete recovery of ATP/ADP in these protocols may be explained by intracellular compartmentalization of ADP rather than a true decline in the adenylate charge of the cytosol. It is clear that we are able to mimic many characteristics of the energy depletion known to occur *in situ* elsewhere in the central nervous system (32). Cellular homeostasis, in particular calcium and protein metabolism, are likely to be affected by such alterations.

## THE CALCIUM HYPOTHESIS AND NEURONAL CELL INJURY

Cellular calcium metabolism has been extensively reviewed (32) and many features are similar in a variety of cell types (26,32).  $\text{Ca}^{2+}$  is maintained at low values



**FIG. 1.** Energy depletion and adenine nucleotides in culture. Cells were harvested from cultures after freezing with liquid nitrogen, homogenized, and processed for high pressure liquid chromatography (HPLC) as before (18). Cells were also assayed for total protein and results are expressed as nmol of metabolites/mg protein. Cultures were sampled at 2 and 10 min after A/CCCP incubation and 5 min post-incubation (washout). Crosshatched area represents ATP changes, open area ADP, and left-diagonal AMP; mean  $\pm$  SEM. The total height of the bars indicates the total adenine nucleotide pool.

( $10^{-7}M$ ) in the cytosol; it can enter via voltage or agonist-dependent channels and reversal of the Na/Ca exchanger. It is reexported after receptor or voltage activation at the expense of metabolic energy. Such energy is either in the form of ATP or the sodium gradient which operates the forward mode of the Na/Ca exchanger. Modes of intracellular regulation of free  $Ca^{2+}$  in neuronal cells are also similar to other excitable cells in that active mitochondria, endoplasmic reticulum, and a series of calcium-binding proteins all play a qualitatively similar role in maintenance of these critically low  $[Ca^{2+}]_i$  levels (14,32 provide detailed reviews). A marked disturbance of this intracellular calcium homeostasis has become one of the basic tenets of the calcium-neuronal damage hypothesis (6,39,40). Major questions, however, remain about elements of this postulate.

The first relates to the magnitude of the  $[Ca^{2+}]_i$  change in individual cells during procedures that mimic cerebral ischemia. Such a question is of some importance if one is to assess the role of intracellular buffering mechanisms during pathological processes. We also do not know whether the diminution of extracellular free calcium, which has been repeatedly described during injury or ischemia in the CNS (6,39), contributes to a calcium paradox phenomenon (39) in the acutely uninjured

neuron population. Without a precise knowledge of the time course of such phenomena, one cannot establish a causal relationship between  $\text{Ca}^{2+}$  entry and subsequent pathological events. The uncertainty of such information continues to be one of the primary problems with the calcium neurotoxicity hypothesis (11,40). Careful attention must also be paid in any cell line to quantitation of the change in  $[\text{Ca}^{2+}]_i$  when measurements of intracellular free  $\text{Ca}^{2+}$  are made in injury or events that mimic cellular ischemia. As discussed below, we have used the fluorescent  $\text{Ca}^{2+}$  indicator fura-2 in single cells and carefully estimated the potential contributors to the  $\text{Ca}^{2+}$ -sensitive signal of fura-2.

## INTRACELLULAR CALCIUM DYNAMICS AS MEASURED BY FURA-2 TECHNOLOGY

The accurate characterization of the free  $\text{Ca}^{2+}$  transients in single cells has been dependent on the recent development of fluorescent probes (e.g., fura-2) that allow the necessary fast time resolution without the non-linearity or potential cell damage that may exist with other techniques (e.g., aequorin or  $\text{Ca}^{2+}$ -selective microelectrodes, 17). The acetoxyethyl ester form of this  $\text{Ca}^{2+}$ -sensitive probe (fura-2/AM), developed by Tsien and his colleagues (17,35), can be easily incorporated into the cell and deesterified by intracellular esterases to form the  $\text{Ca}^{2+}$ -sensitive acid moiety. The high fluorescence yield of fura-2 also permits detection of the signal from a small amount of intracellular dye and thus reduces the potential buffering capacity. The dye is sensitive over the range of calcium values that occur during energy depletion protocols (at least to 5–10  $\mu\text{M}$ ), shows little interference by competing ions, and does not overtly interfere with normal cell function. We have previously used fura-2/AM to calibrate and characterize the slow  $[\text{Ca}^{2+}]_i$  changes that occur in cardiac myocytes after energy depletion (21–24). By using the characteristic shift in fura-2 excitation spectra upon binding  $\text{Ca}^{2+}$  and the associated ratio techniques (35) that theoretically eliminate many of the problems (dye bleaching, changes in cell geometry or pathlength, etc.) found with previous fluorescent dyes of this type (e.g., quin-2), accurate characterization of  $\text{Ca}^{2+}$  transients can be achieved (17,35). The use of such probes is not, however, without potential problems.

The major problems with the use of fluorescent dyes lie in the areas of calibration of the dye signals because of intracellular compartmentalization and/or incomplete deesterification. Our work in energy-depleted cardiac myocytes has provided a method to correct for the highly fluorescent but calcium-insensitive dye components formed by incomplete dye deesterification (22,23). Such studies have produced reliable agreement between *in vivo* and *in vitro* calibration curves under a variety of conditions (23,30). In addition, we have estimated the contribution of the mitochondrial pool to our fluorescence signal after deenergization (13). Finally, it is also clear that excess dye loading can significantly impair intracellular calcium

dynamics (1,3,20,34). By appropriate control of loading procedures (1,3,29), however, such artifacts can be minimized if not eliminated. Under these carefully defined conditions, calcium-induced shifts in fura-2 fluorescence can be used to accurately describe slow and fast calcium transients with reliable accuracy.

### Measurement of Intracellular free $\text{Ca}^{2+}$ in Single Cells: System Design

We have used a PTI Deltascan system to achieve sufficient sensitivity and temporal resolution of the calcium dynamics. It essentially allows the fast time and dual-excitation fluorescence measurements necessary to accomplish different experimental protocols with our cell populations. Excitation wavelengths in the system are provided by a 75W xenon lamp through two excitation monochrometers set at 350 and 380 nm, respectively. The two excitation wavelengths are alternated at 100 Hz (variable; operator selected) by a motor-controlled spinning chopper similar to that described by Danielisova et al. (12). The light beam from either of these sources is directed into the light path of the inverted microscope equipped with a 63X Plan-Neofluar objective, which focuses on the specimen. Emission (420–620 nm) at the two excitation wavelengths from the cell is collected by a photomultiplier tube (PMT). A dichroic mirror (Zeiss FT395) is used to separate the excitation and emission components between the objective and the PMT. The computer program synchronizes the PMT sample mode with the chopper rate to store signals from the two excitation wavelengths into two different channels without crosstalk. Resident programs are used to collect the emission data, plot it in single or dual channel modes, data average, or construct ratios that are used to estimate free calcium. The entire optical path to and from the cell (with the exception of the coverslip at the bottom of the chamber) is through quartz elements to maximize UV transmission and avoid shifts of excitation spectra to higher wavelengths. This is necessary to increase system sensitivity at the low loading magnitude needed to avoid fura-2 buffering (24).

A single cell is placed in the UV-illuminated field (adjusted by a diaphragm) of the 63X objective to avoid signal contamination from neighboring cells. To minimize UV exposure of the cell, a computer-controlled shutter is placed on the excitation side of the light path and is opened only when measurements are being made.

### Calcium Dynamics and Energy Depletion

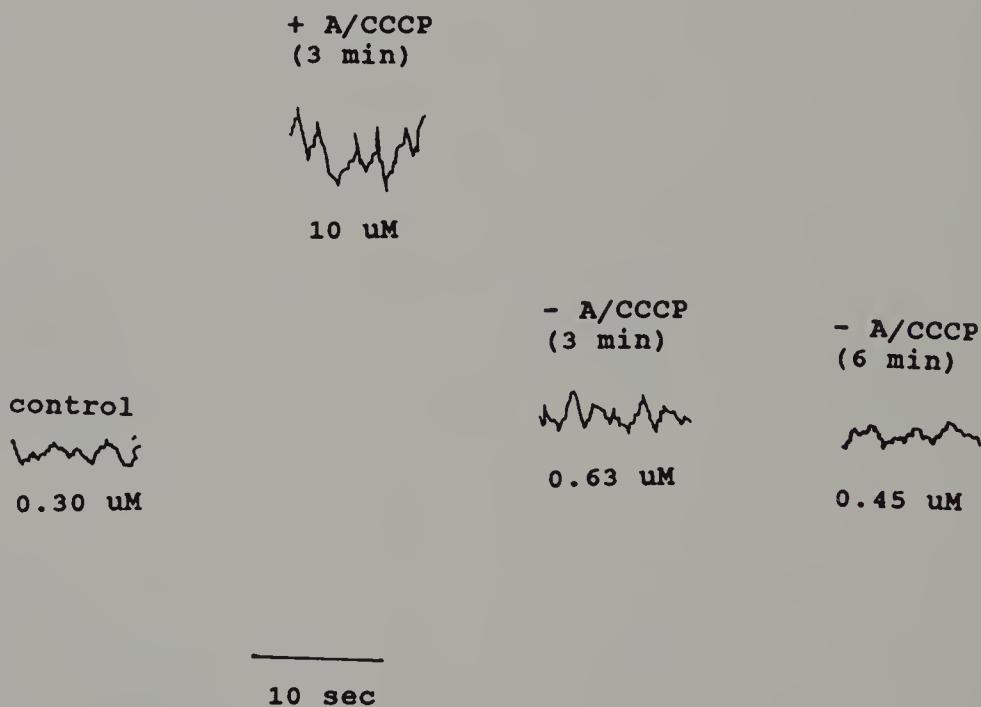
Our previous experience with energy depletion paradigms (10,21,24,28) in other cell lines and use of the fura-2 technology allowed us to examine the consequences of deenergization in our primary culture model.

In a series of spinal cord ventral horn cultures treated in a similar manner with

A/CCCP, we have repeated some of our earlier protocols (21,22,23). From such measurements we estimate the resting free calcium in these cells to be significantly lower ( $n = 7$ ; 150–300 nM) than values found with ion-sensitive microelectrodes but in agreement with other non-invasive techniques (26).

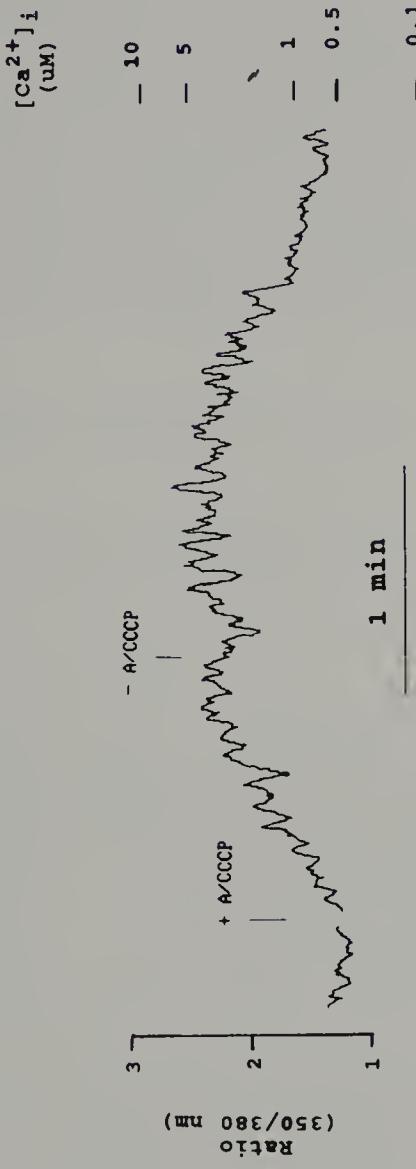
In Fig. 2 and Fig. 3, individual culture plates were loaded with 2  $\mu\text{M}$  fura-2/AM for 5–10 min at room temperature and post-incubated for 1–2 hr. The low concentration and short loading time of the dye are used to minimize the large uptake of fura-2/AM by the cells and the consequent calcium buffering by the deesterified fura-2. Post-incubation is necessary to insure maximal hydrolysis of the fura-2 ester form, which is critical for accurate calibration of the fluorescence signal.

In Figure 2, we show the effects of energy depletion on a single cell. The effects are quite dramatic because free calcium exceeds 10  $\mu\text{M}$  several minutes after ex-



**FIG. 2.** Changes in intracellular free  $\text{Ca}^{2+}$  by metabolic inhibition. Fura-2-loaded cultured neurons were bathed in a closed perfusion chamber and continuously perfused with Krebs-Henseleit (K-H) bicarbonate buffer at 35°C, pH 7.3. Metabolic inhibition was achieved by perfusing the cells with K-H buffer containing 3 mM amytal and 2  $\mu\text{M}$  CCCP (A/CCCP). The fluorescence signal from a single neuron was measured at times indicated. The  $\text{Ca}^{2+}$ -saturated and  $\text{Ca}^{2+}$ -free fura-2 signals were determined intracellularly at the end of the experiment and the fluorescence ratio was converted to  $[\text{Ca}^{2+}]_i$  as described (22). The absolute estimations of  $[\text{Ca}^{2+}]_i$  appear below each of the traces.

## 2ND EXPOSURE



## 4TH EXPOSURE



FIG. 3. Effects of energy depletion and subsequent repletion from the same cell after repeated exposure. Traces from the 2nd and the 4th of such protocols are shown. Notice the further increase in  $[Ca^{2+}]_i$  upon washout before the recovery.

posure. Reversal of these effects is evident as calcium quickly returns to near normal levels within 3 and 6 min after drug removal. This rate of increase is considerably faster than that which occurs in other energy-depleted cell lines as is the ability to quickly exclude the excess free calcium (21–24). In those cells studied ( $n = 9$ ), intracellular calcium quickly rose to over  $2 \mu\text{M}$  within 3 min as a result of the energy depletion paradigm.

In Figure 3, we document the effects of repeated exposure to such regimens. Conceptually, such treatments might be related to intermittent reperfusion phenomena that occur in the injured CNS. Note the delayed recovery during the second exposure as calcium continues to rise after reenergization. Such increases may result from release of calcium from overloaded intracellular stores. Notice also that repeated exposures progressively produce higher calcium levels in the same cell (4th exposure), particularly during recovery from the insult. Further disturbance of intracellular organelles may also contribute to this pattern. Future experiments are needed to establish the mechanisms by which such dynamics in  $[\text{Ca}^{2+}]_i$  take place after reenergization and if "calcium paradox" phenomena occur in cultured neurons.

The final element in the calcium hypothesis is of course to provide some dynamic measure of structural alterations that may occur in the cultured neurons as a result of energy depletion and the consequent elevations in intracellular free calcium.

## THE NEURONAL CYTOSKELETON AND CALCIUM METABOLISM

### General Aspects of the Neuronal Cytoskeleton

The neuronal cytoskeleton is comprised of three fibrous elements: microfilaments (MF), neurofilaments (NF), and microtubules (MT) (8). The major polypeptide components of these substructures have been examined in detail. MFs are polymers of actin with associated actin-regulator proteins. NFs consist of three polypeptides of molecular weights 200,000, 150,000, and 70,000 and microtubules are polymers formed from the heterodimer  $\alpha$  and  $\beta$  tubulin with associated proteins, ie., microtubule-associated proteins (MAPs). The complex interaction of these various components is critical to the maintenance of the great variety of forms illustrated by the hundreds of distinguishable cell types in the vertebrate nervous system.

The intrinsic form and function of the neuronal cytoskeleton is imparted by the associated regulatory proteins such as actin-binding proteins and MAPs. It is not surprising that the organization of the axonal and dendritic cytoskeleton is very different (9). The cell body, which is the site of synthesis of neuronal proteins, is a mixture of both axonal and dendrite-specific cytoskeletal protein. Actin microfilaments are found throughout the neuron; however, actin-binding proteins such as the spectrin isoforms (fodrin) are localized either exclusively in the axon or dendrite. Microtubules are components of all processes, however, tyrosinated  $\alpha$  tubulin is dendrite specific and acetylated  $\alpha$  tubulin is axon specific. This is further complicated by the varied distribution of the MAPs (9).

## Calcium and the Neuronal Cytoskeleton

Virtually all components of the cytoskeleton are either directly or indirectly affected by alterations in calcium metabolism (25). Furthermore, increasing evidence indicates that calcium-activated proteases may also specifically modulate components of the cytoskeleton (15,27). In this regard, calcium-dependent proteases have been implicated in spinal damage and shown to be associated with extensive total calcium accumulation in the hours after traumatic insult (5). In addition to the  $\text{Ca}^{2+}$ -sensitivity of cytoskeleton, it has also been well-established that actin is an ATP-binding protein (25) and several of the actin regulatory proteins are ATP-dependent (25). Thus ATP depletion itself may directly alter the structural components of the neuronal cytoskeleton. It is of interest to point out that numerous major neurodegenerative diseases such as Alzheimer's, Parkinsonian syndrome, amyotrophic lateral sclerosis, as well as a variety of toxic neuropathies are also characterized by changes in the neuronal cytoskeleton (7,16).

## Confocal Microscopy as a Tool to Investigate the Problem

A BioRad Lasersharp MRC-500 scanning laser confocal microscope has been used to conduct the studies on the structural correlates of neuronal deenergization. The MRC-500 converts the standard research microscope into a laser scanning confocal system. Designed particularly for applications in fluorescence microscopy, the system is provided with a multi-line argon ion laser able to excite many commonly used fluorochromes such as fluorescein, rhodamine, and Texas red. In addition, operation is also possible at all reflective wide bandpass optics; the transfer system is thus fully achromatic at all wavelengths from UV to near infrared.

The MRC-500 is controlled by a Nimbus VT desktop computer with purpose-built cards for controlling scanning and image acquisition. The computer-driven focus control allows for optical sectioning. A second independent detector can be used to detect transmitted phase, bright field or Nomarski, and epifluorescence or reflectance microscopy. The software has functions for either displaying the two images simultaneously or "merging" them for direct comparison.

The key element of confocal optics is a spatial filter situated in the reflected light path. The filter is configured so that the region viewed at any one time is coincident with the diffraction-limited illuminating spot. Thus, illumination and detection apertures are "confocal" with each other. The advantage over the conventional microscope is that only a single focal plane is illuminated at any one time.

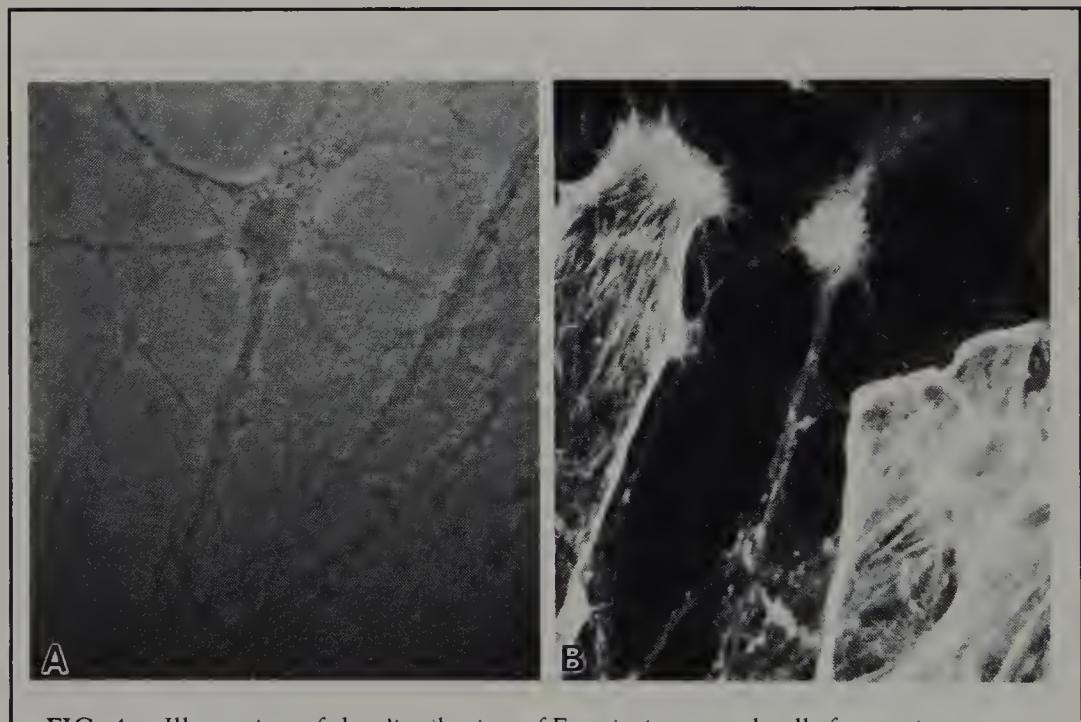
The confocal optical system dramatically changes the contrast properties of the optical microscope. Only those regions of the sample lying within a narrow focal plane are imaged. Regions above and below this plane appear black rather than blurred as in the conventional system. As a result, contrast is not degraded by out of focus flare and it is possible to obtain images with extraordinary clarity and detail. In addition, the acquisition of accurate quantitative data is possible because

of the exclusion of signals from out of focus regions in the tissue cultures. This instrument has been used to assess protein alterations as described below.

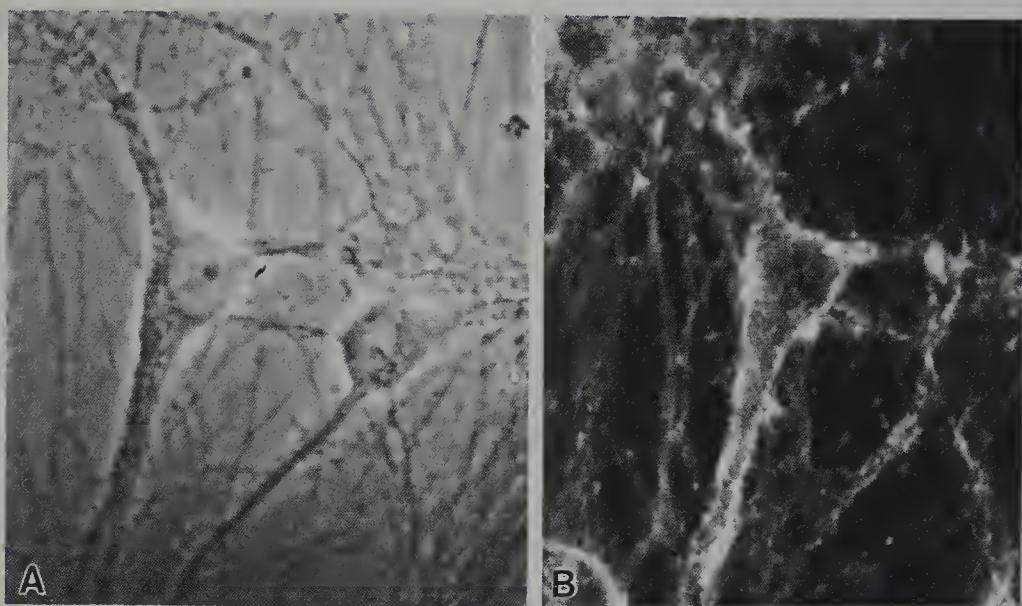
### Energy Depletion and Confocal Microscopy

In Figure 4, we have illustrated the distribution of F-actin in control cultures with the F-actin-specific probe rhodamine phalloidin; (A) phase-contrast transmitted image, (B) confocal image of rhodamine-phalloidin fluorescence. Actin staining in neurons was most concentrated in the cell body, densitometric quantitation of fluorescence revealed that more staining was evident in the cell body (26.4) when compared to the processes (22.1). Actin filament bundles (stress fibers) can also be seen to be prominent features of the non-neuronal cells present in these cultures. In this particular plane of section, the non-neuronal cells (below and left of the neuron) stain more intensely than the neurons (30.00).

Figure 5 is an example (A and B as in Fig. 4 above) of actin-staining patterns in cells that had been ATP-depleted. Actin appears to be clumped into aggregates



**FIG. 4.** Illustration of the distribution of F-actin in control cells from primary ventral-horn cultures. In the phase contrast image (A), a single neuron is evident with several flattened cell profiles (presumably astrocytes) slightly out of the plane of focus. F-actin staining in the same plane of section (B) reveals a dense distribution of the label in neural and non-neuronal elements.



**FIG. 5.** F-Actin distribution in energy-depleted cells from ventral-horn cultures. Cultured cells (**A**-phase contrast) were energy depleted for 10 min as before (see above), fixed and stained for F-actin (**B**). Energy depletion caused a remarkable decrease in cytosolic F-actin.

along the neuron plasma membrane but markedly decreased in the neuronal perikaryon. Actin in non-neuronal cells did not appear to be distributed differently from controls. Quantitative measurements of actin staining revealed that F-actin levels were lower in ATP-depleted neurons (16.3 whole cell, 15.6 cell body, and 12.4 for the processes). Actin measurements of non-neuronal cells, however, were similar to controls. Such measurements are quite repeatable and revealed the power of using confocal microscopy to discriminate between types and individual cell processes.

## SUMMARY

We have outlined an approach to a model system in which the investigation of growth factors may take place. In particular, we have conducted experiments that reveal the neurochemical alterations produced by energy depletion in neural cultures. By coupling these to the free calcium estimations and structural degradation (revealed by the confocal microscope), we are able to add mechanistic arguments

to the calcium-neurotoxicity hypothesis. This is particularly true when free calcium exceeds the necessary levels to activate neutral proteases in the cell interior. Future experiments of this type, and the intervention with protocols designed to ameliorate the ensuing pathological process, will undoubtedly result in more clearly defined hypotheses of how neuronal toxicity occurs.

## REFERENCES

1. Almers W, Neher E. The Ca signal from fura-2 loaded mast cells depends strongly on the method of dye-loading. *FEBS. Lett.* 1985;192:13-18.
2. Anderson DK, Behbehani MM, Means ED, Waters TR, Green ES. Susceptibility of feline spinal cord energy metabolism to severe incomplete ischemia. *Neurology* 1983; 33:722-731.
3. Ashley CC, Potter JD, Strang P, Godber J, Walton A, Griffiths PJ. Kinetic investigations in single muscle fibers using luminescent and fluorescent  $\text{Ca}^{2+}$  probes. *Cell Calcium*. 1985;6:159-181.
4. Balazs R. Metabolic imbalance and nerve cell damage in the brain. *Prog. Brain. Res.* 1988;73:447-463.
5. Banik NL, Hogan EL, Powers JM, Whetstine LJ. Degradation of cytoskeletal proteins in experimental spinal cord injury. *Neurochem. Res.* 1982;7:1465-1475.
6. Beattie MS, Stokes BT. Experimental spinal cord injury: strategies for acute and chronic intervention based on anatomic, physiologic and behavioral studies. In: Stein DG, Sabel BA, eds. *Pharmacologic Approaches to the Treatment of Brain and Spinal Cord Injury*. New York: Plenum Publishing Corp., 1988;59-72.
7. Binet S, Meininger V. Modifications of microtubule proteins in ALS nerve precede detectable histologic and ultrastructural changes. *Neurology* 1988;38:1596-1600.
8. Bray D, Gilbert D. Cytoskeletal elements in neurons. *Annu. Rev. Neurosci.* 1981; 4:505-523.
9. Burgoyne RD, Cambray-Deakin MA. The cellular neurobiology of neuronal development: the cerebellar granule cell. *Brain. Res.* 1988;472:77-101.
10. Caciagli F, Ciccarelli R, Dilorio P, Ballerini P, Tacconelli L. Cultures of glial cells release purines under field electrical stimulation: the possible ionic mechanisms. *Pharm. Res. Comm.* 1988;20(11):935-947.
11. Cheung JY, Bonventre JV, Malis CD, Leaf A. Calcium and ischemic injury. *N. Engl. J. Med.* 1986;314:1670-1676.
12. Danielisova V, Chavko M, Kehr J. Adenine nucleotide levels and regional distribution of ATP in rabbit spinal cord after ischemia and recirculation. *Neurochem. Res.* 1987; 12:241-245.
13. Davis MH, Altschuld RA, Jung DW, Brierley GP. Estimation of intramitochondrial pCa and pH by fura-2 and 2,7 biscarboxyethyl-5(6)-carboxyfluorescein (BCECF) fluorescence. *Biochem. Biophys. Res. Commun.* 1987;149:40-45.
14. DiPolo R, Beauge L.  $\text{Ca}^{2+}$  transport in nerve fibers. *Biochem. Biophys. Acta* 1988; 947:549-569.
15. Gallant PE, Pant HC, Pruss RM, Gainer H. Calcium-activated proteolysis of neurofilament proteins in the squid giant neuron. *J. Neurochem.* 1986;46:1573-1581.

16. Goldman JE, Yen SH. Cytoskeletal protein abnormalities in neurodegenerative diseases. *Ann. Neurol.* 1986;20:9–223.
17. Grynkiewicz G, Poenie M, Tsien RY. A new generation of  $\text{Ca}^{2+}$  indicators with greatly improved fluorescence properties. *J. Biol. Chem.* 1985;260:3440–3450.
18. Hammer DF, Unverferth DV, Kelley RE, Harvan PA, and Altschuld RA. Extraction and measurement of myocardial nucleotides, nucleosides, and purine bases by high-performance liquid chromatography. *Anal. Biochem.* 1988;169:300–305.
19. Hayashi N, Tsubokawa T, Green BA. Changes in energy metabolism and spinal cord blood flow following severe spinal cord injury. *No. Shinkei. Geka.* 1984;12:923–930.
20. Lambert MR, Johnson JD, Lamka KG, Brierley GP, Altschuld RA. Intracellular free  $\text{Ca}^{2+}$  and the hypercontracture of adult rat heart myocytes. *Arch. Biochem. Biophys.* 1986;245:426–435.
21. Li Q, Altschuld RA, Biagi BA, Stokes BT. Cation and membrane potential alterations in energy depleted cardiac myocytes. In: Clark WA, Decker RS, Borg TK, eds. *Biology of Isolated Adult Cardiac Myocytes*. New York: Elsevier Science Publishing Co., 1988; 342–345.
22. Li Q, Altschuld RA, Stokes BT. Quantitation of intracellular free calcium in single adult cardiomyocytes by fura-2 fluorescence microscopy: calibration of fura-2 ratios. *Biochem. Biophys. Res. Commun.* 1987;147:120–126.
23. Li Q, Altschuld RA, Stokes BT. Myocyte deenergization and intracellular free calcium dynamics. *Am. J. Physiol.* 1988;255:C162–C168.
24. Li Q, Hohl CM, Altschuld RA, Stokes BT. Energy depletion/repletion and calcium transients in single cardiomyocytes. *Am. J. Physiol.* 1989;257(Cell Physiol. 26):C427–C434.
25. Mooseker MS. Organization, chemistry, and assembly of the cytoskeletal apparatus of the intestinal brush border. *Annu. Rev. Cell Biol.* 1985;1:209–241.
26. Morris ME, Friedlich JJ, MacDonald JF. Intracellular calcium in mammalian brain cells: fluorescence measurements with quin2. *Exp. Brain. Res.* 1987;65:520–526.
27. Nixon RA. Fodrin degradation by calcium-activated neutral proteinase (CANP) in retinal ganglion cell neurons and optic glia: preferential localization of CANP activities in neurons. *J. Neurosci.* 1986;6:1264–1271.
28. Panula P, Emson P, Wu JY. Demonstration of enkephalin-, substance P- and glutamate decarboxylase-like immunoreactivity in cultured cells derived from newborn rat neostriatum. *Histochemistry.* 1980;69:169–179.
29. Peeters GA, Hlady V, Bridge JH, Barry WH. Simultaneous measurement of calcium transients and motion in cultured heart cells. *Am. J. Physiol.* 1987;253:H1400–H1408.
30. Scanlon M, Williams DA, Fay FS. A  $\text{Ca}^{2+}$ -insensitive form of fura-2 associated with polymorphonuclear leukocytes. Assessment and accurate  $\text{Ca}^{2+}$  measurement. *J. Biol. Chem.* 1987;262:6308–6312.
31. Schultz V, Lowenstein JM. The purine nucleotide cycle. Studies of ammonia production and interconversions of adenine and hypoxanthine nucleotides and nucleosides by rat brain *in situ*. *J. Biol. Chem.* 1978;1938–1943.
32. Siesjo BK. Historical overview. Calcium, ischemia, and death of brain cells. *Ann. N.Y. Acad. Sci.* 1988;522:638–661.
33. Siesjo BK. Hypoglycemia, brain metabolism, and brain damage. *Diabetes. Metab. Rev.* 1988;4:113–144.
34. Thomas AP, Selak M, Williamson JR. Measurement of electrically induced  $\text{Ca}^{2+}$  transients in Quin2-loaded cardiac myocytes. *J. Mol. Cell Cardiol.* 1986;18:541–545.

35. Tsien RY, Rink TJ, Poenie M. Measurement of cytosolic free  $\text{Ca}^{2+}$  in individual small cells using fluorescence microscopy with dual excitation wavelengths. *Cell Calcium*. 1985;6:145–157.
36. Vink R, McIntosh TK, Weiner MW, Faden AI. Effects of traumatic brain injury on cerebral high-energy phosphates and pH: a  $^{31}\text{P}$  magnetic resonance spectroscopy study. *J. Cereb. Blood Flow. Metab.* 1987;7:563–571.
37. Vink R, McIntosh TK, Yamakami I, Faden AI.  $^{31}\text{P}$  NMR characterization of graded traumatic brain injury in rats. *Magn. Reson. Med.* 1988;6:37–48.
38. Walker JG, Yates RR, Yashon D. Regional canine spinal cord energy state after experimental trauma. *J. Neurochem.* 1979;33:397–401.
39. Young W. Ca paradox in neural injury: a hypothesis. *CNS Trauma* 1986;3:235–251.
40. Young W. The post-injury responses in trauma and ischemia: secondary injury or protective mechanisms? *CNS Trauma* 1987;4:27–51.

# Recovery of Dopaminergic Function Following MPTP-induced Neurodegeneration by Exogenous GM<sub>1</sub> Ganglioside

M. Hadjiconstantinou<sup>1,2</sup>, F. B. Weihmuller<sup>3</sup>, J. P. Bruno<sup>2,3</sup>,  
A. P. Mariani<sup>4</sup> and N. H. Neff<sup>1</sup>

<sup>1</sup>Department of Pharmacology, <sup>2</sup>Department of Psychiatry, College of Medicine

<sup>3</sup>Department of Psychology, College of Social and Behavioral Sciences  
The Ohio State University, Columbus, OH 43210; <sup>4</sup>Laboratory of Neurophysiology,  
NINCDS, NIH, Bethesda, MD 20892

**D**uring the past several years major advances have been made in our understanding of brain plasticity and the role of neurotrophic factors (see 24). The possibility of stimulating plastic responses after an insult in the mature brain has been at the epicenter of intensive research. The motivations for this interest are the numerous neurodegenerative diseases and the possible disastrous consequences of trauma to the central nervous system (CNS).

Parkinson's disease is an idiopathic neurodegenerative disease, characterized by a selective loss of dopamine (DA) in the striatum. Treatment with L-3,4-dihydroxyphenylalanine (L-DOPA) to replace DA has been rather disappointing because of the many side effects and the loss of potency of L-DOPA as the disease progresses. Slowing or arresting the degenerative process would be of significant therapeutic importance.

The neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) destroys dopaminergic neurons in several species, and in humans and other primates it causes clinical symptoms that resemble Parkinson's disease (4,23). The MPTP-treated mouse is a useful small animal for studying the biochemical changes associated with the neurodegenerative process (15). After treating mice with MPTP, there is a loss of DA-containing neurons from the substantia nigra (14), a loss of tyrosine hydroxylase (TH) and DA from the striatum, and diminution of locomotor activity, which is most prominent after challenge with neuroleptic drugs or stress (35). The mechanism for MPTP neurotoxicity is unclear. Experimental evidence suggest that *in vivo* MPTP is converted to 1-methyl-4-phenyl-pyridinium ion ( $MPP^+$ ) by monoamine oxidase (MAO) B (16).  $MPP^+$ , which appears to be the ultimate toxin, uses the DA transporter to enter dopaminergic neurons (18). Within dopaminergic neurons  $MPP^+$  may generate free radicals (28), suppress mitochondrial respiration (17,26) or ATP formation (25), resulting in neuronal death. Based on the phar-

macology of MPTP, its toxicity can be prevented by pretreating mice with a MAO B inhibitor (3,16) or a DA transport-blocking drug (27).

Administration of GM<sub>1</sub> ganglioside (II<sup>3</sup>NeuAc-GgOse<sub>4</sub>Cer) stimulates the recovery of dopaminergic (2,12,13,33,34), serotonergic (9,11,19), noradrenergic, (22) and cholinergic neurons (5,7,29,30,36) after surgical or neurotoxin lesions. The basis for recovery is unknown, however various hypotheses have been suggested: 1) GM<sub>1</sub> ganglioside may have a regrowth-stimulating effect, a protective action against secondary degenerative changes or both and thus acts like a neurotrophic factor (24); 2) GM<sub>1</sub> ganglioside may not have neurotrophic activity itself but may modulate the action of endogenous neurotrophic factors; 3) GM<sub>1</sub> ganglioside may stabilize membrane function (20).

We have evaluated whether treatment with GM<sub>1</sub> ganglioside or the internal ester AGF2 can restore striatal dopaminergic function in the MPTP-treated mouse.

### MPTP NEURODEGENERATION AND GM<sub>1</sub> GANGLIOSIDE

Treatment with MPTP for 7 days results in depletion of striatal DA, which is evident in mice killed either 1 (MPTP 7) or 24 (MPTP 30) days after completing the MPTP treatment (Table 1). Administration of GM<sub>1</sub> ganglioside has no apparent effect on the content of DA or 3,4-dihydroxyphenylacetic acid (DOPAC) (Table 1) in the striatum of normal mice. In initial studies we determined that GM<sub>1</sub> ganglioside did not directly interfere with MPTP neurotoxicity. Indeed, it did not

**TABLE 1.** Treatment with GM<sub>1</sub> or AGF2 restores striatal DA and DOPAC content in mice lesioned with MPTP

Treatment	DA		DOPAC	
		(pmol/mg prot ± SEM)		%
Saline	621 ± 30	%	179 ± 12	%
MPTP 7	282 ± 12*	-54	88 ± 7*	-51
MPTP 30	312 ± 18*	-50	132 ± 9*	-26
GM <sub>1</sub>	604 ± 27		176 ± 11	
AGF2	660 ± 34		232 ± 19	
MPTP 30 + GM <sub>1</sub>	570 ± 24†	-8	158 ± 13†	-11
MPTP 30 + AGF2	495 ± 36	-20	217 ± 20†	+21

For all studies, MPTP, 30 mg/kg ip was administered for 7 days and the animals euthanized either 8 (MPTP 7) or 31 (MPTP 30) days after the initiation of MPTP treatment. GM<sub>1</sub> ganglioside, 30 mg/kg ip, or AGF2, 10 mg/kg ip, were administered beginning on day 8 and continued for 23 days (MPTP 30 + GM<sub>1</sub>) and (MPTP 30 + AGF2). Animals treated with saline, GM<sub>1</sub>, or AGF2 served as controls. All animals were euthanized 24 hr after completing the treatments. The catecholamines were assayed by HPLC with an electrochemical detector.

\* p < 0.05 compared with Saline.

† p < 0.05 compared with MPTP 30.

Percentage differences have been calculated with respect to the Saline group. n = 15–20.

inhibit the transport of DA into synaptosomes *in vitro* and it did not block the conversion of DA to DOPAC by MAO *in vivo* (12). Furthermore, administration of the ganglioside 30 min prior to MPTP injection did not prevent the depletion of DA and DOPAC in both acute and chronic experiments (12).

Studies were designed to evaluate whether chronic administration of GM<sub>1</sub> could restore the content of DA and DOPAC in the striata of MPTP lesioned mice. Three treatment programs were tested. 1) Cotreatment: GM<sub>1</sub> was administered together with MPTP. On day 8, MPTP was discontinued but GM<sub>1</sub> administration continued until day 30. 2) Pretreatment: Animals were pretreated with GM<sub>1</sub> for 5 days and then MPTP administered together with GM<sub>1</sub> for an additional 7 days. On day 8, MPTP was discontinued but GM<sub>1</sub> administration was continued until day 30. 3) Posttreatment: Animals were treated with MPTP for 7 days and on day 8, GM<sub>1</sub> administration was initiated and continued until day 30.

In all three studies chronic administration of GM<sub>1</sub> resulted in the restoration of DA and DOPAC in the striatum of the MPTP-lesioned mice. Moreover, the percentage recovery of the catechols did not differ among the various treatments (12). We conclude, therefore, that chronic treatment with GM<sub>1</sub> ganglioside is necessary to restore DA content in MPTP-lesioned mice. Because the outcome for all three treatment protocols was essentially the same, we used the post-treatment paradigm for subsequent studies as it more closely mirrors the clinical situation where parkinsonism is diagnosed and treatment initiated.

## OPTIMAL GM<sub>1</sub> GANGLIOSIDE TREATMENT

The optimal dose and duration of GM<sub>1</sub> ganglioside treatment for restoring DA in the striatum of MPTP-lesioned mice was evaluated in subsequent studies. A dose-response study (5–60 mg/kg ip) revealed that DA and DOPAC content in the striatum of the MPTP-lesioned mice responded optimally after a dose of 30 mg/kg ip. This dose of GM<sub>1</sub> resulted in a maximal return of DA and DOPAC content after about three weeks of treatment (12). Based on these results, a dose of 30 mg/kg for 23 days was used for the studies that follow.

Chronic administration of GM<sub>1</sub>, starting 24 hr after completing the MPTP treatment resulted in an almost complete repletion of DA (92% of the control value) and DOPAC (89% of the control value) in the striatum of the MPTP-lesioned animals (Table 1). The internal ester of GM<sub>1</sub>, AGF2, also facilitates the recovery of DA and DOPAC in the striatum of MPTP-lesioned mice.

## DOPAMINE TURNOVER AND GM<sub>1</sub> GANGLIOSIDE TREATMENT

The finding that DOPAC content returned to normal or greater after administering the gangliosides to MPTP-lesioned mice suggested that the rate of DA

formation had accelerated. This notion has been tested directly (Table 2) and the rate of DA formation for the MPTP plus GM<sub>1</sub>-treated mice was found to be comparable with control mice and about twice the rate estimated for mice treated with the neurotoxin alone.

### BIOCHEMICAL MEASUREMENT OF NERVE TERMINAL SPROUTING

Neurotransmitter transport into synaptosomes is often used as a biochemical marker for the presence of functional nerve terminals. Chronic administration of GM<sub>1</sub> or AFG2 did not alter the DA uptake into striatal synaptosomes when compared with the saline-treated animals (Table 3). MPTP induced about a 47% reduction of DA uptake when studied 31 days later. Administration of GM<sub>1</sub> or AGF2 to the MPTP-lesioned animals did not correct the loss of uptake function.

### TYROSINE HYDROXYLASE IMMUNOREACTIVE CELLS IN THE SUBSTANTIA NIGRA

The effects of MPTP and GM<sub>1</sub> ganglioside treatment on the dopaminergic nigrostriatal neurons were studied immunohistologically using antibodies raised against TH. The results of these studies are presented in Table 4. TH-immunoreactive neuronal cell bodies in the substantia nigra of saline-treated animals were present at a density of 11,541 cells/mm<sup>3</sup> and had an average diameter of 11.48  $\mu\text{m}$ . Administration of GM<sub>1</sub> ganglioside did not change the density (number) of TH-immunoreactive cell bodies but did increase the cell body size. The MPTP treatment alone resulted in a decrease of both the number (down about 40%) and

**TABLE 2.** GM<sub>1</sub> treatment increases DA synthesis rate in the striatum of MPTP-treated mice

Treatment	DA (pmol/mg prot)	k (hr <sup>-1</sup> )	t <sub>½</sub> (hr)	DA turnover rate (pmol/mg prot/hr)
Saline	632 $\pm$ 45	0.312	2.2	197
GM <sub>1</sub>	698 $\pm$ 70	0.246	2.8	172
MPTP 30	261 $\pm$ 40	0.456	1.5	119
MPTP + GM <sub>1</sub>	481 $\pm$ 37	0.432	1.6	208

Treatments were administered as follows: MPTP, 30 mg/kg ip, for 7 days and animals euthanized 31 (MPTP 30) days later; GM<sub>1</sub>, 30 mg/kg ip, and animals euthanized 23 (GM<sub>1</sub>) days later; MPTP, 30 mg/kg ip, for 7 days. On day 8 GM<sub>1</sub>, 30 mg/kg ip, was initiated and continued for 23 days, and animals euthanized 24 hr after the last dose of GM<sub>1</sub> (MPTP + GM<sub>1</sub>). DA turnover studies were performed 24 hr after the last treatment. Alpha-methyl-p-tyrosine, 300 mg/kg ip, was administered and animals euthanized 1, 2, and 3 hr later. Striatal DA was estimated by HPLC with an electrochemical detector and turnover calculated from the decline of DA with time.

**TABLE 3.** Treatment with GM<sub>1</sub> or AGF2 does not restore the loss of DA transport into synaptosomes prepared from MPTP-lesioned mice

Treatment	DA uptake (pmol/mg/prot/6 min ± SEM)
Saline	232 ± 13 %
GM <sub>1</sub>	193 ± 13
AGF2	217 ± 11
MPTP 30	130 ± 6* -47
MPTP 30 + GM <sub>1</sub>	109 ± 7* -52
MPTP 30 + AGF2	117 ± 8* -50

Mice were treated with MPTP, 30 mg/kg ip, for 7 days and killed on day 31 (MPTP 30). GM<sub>1</sub>, 30 mg/kg ip, or AGF2, 10 mg/kg ip, were initiated on day 8 after MPTP treatment completion and continued for 23 days (MPTP 30 + GM<sub>1</sub> or MPTP 30 + AGF2). Animals treated with saline, GM<sub>1</sub>, or AGF2 served as controls. Uptake studies were performed using striatal synaptosomes prepared from animals killed 24 hr after the last day of treatment. Data are presented as the mean ± SEM of three separate experiments. n = 15–20.

\* p < 0.05 compared with saline-treated mice.

the size (down about 15%) of the TH-immunoreactive cells in substantia nigra. When GM<sub>1</sub> was administered after MPTP treatment there was no change in the number of cell bodies, the same low density as in MPTP alone, but there was a significant restoration in the cell body size.

**TABLE 4.** Cell body size and density of TH-immunoreactive neurons in the substantia nigra of mice treated with MPTP alone or followed by GM<sub>1</sub> ganglioside

Treatment	Cell body diameter (μm ± SEM N = 20)	Cell density (Cells/mm <sup>3</sup> ± SEM N = 4)
Saline	11.48 ± 0.33	11,541 ± 472
GM <sub>1</sub>	12.96 ± 0.32*	11,269 ± 259
MPTP	9.72 ± 0.25*	8,601 ± 542*
MPTP + GM <sub>1</sub>	13.02 ± 0.30*,**	7,549 ± 214*

Treatments were performed as described in Table 3. Animals from each group were perfused with ice-cold 4% w/v paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4, containing 15% w/v sucrose. Sagittal sections, 30 μm, of hemisected brain were cut, mounted and incubated with specific TH antisera overnight. The primary antibodies were visualized by the peroxidase-antiperoxidase (PAP) method (31). Substantiae nigra were examined, studied and photographed in a light microscope. Measurements were made with an ocular reticle calibrated against the stage micrometer at a magnification of 630x. Cell counts were made within the confines of a square ocular reticle 388 μm on the edge at the magnification used. The density of TH-immunoreactive neurons was estimated by method 1 of Abercrombie (1) correcting for cell body size. Data were evaluated with one-way ANOVA with a t-test for comparison of pairs of groups.

\* p < 0.05 compared with saline treatment.

\*\* p < 0.05 compared with MPTP treatment.

## GM<sub>1</sub> TREATMENT PARAMETERS AND RECOVERY OF DOPAMINERGIC FUNCTION

Early initiation of GM<sub>1</sub> treatment appears to be necessary to benefit damaged cholinergic neurons (30). We studied the conditions required for successful GM<sub>1</sub> treatment of the MPTP-lesioned mice.

First, we studied the latency between the MPTP lesion and the initiation of GM<sub>1</sub> therapy for the recovery of DA neurons. GM<sub>1</sub> ganglioside, 30 mg/kg ip, was initiated at various times, 1–10 days, after the completion of MPTP treatment. The ganglioside was administered for a total of 23 days and DA and DOPAC assayed in the striatum. Only the animals treated within 4 days post-MPTP showed significant recovery of striatal DA and DOPAC.

We then investigated the magnitude of the nigrostriatal lesion as it relates to the response to treatment with GM<sub>1</sub> ganglioside. Mice were treated with various doses of MPTP, 10–50 mg/kg ip, to induce a range of lesions; then GM<sub>1</sub> treatment, 30 mg/kg ip, was introduced for 23 days. There was a significant negative correlation,  $r = 0.985$ , between the percentage of DA depletion by MPTP and the percentage of DA recovery to control values after treatment with GM<sub>1</sub>. Indeed, the larger the lesion the poorer the prospect for recovery of dopamine in the striatum.

We have explored whether the GM<sub>1</sub>-induced restoration of DA function in the striatum of MPTP-treated animals is permanent. GM<sub>1</sub>, 30 mg/kg ip, was administered to MPTP-lesioned animals for 23 days and then discontinued. A fall of the GM<sub>1</sub>-restored DA and DOPAC content in the striatum was observed with time. After 30 days the levels of catechols reached those found in animals that received MPTP alone.

In preliminary studies, we evaluated whether an animal's age is a factor in the return of dopaminergic function after GM<sub>1</sub> treatment. For this purpose, 20-month-old mice were treated with MPTP and then GM<sub>1</sub> was administered as previously described. MPTP administration, 30 mg/kg ip, for 7 days, to these mice resulted in about a 96% reduction of DA and DOPAC content. Furthermore, GM<sub>1</sub> treatment, 30 mg/kg for 23 days, was without effect. Given that the GM<sub>1</sub> response is negatively correlated with the extent of the MPTP lesion, it is difficult to conclude from these studies whether GM<sub>1</sub> is less active in old animals or whether the nigrostriatal lesion was too extensive to correct dopaminergic function. The results of the afore-mentioned studies are summarized in Table 5.

TABLE 5. Requirements for successful GM<sub>1</sub> treatment of the MPTP-treated mice

- 
1. Early, within 4 days post-MPTP, initiation of GM<sub>1</sub> administration
  2. Small to moderate nigrostriatal lesions
  3. Continuous administration of GM<sub>1</sub>
  4. Young animals?
-

## GM<sub>1</sub> BEHAVIORAL MODIFICATION AND RECEPTORS

Lesions of nigrostriatal neurons result in the development of abnormal behaviors and receptor denervation supersensitivity as evidenced by postsynaptic receptor changes. Under normal testing conditions, our MPTP-treated mice do not exhibit sensorimotor deficits. Indeed, there is about a 40–60% loss of DA in our animals, whereas 80% or greater loss is apparently required to observe behavioral changes. However, the administration of a low dose of the non-selective DA receptor antagonist haloperidol, 0.2 mg/kg ip, produces marked behavioral dysfunction in mice treated with MPTP (35). In addition, after treatment with MPTP there is an increase of the number of D-2 receptors in the striatum when examined 30 days after the lesion (M. Hadjiconstantinou, F.B. Weihmuller, N.H. Neff, *unpublished observations*).

We have explored the consequences of the GM<sub>1</sub>-induced restoration of DA in the MPTP-treated animals as it relates to behavior and receptor binding sites. Figure 1 illustrates the duration of akinesia while Fig. 2 illustrates somatosensory orientation scores before and 1 hr after haloperidol in the various treatment groups. After haloperidol, the duration of akinesia is about 15 times longer in mice that received MPTP alone. Haloperidol also impaired somatosensory orientation in mice treated with MPTP. Treatment with GM<sub>1</sub> eliminated the haloperidol-induced akinesia and somatosensory neglect in the MPTP-treated mice.

Thirty days after treating mice with MPTP there was about a 47% increase in the number of D-2 receptor binding sites, up-regulation or receptor supersensitivity, as measured by [<sup>3</sup>H]spiperone binding (Table 6). There were no changes of receptor

**TABLE 6.** Prevention of D-2 receptor up-regulation in the striatum of MPTP-treated mice after GM<sub>1</sub> administration

Treatment	B <sub>max</sub> (fmol/mg prot ± SEM)	K <sub>d</sub> (nM ± SEM)
Saline	247 ± 8	0.26 ± 0.002
MPTP 30	367 ± 13*	0.27 ± 0.002
GM <sub>1</sub>	250 ± 3	0.26 ± 0.015
MPTP 30 ± GM <sub>1</sub>	254 ± 4†	0.28 ± 0.007

Treatments were performed as described. Animals were killed 24 hr after the last injection, striata dissected, immediately frozen on dry ice and kept in -70°C until assayed. Membranes were prepared by homogenizing the tissues in Tris-HCl buffer 50 mM, pH 7.5 and centrifuging at 20,000g for 10 min. The particulate fraction was washed with buffer and then suspended in Tris-HCl containing MgCl<sub>2</sub>, 1 mM, NaCl, 120 mM, KCl, 5 mM and CaCl<sub>2</sub>, 2 mM. [<sup>3</sup>H]Spiperone was used as the ligand for D-2 receptors. Incubations were for 15 min at 37°C and reaction stopped by rapid filtration. Specific binding was defined as the difference between total binding and that found in the presence of 2 μM of ketanserin and sulpiride. B<sub>max</sub> and K<sub>d</sub> values were calculated from Scatchard curve analysis.

N = 3–5 separate experiments.

\* p < 0.05 compared with saline.

† p < 0.05 compared with MPTP.

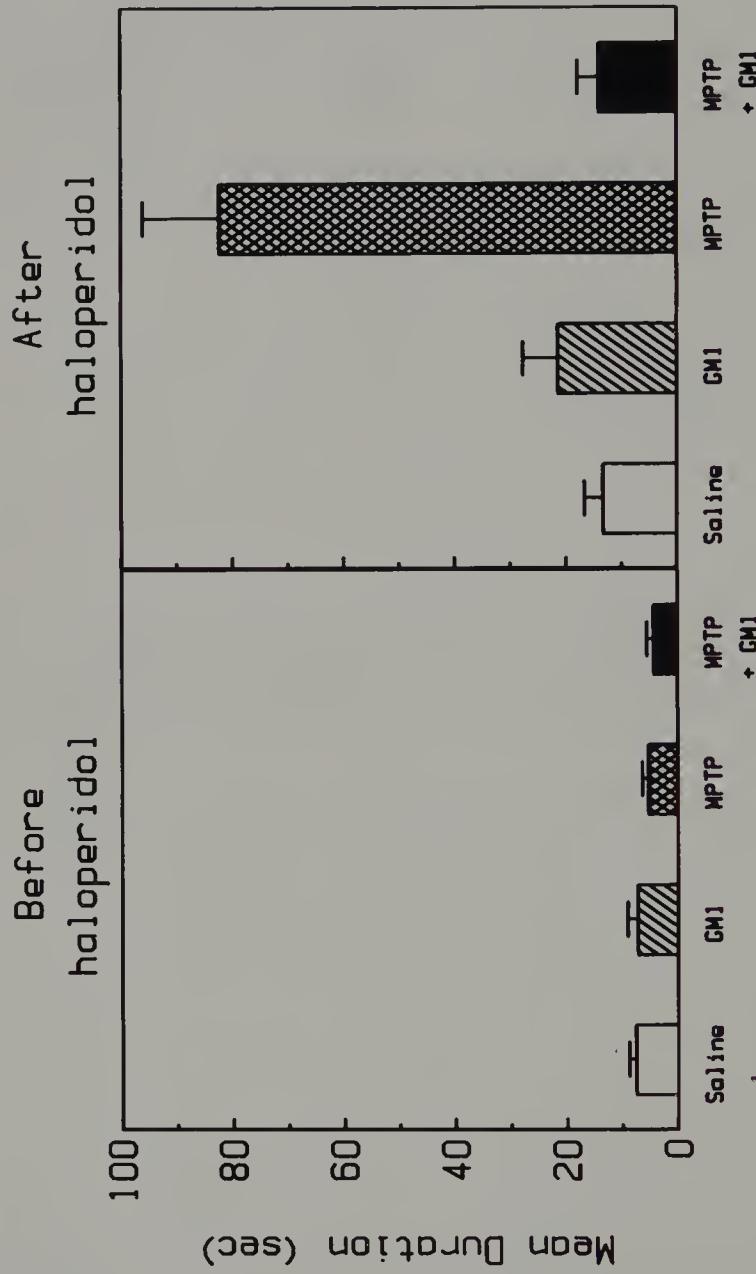
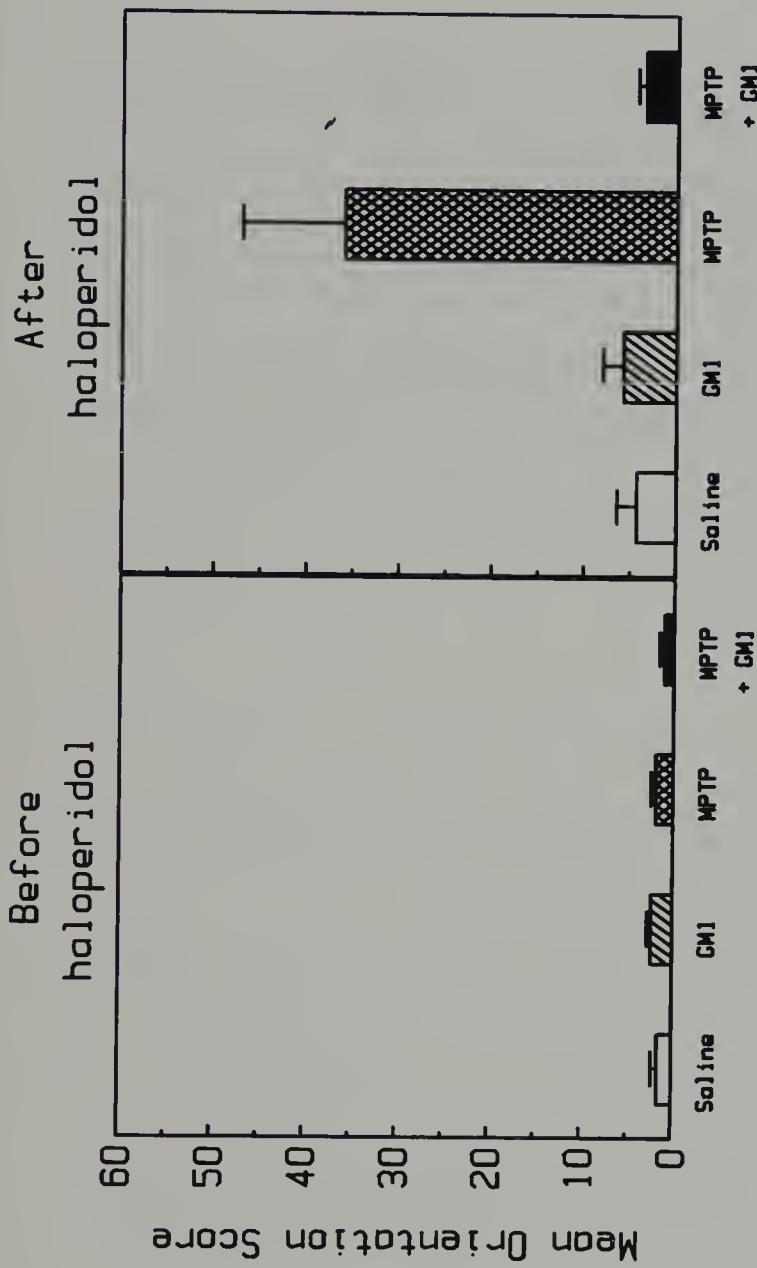


FIG. 1. Duration of akinesia induced by haloperidol in MPTP-lesioned mice. The mice were treated as described in Table 1. For the akinesia studies they were placed on a flat surface and the latency to move all four limbs were recorded in sec. One hour after the administration of haloperidol, 0.2 mg/kg ip, the animals were tested again and the scores recorded. Data are reported as the mean  $\pm$  SEM for 5–7 mice/group.



**FIG. 2.** Somatosensory orientation scores induced by haloperidol in MPTP-lesioned mice. The mice were treated as described in Table 1. For somatosensory orientation scores a Von Frey hair (Lafayette Instruments, Lafayette, IN) was used to determine both a behavioral and a sensitivity score for bilateral stimulation of the head. This test was performed before and after administering haloperidol, 0.2 mg/kg ip. Stimulation was continued at progressively increasing force (0.3–15 g pressure) until a behavioral response was elicited. Behavioral responses were rated as belonging to one of the following four categories: Type 1, locomotion away from the hair or sniffing and biting of the hair when stimulated; Type 2, scratching of the stimulated site; Type 3, a head turn in the direction of the stimulated site; and Type 4, no obvious orientation. For purposes of statistical analysis and graphic representation, somatosensory orientation was expressed as the product of the behavioral response (i.e., 1–4) and the threshold of force applied (i.e., 0.3–15 g). This resulted in scores ranging from 0.3 to 60, with increasing scores indicative of decreasing somatosensory orientation. Data are reported as the mean  $\pm$  SEM for 5–7 mice/group.

affinity. GM<sub>1</sub> treatment alone had no effect on the D-2 receptor binding characteristics. However, when the ganglioside was administered after MPTP, it prevented the development receptor up-regulation.

## CONCLUSIONS

There are now many reports that the administration of exogenous GM<sub>1</sub> ganglioside facilitates neuronal recovery after injury (24). Recovery of neuronal function after surgical, neurotoxin, or ischemic insult has been documented by various laboratories using biochemical, morphological, and behavioral measures. Our studies are consistent with these findings and they suggest that GM<sub>1</sub> ganglioside might be of significant value for treating parkinsonism. Based on the pharmacology of GM<sub>1</sub>, it appears that early recognition of the disease and initiation of treatment are critical. Early diagnosis might insure that sufficient dopaminergic neurons remain for a therapeutic effect. Chronic treatment with GM<sub>1</sub> appears essential as the response deteriorates when treatment is terminated. Whether the progression of parkinsonism might be reversed or stabilized remains to be evaluated.

Gangliosides are natural components of the outer surface of neuronal membranes and apparently play a role in membrane phenomena. They may be involved with receptor events and biotransformation of membrane-mediated information (8). Gangliosides contain a hydrophilic portion, the sialic acid oligosaccharide, which protrudes from the outer membrane surface, and a hydrophobic portion, the ceramide, which is inserted into the lipid core of neuronal membranes. The oligosaccharide portion offers a site for the selective binding of materials in the extracellular environment (10). For example, cholera toxin selectively binds to neuronal membrane GM<sub>1</sub> ganglioside (6). A subunit of the toxin then enters the cell and activates adenylate cyclase. Perhaps an endogenous neurotrophic factor structurally similar to the toxin binds to GM<sub>1</sub> and induces the recovery of function we have observed in the MPTP-lesioned mice.

The mechanism of action of GM<sub>1</sub> ganglioside for promoting the recovery of function after neuronal injury is unclear. A small fraction of exogenously administered GM<sub>1</sub> is incorporated into neuronal membranes (32). Based on *in vitro* and *in vivo* studies it is possible that it enhances the sprouting of regenerating nerves and/or accelerates repair of damaged neurons (24). GM<sub>1</sub> ganglioside may also reduce edema in a lesioned area and limit damage (21). Even though the mechanism is obscure, it is clear that the administration of GM<sub>1</sub> ganglioside facilitates the recovery of neuronal function after a variety of surgical and neurotoxin lesions.

## ACKNOWLEDGMENTS

The studies presented in this manuscript were supported in part by The American Parkinson's Disease Association, FIDIA Research Laboratories, and NIH grant

NS23627. GM<sub>1</sub> ganglioside was a gift from FIDIA Research Laboratories. Expert technical assistance was provided by Trina Wemlinger and Lori Isaacs.

## REFERENCES

1. Abercrombie M. Estimation of nuclear population from microtome sections. *Anat. Rec.* 1946;94:239-247.
2. Agnati LF, Fuxe K, Calza L, Goldstein M, Toffano G, Giardino L, Zoli M. Further studies on the effects of the GM<sub>1</sub> ganglioside on the degenerative and regenerative features of mesostriatal dopamine neurons. *Acta Physiol. Scand. [Suppl]* 1984;432:37-44.
3. Bradbury AJ, Costall B, Domeney AM, Jenner P, Kelly ME, Marsden CD, Naylor RJ. 1-methyl-4-phenylpyridine is neurotoxic to the nigrostriatal dopamine pathway. *Nature* 1986;319:56-57.
4. Burns SP, Chiueh CC, Markey SP, Ebert MH, Jacobowitz DM, Kopin IJ. A primate model of parkinsonism: selective destruction of dopaminergic neurons in the pars compacta of the substantia nigra by N-methyl-1,2,3,6-tetrahydropyridine. *Proc. Natl. Acad. Sci. USA* 1983;80:4546-4550.
5. Casamenti F, Bracco L, Bartolini L, Pepeu G. Effects of ganglioside treatment in rats with a lesion of the cholinergic forebrain nuclei. *Brain Res.* 1985;338:45-52.
6. Cuatrecasas P. Ganglioside and membrane receptors for cholera toxin. *Biochemistry* 1973;12:3558-3566.
7. Cuello AC, Stephens PH, Tagari PC, Sofroniew MV, Pearson RC. Retrograde changes in the nucleus basalis of the rat, caused by cortical damage, are prevented by exogenous ganglioside GM<sub>1</sub>. *Brain Res.* 1986;376:373-377.
8. Fishman PW. Role of membrane gangliosides in the binding and actions of bacterial toxins. *J. Membr. Biol.* 1982;69:85-98.
9. Fusco M, Dona M, Tessari F, Hallman H, Jonsson G, and Gorio A. GM<sub>1</sub> ganglioside counteracts selective neurotoxin-induced lesion of developing serotonin neurons in rat spinal cord. *J. Neurosci. Res.* 1986;15:467-479.
10. Gazzotti G, Sonnino S, Ghidoni R, Kirschner G, and Tettamanti G. Analytical and preparative high-performance liquid chromatography of gangliosides. *J. Neurosci. Res.*, 1984;12:179-192.
11. Hadjiconstantinou M, Neff NH. Treatment with GM<sub>1</sub> ganglioside increases rat spinal cord indole content. *Brain Res.* 1986;366:343-345.
12. Hadjiconstantinou M, Neff NH. Treatment with GM<sub>1</sub> ganglioside restores striatal dopamine in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated mouse. *J. Neurochem.* 1988;51:1190-1196.
13. Hadjiconstantinou M, Rossetti ZL, Paxton RC, Neff NH. Administration of GM<sub>1</sub> ganglioside restores the dopamine content in striatum after chronic treatment with MPTP. *Neuropharmacology* 1986;25:1075-1077.
14. Heikkila RE, Cabbat FS, Manzino L, Duvoisin RC. Effects of 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine on neostriatal dopamine in mice. *Neuropharmacology* 1984; 23:711-713.
15. Heikkila RE, Hess A, Duvoisin RC. Dopaminergic neurotoxicity of 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine in mice. *Science* 1984;224:1451-1453.

16. Heikkila RE, Manzino L, Cabbat FS, Duvoisin RC. Protection against the dopaminergic neurotoxicity of 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine by monoamine oxidase inhibitors. *Nature* 1984;311:467–469.
17. Heikkila RE, Nicklas WJ, Vyas I, Duvoisin RC. Dopaminergic toxicity of rotenone and the 1-methyl-4-phenylpyridinium ion after their stereotaxic administration to rats: implication for the mechanism of 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine toxicity. *Neurosci. Lett.* 1985;62:389–394.
18. Javitch JA, D'Amato RJ, Strittmatter SM, Snyder SH. Parkinsonism-inducing neurotoxin, N-methyl-4-phenyl-1,2,3,6 tetrahydropyridine: uptake of the metabolite N-methyl-4-phenylpyridine by dopamine neurons explains selective toxicity. *Proc. Natl. Acad. Sci. USA.* 1985;82:2173–2177.
19. Jonsson G, Gorio A, Hallman H, Janigro D, Kojima H, Zanoni R. Effect of GM<sub>1</sub> ganglioside on neonatally neurotoxin induced degeneration of serotonin neurons in the rat brain. *Brain Res.* 1984;318:171–180.
20. Karpiak SE. Exogenous gangliosides enhance recovery from CNS injury. *Adv. Exp. Med. Biol.* 1984;174:489–497.
21. Karpiak SE, Mahadik SP. Reduction of cerebral edema with GM<sub>1</sub> ganglioside. *J. Neurosci. Res.* 1984;12:485–492.
22. Kojima H, Gorio A, Janigro D, Jonsson G. GM<sub>1</sub> ganglioside enhances regrowth of noradrenaline nerve terminals in rat cerebral cortex lesioned by the neurotoxin 6-hydroxydopamine. *Neuroscience* 1984;13:1011–1022.
23. Langston JW, Langston EB, Irwin I. MPTP-induced parkinsonism in human and non-human primates: clinical and experimental aspects. *Acta Neurol. Scand. [Suppl]* 1984; 100:49–54.
24. Ledeen RW. Biology of gangliosides: neuritogenic and neuronotrophic properties. *J. Neurosci. Res.* 1984;12:147–159.
25. Nicklas WJ, Vyas I, Heikkila RE. Inhibition of NADH-linked oxidation in brain mitochondria by 1-methyl-4-phenyl-pyridine, a metabolite of the neurotoxin, 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine. *Life Sci.* 1985;36:2503–2508.
26. Nicklas WJ, Youngster SK, Kindt MV, Heikkila RE. MPTP, MPP<sup>+</sup> and mitochondrial function. *Life Sci.* 1987;40:721–729.
27. Ricaurte GA, Langston JW, Delaney LE, Irwin I, Brooks JD. Dopamine uptake blockers protect against the dopamine depleting effect of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in the mouse striatum. *Neurosci. Lett.* 1985;59:259–264.
28. Rossetti ZL, Sotgiu A, Sharp DE, Hadjiconstantinou M, Neff NH. 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and free radicals *in vitro*. *Biochem. Pharmacol.* 1988;37:4573–4574.
29. Sofroniew MV, Pearson RC, Cuello AC, Tagari PC, Stephens PH. Parenterally administered GM<sub>1</sub> ganglioside prevents retrograde degeneration of cholinergic cells of the rat basal forebrain. *Brain Res.* 1986;398:393–396.
30. Stephens PH, Tagari PC, Garofalo L, Maysinger D, Piotte M, Cuello AC. Neural plasticity of basal forebrain cholinergic neurons: effects of gangliosides. *Neurosci. Lett.* 1987;80:80–84.
31. Sternberger, LA. (1979): *Immunocytochemistry*. Wiley, Chichester, England; 104–169.
32. Tettamanti G, Venerando B, Roberti S, Chigorno V, Sonnino S, Ghidoni R, Orlando P, Massari P. The fate of exogenously administered brain gangliosides. In: Rappaport

- MM, Gorio A, eds. *Gangliosides in Neurological and Neuromuscular Function*. New York: Raven Press, 1981;225–240.
- 33. Tilson HA, Harry GJ, Nanry K, Hudson PM, Hong JS. Ganglioside interactions with the dopaminergic system of rats. *J. Neurosci. Res.* 1988;19:88–93.
  - 34. Toffano G, Agnati LF, Fuxe K, Aldinio C, Consolazione A, Valenti G, Savoini G. Effect of GM<sub>1</sub> ganglioside treatment on the recovery of dopaminergic nigro-striatal neurons after different types of lesion. *Acta Physiol. Scand.* 1984;122:313–321.
  - 35. Weihmuller FB, Hadjiconstantinou M, Bruno JP. Acute stress on neuroleptics elicit sensorimotor deficits in MPTP-treated mice. *Neurosci. Lett.* 1988;85:137–142.
  - 36. Wojcik M, Ulas J, Oderfeld-Nowak B. The stimulating effect of ganglioside injections on the recovery of choline acetyltransferase and acetylcholinesterase activities in the hippocampus of the rat after septal lesions. *Neuroscience* 1982;7:495–499.



# Degeneration and Regeneration of Basal Forebrain Cholinergic Neurons

A. C. Cuello, L. Garofalo, R. L. Kenigsberg, D. Maysinger,  
E. P. Pioro and A. Ribeiro-da-Silva

*Department of Pharmacology and Therapeutics, McGill University,  
Montreal, Quebec, H3G 1Y6 Canada*

The pioneering work of Ramon y Cajal (69) on the peripheral nervous system's capacity to regenerate after injury has been amply confirmed by subsequent investigators. This regenerative capacity of nervous tissue was thought for many decades to be restricted to peripheral neurons. However, recent experimental evidence has seriously challenged that contention. In this regard, Aguayo (2) and collaborators have unequivocally demonstrated the regenerative potential of central nervous system (CNS) neurons in a number of experimental situations. There is now a growing opinion that regenerative potential in the nervous system is governed by endogenously produced and released trophic factors. Nerve growth factor ( $\beta$ -NGF) can be taken as the prototype for such factors. NGF exerts well-defined trophic effects on distinct cells of the nervous system *in vivo* and *in vitro* (55). In addition to  $\beta$ -NGF's peripheral actions, it acts on subsets of centrally located neurons (49,56). This latter finding has added a new dimension to investigations on the role of putative trophic factors in facilitating brain repair mechanisms. Central forebrain cholinergic neurons which represent a subset of central NGF responsive neurons are suitable candidates for these investigations. There is a great deal of literature on their organization (for reviews see 7,15,24) and they appear to be affected in brains afflicted with Alzheimer's disease (6,17), a condition possibly caused by diminished trophic factor function (4,37). Forebrain cholinergic neurons contain NGF-binding sites (85) and their terminal targets produce the trophic factor which can be transported retrogradely to cell bodies of these neurons (85). Furthermore, in the adult, these cholinergic neurons respond to exogenous  $\beta$ -NGF after partial or total damage of the septo-hippocampal connections (36,50,93). Furthermore,  $\beta$ -NGF has also been found to affect forebrain cholinergic neurons *in vitro* (35,39).

In addition to NGF, a number of other endogenous substances display neurotrophic activity in the CNS in a variety of experimental situations (for review see 90). Gangliosides, particularly GM<sub>1</sub>, exert trophic-like activity, both *in vivo* and *in vitro* and resemble "bona fide" trophic factors in many ways (for review see 52).

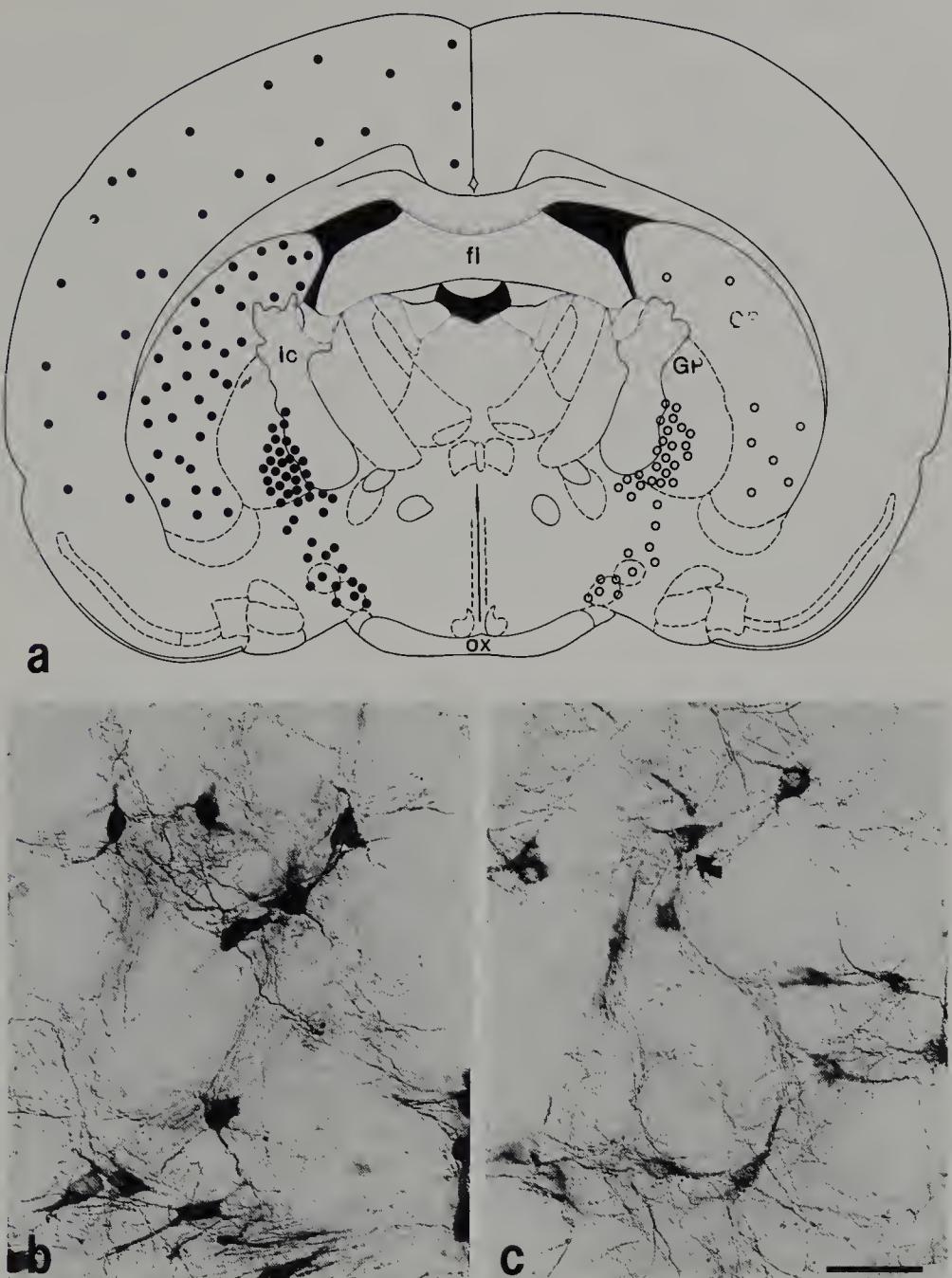
Applied *in vivo*, they promote the anterograde regeneration of acetylcholinesterase reactive fibers in the hippocampus after partial fimbria transections (67). Administration of GM<sub>1</sub> also prevents the retrograde cell shrinkage of cholinergic neurons of the NBM which follows cortical infarction (16), or cell death in the medial septum after unilateral hippocampal ablation (78). The mechanism for which gangliosides elicit these effects on cholinergic neurons and their possible interactions with NGF remain to be established. This chapter deals with the localization of NGF receptors in cholinergic neurons of the CNS; the actions of NGF and gangliosides on these neurons, both *in vivo* and *in vitro*; and discusses potential therapeutic strategies to preserve the integrity of CNS neurons in circumstances which would ultimately lead to cell degeneration.

## PRESENCE OF NGF-RECEPTOR IMMUNOREACTIVITY IN FOREBRAIN CHOLINERGIC NEURONS

Since the development of monoclonal antibodies that identify the NGF receptor (NGFR) in the nervous system of the rat (8) and primate (73), numerous studies have described the presence of NGFR immunoreactivity (IR) in magnocellular basal forebrain neurons of both these species (18,20,30,40,46,48,59,65,74,77,80, 84,94). The cellular pattern of NGFR immunostaining appears to correlate very well with the distribution of NGF-binding sites in autoradiographic studies (70,71,72), although co-localization studies have not been performed. Nevertheless, the responsiveness of these neurons to NGF in the developing (28,64), aged (25), and lesioned (12,26,38,50,93) rat indicates they possess the high affinity form of the NGFR which is necessary for internalization of NGF (42).

Most NGFR immunoreactive basal forebrain neurons, e.g., in the NBM, appear to be cholinergic (Fig. 1a). This has been demonstrated using acetylcholinesterase histochemistry (40,80) and choline acetyltransferase (ChAT) immunocytochemistry (18,48). In contrast, only few NGFR-positive neurons occur in the striatum of the rat (30,46) and monkey (48) where numerous cholinergic neurons occur (15,43) (Fig. 1a). Multiple cholinergic neurons of the rat caudate putamen, however, possess NGF-binding sites (70,71) and are responsive to exogenous NGF in the developing (63) and injured adult (27) animal. No NGFR immunoreactive neurons have been described in the rat neocortex, whereas ChAT-containing interneurons are plentiful (43) (Fig. 1a).

The light microscopic appearance of ChAT and NGFR immunoreactivities in neurons of the rat NBM are distinct (Fig. 1b and 1c) and have been recently reported (46). The reaction product for ChAT is homogeneously distributed throughout the cell somata and neurites with occasional visualization of the non-immunoreactive nuclear region (Fig. 1b). In contrast, NGFR-IR which is also diffusely distributed throughout the cytoplasm, occurs mostly in granular form over the cell somata and processes (Fig. 1c). A distinctive feature of NGFR-containing basal forebrain neurons is the frequent concentration of reaction product in the immediate perinuclear area.



**FIG. 1.** **a:** Schematic representation of coronally sectioned rat forebrain at the level of the mid NBM. Choline acetyltransferase immunoreactive neurons, represented as solid dots in the left half of the brain, are numerous throughout the NBM (innermost part of globus pallidus: GP), caudate putamen (CPu), and neocortex (Cx). Open circles in the right half of the brain represent NGFR immunoreactive neurons which are also numerous in the NBM but rare in the CPu and absent in the Cx. Fimbria, fl; internal capsule, ic; optic chiasm, ox. Schematic diagram modified from Paxinos & Watson (68). **b** and **c:** Light microscopic appearance of ChAT immunoreactive (**b**) and NGFR immunoreactive (**c**) neurons in the NBM. ChAT immunoreactivity occurs as dense homogeneous staining throughout the cell somata and proximal neurites whereas the NGFR reaction product appears mostly granular with a prominent perinuclear rim of staining seen in some neurons (*curved arrow in c*). Interference contrast transmission optics. Scale bars in **b** and **c**, 60  $\mu\text{m}$ .

At the electron microscopic level, NGFR in putatively cholinergic NBM neurons is found along the plasma membrane of the cell soma and neurites (Fig. 2a). Intracellular immunostaining occurs in cisterns of the rough endoplasmic reticulum and over the nuclear membrane (Fig. 2b). Other intracytoplasmic structures where NGFR-IR is seen include the Golgi apparatus (Fig. 2c), small vesicles which are sometimes in contiguity with the plasma membrane (Fig. 2d) and multivesicular bodies (Fig. 2e). This distribution of NGFR-IR reaction product is compatible with sites of protein synthesis from the initial ribosomal step of polypeptide synthesis to subsequent modification in the Golgi apparatus and packaging into vesicles for transport to the cell membrane (3). Some membrane-related immunoreactive vesicles possess a coating which suggests an endocytotic origin (29). Studies with PC12 cells have shown that radioactive NGF is internalized and transported in membrane-bound compartments that fuse with lysosomes (5,41). It is therefore possible that the NGFR-IR coated vesicles observed correspond to NGF-NGFR complexes being internalized, while the immunoreactive multivesicular bodies represent the intralysosomal degradative stage of such complexes.

## **NERVE GROWTH FACTOR AND GANGLIOSIDE IN VIVO EFFECTS ON DAMAGED CHOLINERGIC NEURONS**

Retrograde cell shrinkage and loss of neurites of the rat NBM cholinergic neurons becomes apparent in the rat 30 days after cortical damage (79). The immunohistochemical signs of neuronal degeneration are accompanied by a pronounced decrease in the levels of ChAT activity in microdissected samples of the NBM (82) while no changes are observed in other CNS nuclei examined. These biochemical and anatomical degenerative changes can be completely prevented in cortically lesioned rats by administering large doses (30 mg/kg/day, ip) of the ganglioside GM<sub>1</sub> for 30 days, beginning immediately post-lesion. Transsection of the fimbria-fornix or removal of the hippocampus leads to the apparent loss of cholinergic neurons of the medial septum. These cell losses can be prevented to a large extent by the intracerebroventricular (i.c.v.) administration of NGF (36,50,93). A similar effect can be observed in the same animal model with the intraperitoneal administration of the ganglioside GM<sub>1</sub> (78).

More recently, we have compared the actions of NGF with those of gangliosides in the NBM-cortex model of cholinergic injury. We have observed that the i.c.v. administration of  $\beta$ -NGF for 7 days, beginning at the time of lesioning in doses of 12  $\mu$ g/day, prevents the decrease in ChAT activity in the NBM after partial cortical infarction (13,14). The magnitude of this protective effect was shown to be comparable to that obtained with the i.c.v. administration of GM<sub>1</sub> alone (5 mg/kg/day) (Table 1). The combined administration of  $\beta$ -NGF and GM<sub>1</sub> in the decorticated animals increased ChAT activity in the ipsilateral NBM above control levels (Table 1). Immunocytochemical analysis revealed not only full protection

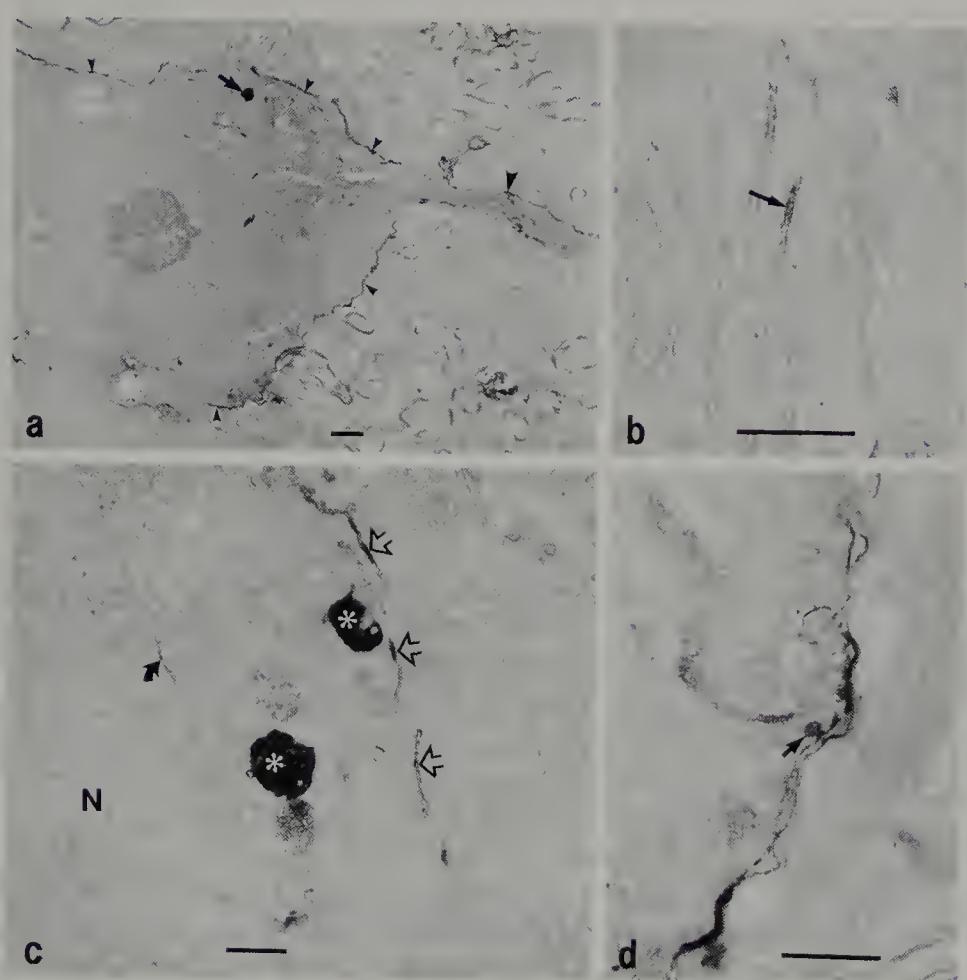


FIG. 2. Electron microscopic detection of NGFR immunoreactivity in putative cholinergic neurons of the rat nucleus basalis magnocellularis. a: Low magnification micrograph demonstrating immunoprecipitate on the plasma membrane of the cell body (small arrowheads) and its neuritic process (larger arrowhead). At higher magnification, immunostaining is seen in cisternae of the rough endoplasmic reticulum (arrow in b), along the nuclear membrane (curved arrow in c), in Golgi cisternae (open arrows in c), and multivesicular bodies (asterisks in c). d: An NGFR-immunoreactive vesicle, which is likely coated (arrow), appears contiguous with the immunostained plasma membrane. All micrographs in Figs. 1 and 2 were obtained from rats perfused with a 4% paraformaldehyde-1.0% glutaraldehyde mixture and immunostained with a peroxidase anti-peroxidase protocol. Monoclonal antibodies against rat NGFR (8) and ChAT (21) were used. Scale bars; a, 1  $\mu\text{m}$ ; b, c and d, 0.5  $\mu\text{m}$ .

**TABLE 1.** Effect of NGF administered in combination with an effective dose of GM<sub>1</sub> (5 mg/kg/day, 7 days) on ChAT activity in the NBM and cortex of mature rats, 30 days after unilateral decortication

Group	n	Ipsilateral NBM		Ipsilateral Cortex	
		ChAT activity	%Control	ChAT activity	%Control
Control	6	57.67 ± 3.86	—	35.81 ± 2.39	—
Lesion + Vehicle	6	31.16 ± 3.17	54*	35.85 ± 1.74	100
Lesion + GM <sub>1</sub>	5	61.94 ± 6.55	107	50.70 ± 2.44	142*
Lesion + NGF	5	50.94 ± 3.75	88	47.63 ± 3.12	132*
Lesion + GM <sub>1</sub> + NGF	5	69.41 ± 1.06	120*	84.82 ± 10.42	237*

Values for ChAT activity are the mean ± SEM, and expressed as nmol/mg protein/hr; n indicates number of cases, \* significantly different from control at  $p < 0.01$ , ANOVA followed by a post-hoc Dunnett's test. (From ref. 12.)

Abbreviations: NGF, nerve growth factor; GM<sub>1</sub>, ganglioside GM<sub>1</sub>; ChAT, choline acetyltransferase; NBM, nucleus basal magnocellularis.

of the cholinergic neurons from retrograde cell shrinkage and loss of neuritic extensions but also showed an apparent increase in the number of ChAT immunoreactive processes in the neuropile.

In the aforementioned experiments, ChAT activity in the remaining ipsilateral cortex of lesioned untreated animals did not differ significantly from that on the unlesioned contralateral side. Treatment with either  $\beta$ -NGF or GM<sub>1</sub> increased ChAT activity in the remaining ipsilateral cortex over that of control levels. Furthermore, when their administration was combined, ChAT activity in the remaining ipsilateral neocortex increased to over 200% of control values, suggesting a cooperativity between these two factors (Table 1).

The view of a cooperative interaction of NGF and ganglioside is reinforced by observations made in cortically-lesioned animals that were treated with low doses of GM<sub>1</sub> (0.5 mg/kg/day, i.c.v., 7 days) which did not protect ipsilateral NBM cholinergic neurons (see Table 2). However, if these subtherapeutic amounts of ganglioside were administered concurrently with effective doses of  $\beta$ -NGF to lesioned animals, an increase in ChAT activity over that shown when  $\beta$ -NGF was given alone was evident both in the affected forebrain cholinergic neurons and ipsilateral neocortex (12).

## NERVE GROWTH FACTOR AND GANGLIOSIDE IN VITRO EFFECTS ON DISSOCIATED CHOLINERGIC SEPTAL NEURONS

In an attempt to investigate in a more controlled system the effects of two putative trophic substances (NGF and GM<sub>1</sub>), and observe any possible interactions

**TABLE 2.** Effect of NGF administered in combination with an ineffective dose of GM<sub>1</sub> (0.5 mg/kg/day, 7 days) on ChAT activity in the NBM and cortex of mature rats, 30 days after unilateral decortication

Group	n	Ipsilateral NBM		Ipsilateral Cortex	
		ChAT activity	%Control	ChAT activity	%Control
Control	6	69.06 ± 4.67	—	39.20 ± 3.77	—
Lesion + Vehicle	6	44.87 ± 6.60	65*	38.20 ± 4.69	97
Lesion + GM <sub>1</sub>	5	46.92 ± 2.80	68*	36.93 ± 2.80	94
Lesion + NGF	5	73.07 ± 3.30	109	59.06 ± 2.90	151*
Lesion + GM <sub>1</sub> + NGF	5	83.87 ± 6.56	121*	72.98 ± 4.08	186*

Values for ChAT activity are the mean ± SEM, and expressed as nmol/mg protein/hr; n indicates number of cases, \* significantly different from control at  $p < 0.01$ , ANOVA followed by a post-hoc Dunnett's test. For abbreviations see Table 1. (From ref. 12.)

between them, we employed cell culture techniques. Dissociated septal cells maintained in monolayer culture in the presence of serum-supplemented media represent a mixed neuronal-glial cell population (39). This was confirmed immunocytochemically by applying markers such as anti-GFAP (glial fibrillary acidic protein) and anti-neurofilament antiserum. The presence of ChAT-immunoreactive neurons in the culture system was also confirmed with the use of an anti-ChAT monoclonal antibody (21). ChAT activity in these septal cells was found to be modulated by either  $\beta$ -NGF or GM<sub>1</sub>. A dramatic increase in ChAT activity was noted after a 7-day exposure to exogenous  $\beta$ -NGF. These observations were in agreement with those of Hatanaka and Tsukui (35) in that the increase in enzyme activity was dose-related. The increase in ChAT activity was detectable at  $\beta$ -NGF concentrations as low as  $10^{-13}$  and was maximal in the nanomolar range (12).

In this mixed glial-neuronal culture, GM<sub>1</sub> produced a moderate (15–30% over control) increase in ChAT activity. In the serum-supplemented mixed cultures, enzyme induction was only detected when cells were exposed to  $10^{-6}$ – $10^{-5}$  M GM<sub>1</sub>. Lower (e.g.,  $10^{-7}$  M) concentrations of GM<sub>1</sub> were found to be ineffective when applied alone to these cultures.

When effective concentrations of GM<sub>1</sub> ( $10^{-5}$  M) were added in combination with  $\beta$ -NGF, an obvious potentiation of the effects of the latter factor was observed (Fig. 3a). In combination with  $10^{-5}$  M GM<sub>1</sub>, submaximal ( $10^{-13}$  M) and maximal ( $10^{-9}$  M) concentrations of  $\beta$ -NGF produced an increase in ChAT enzymatic activity which was significantly greater than that obtained with  $\beta$ -NGF alone. This potentiating effect of GM<sub>1</sub> was most evident when applied in combination with submaximal concentrations of  $\beta$ -NGF. The idea of cooperativity between these factors was further supported by the finding that ineffective GM<sub>1</sub> ( $10^{-7}$  M) concentrations potentiated the  $\beta$ -NGF-induced increase in ChAT activity (Fig. 3b).

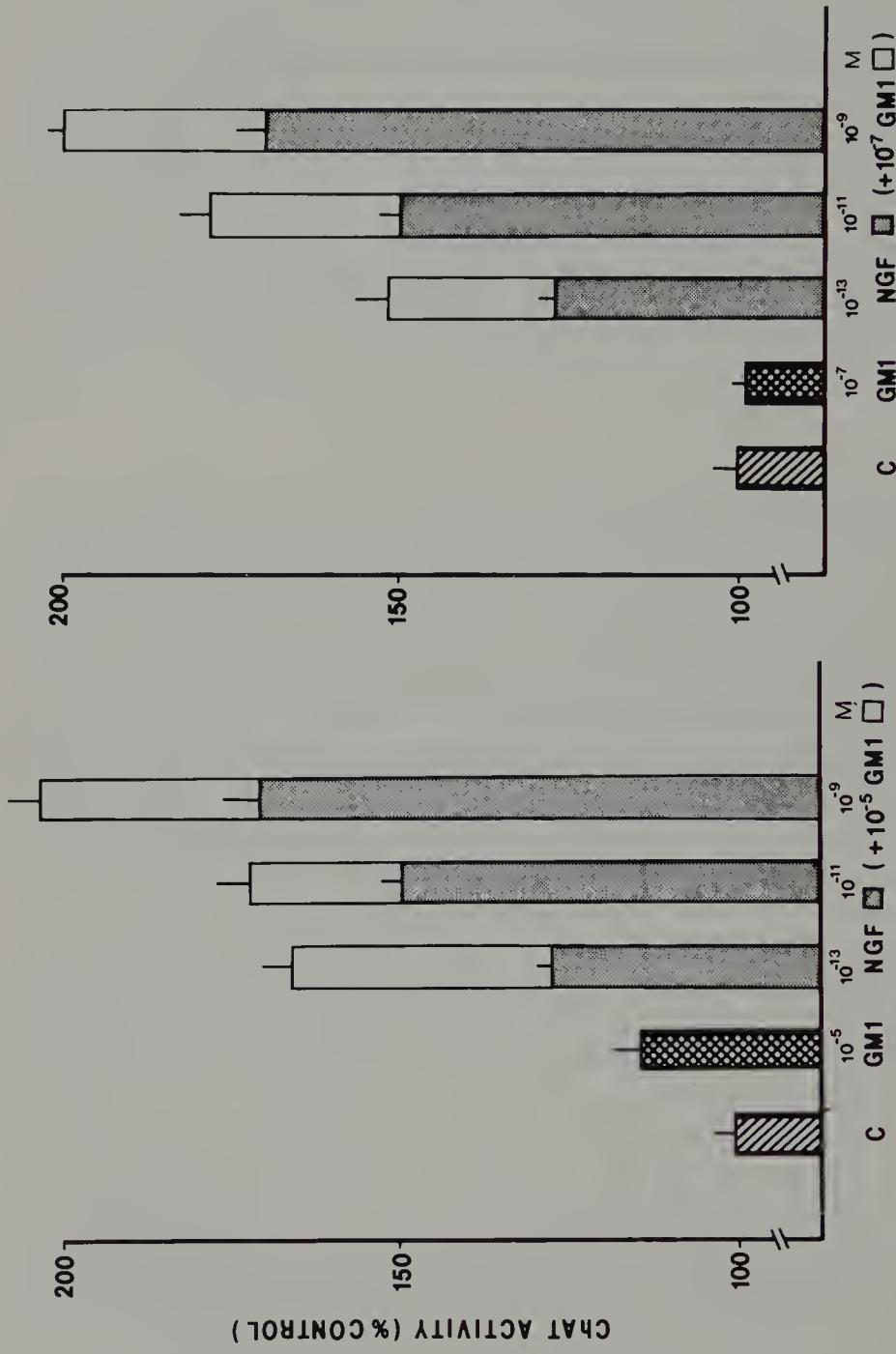


FIG. 3. Effects of GM<sub>1</sub>, alone and in combination with various concentrations of  $\beta$ -NGF on ChAT activity in cultures of dissociated septal cells. Septal cultures were grown in the absence (control), in the presence of GM<sub>1</sub> (▨), or in varying concentrations of  $\beta$ -NGF alone (▨) or in combination with GM<sub>1</sub> (▨) for 7 days. Values represent the means  $\pm$  SEM from quadruplicate culture wells from sister culture preparations. Control absolute values were 4.5 nmol of acetylcholine/mg protein/hr.

## EXPERIMENTAL THERAPEUTICS OF DAMAGED CHOLINERGIC NEURONS: PERMISSIVE CONDITIONS AND APPLICATION OF MICROENCAPSULATED GANGLIOSIDES

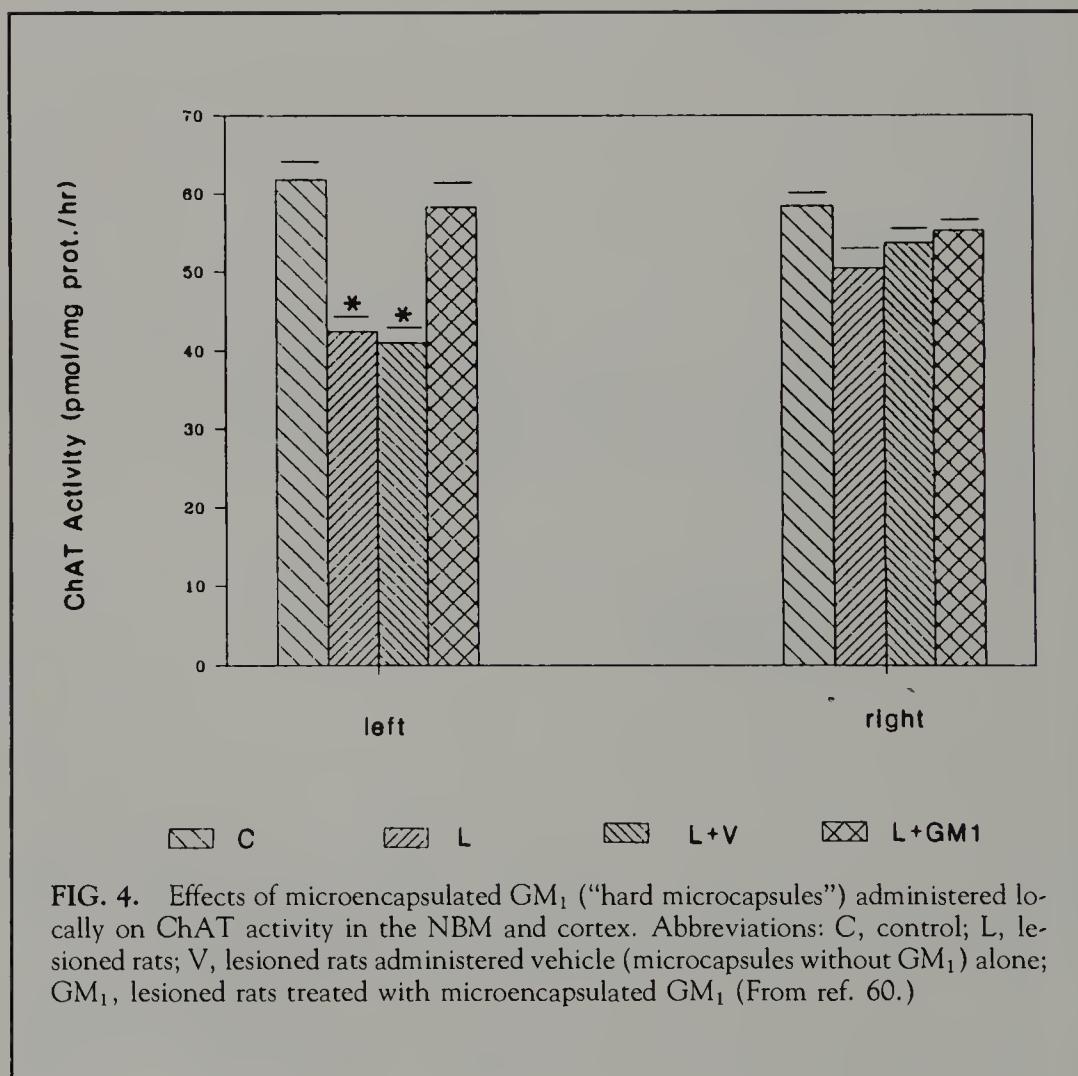
It is interesting to note that certain conditions are required for gangliosides to show a trophic effect *in vivo* or *in vitro*. These have been referred to as "permissive conditions" for the *in vivo* effects (12,83) or a "window of opportunity" for the *in vitro* effects (91). It is conceivable that, under certain conditions, the availability of endogenous trophic factors is affected and consequently the ability of cells to respond to these factors is influenced. In the *in vivo* experimental model the early initiation of ganglioside treatment is essential for prevention and repair of the cholinergic neurons (83). Thus, we have observed that a delay of 10 days renders the ganglioside ineffective in preventing retrograde cholinergic degeneration (83). An analogous observation has been made for the anterograde degeneration of dopaminergic neurons of the substantia nigra in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model (32). These observations are supported by the findings that, in response to injury, the brain produces low amounts of endogenous trophic factors immediately after the insult (for example see 66). Therefore, in instances of extensive neural lesions, cells are probably in an extremely vulnerable state which could result in irreversible anterograde and retrograde cellular damage. In central cholinergic neurons, retrograde degenerative changes can be partly reversed with the timely administration of  $\beta$ -NGF (36,50,93) even if initiation of treatment is delayed for 14 days after injury (33). In view of the above, it can be proposed that the *in vivo* administration of gangliosides prevents neuronal degeneration by potentiating the actions of the low amounts of endogenous trophic factors produced in the first few days after a lesion. The lack of protective effects by exogenous gangliosides on cholinergic neurons in lesioned aged rats (83) can be explained in the same way since aging is accompanied by an apparent loss of  $\beta$ -NGF receptors (47) and a diminished production of endogenous factors after injury (92).

In order to maximize the constant release of putative trophic factors, we have tested the administration of GM<sub>1</sub> and  $\beta$ -NGF via permanent cannulae implanted into the lateral ventricle (experiments discussed above). Furthermore, with the prospect of a potential application in human therapy, we have assessed the use of microencapsulated gangliosides (60). Animals which had been cortically lesioned were immediately treated with either microencapsulated GM<sub>1</sub> or human serum albumin (HSA)-microcapsules containing no drug. In these experiments, both readily degradable (soft) and more resistant (hard) microcapsules were assessed. Ten and 30 days after unilateral cortical devascularizing lesions, animals were sacrificed and ChAT enzymatic activity was measured in various microdissected brain areas. GM<sub>1</sub> prepared in "soft" capsules at 90°C did not protect forebrain cholinergic neurons. The ultrastructural morphology of these microcapsules resembled that of "hard" capsules obtained at 150°C, but their stability was considerably lower. They decomposed in a physiological medium and phosphate buffer (at 37°C) in less than

24 hr. In our preliminary tests, the "soft" and rapidly degradable GM<sub>1</sub> preparation did not prevent the decrease of ChAT activity in the NBM and had no effect in the remaining ipsilateral cortex either at 10 or 30 days post-operatively.

Comparable amounts of GM<sub>1</sub> incorporated into "hard" HSA-microcapsules prepared at 150°C prevented the decrease of ChAT activity in the NBM after cortical lesions. The effect of this more stable HSA-microencapsulated GM<sub>1</sub> on ChAT enzymatic activity in the NBM, and the remaining cortex adjacent to the lesion, is illustrated in Fig. 4.

In those studies, immunocytochemistry confirmed that ChAT-positive neurons in the NBM of lesioned animals were significantly smaller than in the control group or lesioned animals which received GM<sub>1</sub> locally in the encapsulated form, as measured by an automatic image analysis system. In unlesioned animals, the mean cross-sectional area of such cells was approximately  $285 \pm 10.6 \mu\text{m}^2$ , whereas in animals with unilateral cortical lesions, the average cross-sectional area of these



**FIG. 4.** Effects of microencapsulated GM<sub>1</sub> ("hard microcapsules") administered locally on ChAT activity in the NBM and cortex. Abbreviations: C, control; L, lesioned rats; V, lesioned rats administered vehicle (microcapsules without GM<sub>1</sub>) alone; GM<sub>1</sub>, lesioned rats treated with microencapsulated GM<sub>1</sub> (From ref. 60.)

neurons ipsilaterally was significantly smaller ( $223 \pm 8.7 \mu\text{m}^2$ ) in lesioned rats treated with HSA-microcapsules without GM<sub>1</sub>). However, after treatment with encapsulated GM<sub>1</sub>, cholinergic neurons in the NBM retained a relatively normal shape and size (the average cross-sectional area:  $277 \pm 11.5 \mu\text{m}^2$ ) and were comparable with those observed in control animals. These initial experiments on delivery of putative trophic factors from biodegradable microcapsules, and the current information on "permissive circumstances" for trophic factor therapy, offer a useful basis to consider possible treatment strategies in humans.

## CONCLUDING REMARKS

The trophic effects of  $\beta$ -NGF on central and peripheral neurons are, in all likelihood, receptor mediated. Although, there is ample evidence for the presence of NGF receptors on cholinergic neurons of the NBM and medial septum (18,30,40,46,48,59,70,71,80,94), there is little information on the cellular and molecular events which take place after the interaction of  $\beta$ -NGF with its receptor (for review, see 57,81). The sequence of events which follow this receptor-ligand interaction may include induction of specific genes (31,51,54,62) and second messenger systems (11,34) which results in the translation and/or modification of specific proteins involved in trophic responses. The interaction of gangliosides with endogenous trophic factors, and with  $\beta$ -NGF in particular, could occur at various levels. Nevertheless, it is very likely that this interaction occurs at the level of the cell membrane where both NGF (42) and GM<sub>1</sub> (86) are incorporated. In this regard, it is interesting to note that immobilized GM<sub>1</sub> is capable of binding  $\beta$ -NGF with low affinity (76). Thus, gangliosides could provide additional binding sites for growth factors or, alternatively, modify the state of the growth factor receptor as is the case for the ganglioside GD<sub>2</sub> and the vitronectin receptor in the plasma membrane of human melanoma cells (10).

The opportunity for a cooperative interaction between gangliosides and  $\beta$ -NGF in the *in vivo* cholinergic model is highlighted by the occurrence of an increase in  $\beta$ -NGF levels in the target areas of basal forebrain cholinergic neurons after mechanical lesions (for review see 92). However, ganglioside actions on dopaminergic neurons (1,87) cannot be explained simply on this basis since NGF is apparently not a trophic agent for dopaminergic neurons (75). It is possible that one or more factors are trophic for this subset of CNS neurons. In fact, one such factor has already been postulated (23,88). As a preliminary hypothesis, one can propose that diverse subsets of CNS neurons may be under the specific trophic influence of very selective factors such as NGF, while certain nonselective endogenous substances such as gangliosides could influence their actions. The limited specificity and selectivity of gangliosides as compared to  $\beta$ -NGF is highlighted by the fact that in the *in vivo* model the GM<sub>1</sub>/NGF molar ratio for equivalent protection of CNS cholinergic neurons is 4,050:1. The examples discussed here are of "positive"

cooperativity between these selective (NGF) and nonselective (gangliosides) trophic factors. Nonetheless, the possibility exists for "negative" cooperativity. In other words, gangliosides may act in "enabling" or "disabling" a number of trophic factor receptor-mediated responses.

From the pharmacological point of view, the concept that gangliosides potentiate  $\beta$ -NGF-mediated effects on cholinergic neurons is further supported by *in vitro* studies where concentrations of the two factors can be accurately controlled. It has been shown in some cell culture systems that the trophic actions of gangliosides are dependent upon the presence of  $\beta$ -NGF (19). Glial cells might play a role in this interaction. For example, neuroglia have been found to produce neurotoxic factors (53) and neurotrophic (NGF-like) substances (35). Release of neurotoxic substances can be affected by ion fluxes (53) and the same may be true for neurotrophic factors. In this regard, gangliosides, which have been shown to affect ion fluxes, may regulate the release of such factors from neuroglia. *In vivo*, neural damage results in reactive gliosis (44), a phenomenon which may contribute to the increased availability of endogenous trophic factors or, alternatively, neural damaging factors. Consequently, the contribution of glial cells is important both *in vivo* and *in vitro*.

In our *in vivo* cholinergic model, the effects of  $\beta$ -NGF and/or GM<sub>1</sub> on ChAT activity in the remaining neocortex may be due either to increased production of the biosynthetic enzyme or sprouting of terminals with resultant reorganization of cholinergic fibers. However, it should be noted that, in cortically damaged rats which have been treated with gangliosides, release of acetylcholine is enhanced from the remaining cortex (61). Such animals also show an improved behavioral performance over lesioned untreated animals in passive avoidance or Morris water maze tests (22). These aforementioned points are of potential clinical relevance in the quest to improve cortical cholinergic function in cases of senile dementia. Whether victims of Alzheimer's disease could benefit from such a treatment strategy remains to be seen. Clinical trials based on principles discussed here, which are currently underway in Göteborg, Sweden, would shed some light on this issue.

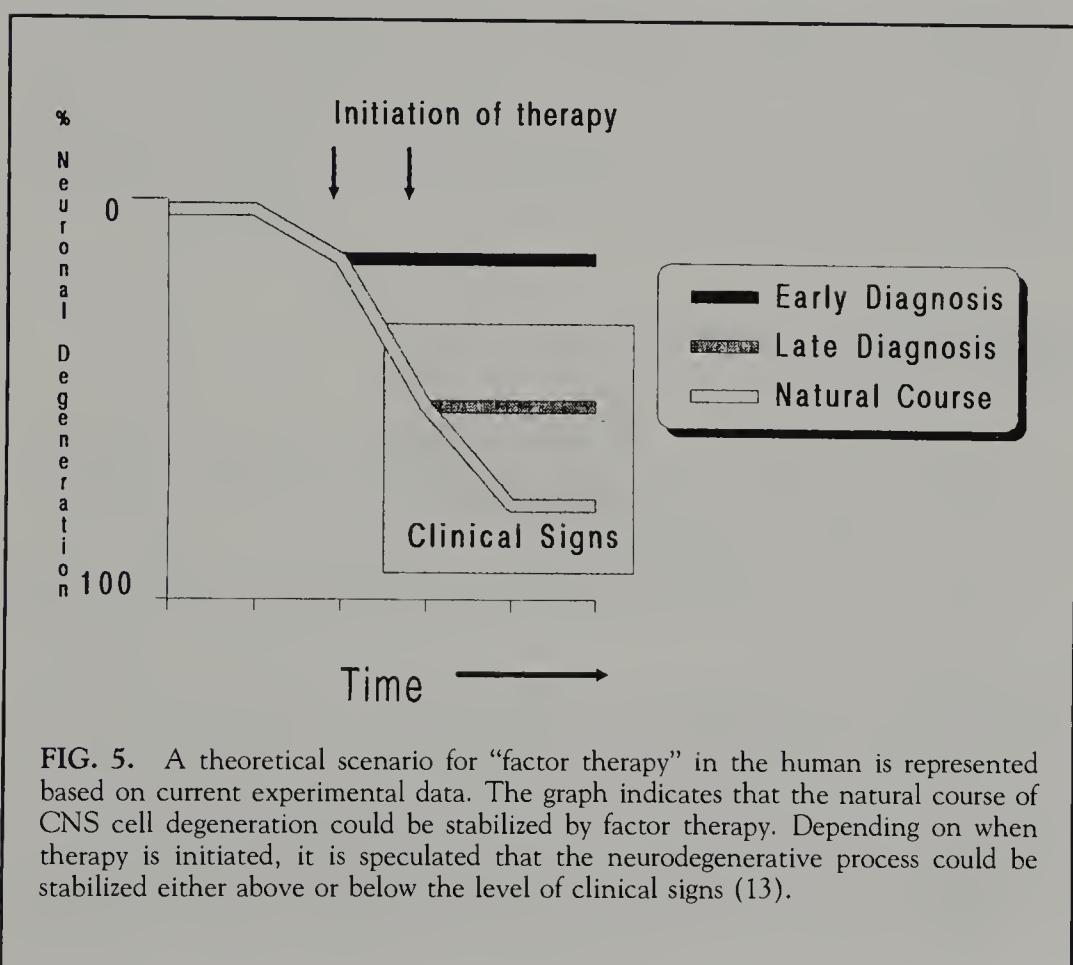
To validate the ganglioside-trophic factor cooperativity hypothesis, further investigations on the molecular mechanisms underlying their interactions in the central and peripheral nervous systems are required. There is already evidence for the cooperativity of  $\beta$ -NGF and gangliosides in the peripheral nervous system (89). Investigation of the interaction of gangliosides with  $\beta$ -NGF and other trophic factors may provide valuable insight for the establishment of novel therapeutic regimes in neurodegenerative diseases. In this regard, the experiments on microencapsulated putative trophic factors affecting CNS structures and functions could provide a less invasive method of administering drugs with the additional advantage that readily hydrolysable drugs would be protected from rapid degradation. A variety of microencapsulation techniques have been developed (9,58,95). Biodegradable microcapsules offer certain advantages over those which are non-biodegradable since removal of the encapsulation material is unnecessary once the drug is spent. Our efforts have focused on developing and testing a preparation of HSA-micro-

capsules for the sustained and controlled release of gangliosides *in vivo*. This strategy could, in the future, be used clinically for the prolonged delivery of trophic factors which would play a preventive or reparative role in brain diseases.

A theoretical scenario for "trophic factor therapy" is proposed based on experimental data already available (Fig. 5). In such a scheme, it is assumed that clinical signs of certain degenerative diseases of the nervous system, for example Alzheimer's or Parkinson's diseases, are dependent on the degeneration or loss of a critical number of specific neurons. Clinical signs and symptoms usually appear when these losses have reached considerable proportions. Initiation of "trophic factor therapy" at that point could, at best, prevent further progression of the disease. Ideally, the achievement of early diagnosis would allow initiation of treatment prior to the onset of obvious clinical disease, thereby providing the greatest opportunity for neural recovery and normal functioning.

## SUMMARY

This chapter revises evidence for the cellular localization of a nerve growth factor (NGF) receptor in cholinergic neurons of the rat forebrain, and provides novel



evidence for its subcellular localization in the cell membrane of neurons of the nucleus basalis magnocellularis (NBM) and in the organelles of these cells. This evidence supports the idea of a physiological role of NGF in adult rats. Studies on the retrograde degeneration of forebrain cholinergic neurons discussed in this chapter have provided convincing experimental data consistent with the sustaining and reparative role of NGF in these neurons after the loss of target site and/or distal axonotomy. Besides NGF, the ganglioside GM<sub>1</sub> has been shown to protect cholinergic neurons from retrograde degeneration. The protective effects of GM<sub>1</sub> are conditional upon factors such as dosage, timing of administration, and age of recipient animals. The studies presented here also provide evidence for a positive cooperativity between gangliosides over central nervous system cholinergic neurons, both *in vivo* and *in vitro*. Finally, strategies for administration of trophic factors in hypothetical therapeutic scenarios are discussed.

## ACKNOWLEDGMENTS

The authors would like to acknowledge support from the Medical Research Council of Canada, the office of the Dean, Faculty of Medicine (McGill University), and partial assistance from FIDIA Research Laboratories. The editorial and secretarial assistance of Jennifer Seguin, Diane Leggett, and Mary Babineau, as well as the technical expertise of Sylvain Côté and Alan Forster, is gratefully appreciated. Lorella Garofalo is the recipient of a studentship provided by Fonds de la Recherche Scientifique (Quebec).

## REFERENCES

1. Agnati LF, Fuxe K, Calza L, Benfenati F, Cavicchioli L, Toffano G, Goldstein M. Gangliosides increase the survival of lesioned nigral dopamine neurons and favour the recovery of dopaminergic synaptic function in striatum of rats by collateral sprouting. *Acta Physiol Scand.* 1983;119:347-363.
2. Aguayo AJ. Axonal regeneration from injured neurons in the adult mammalian central nervous system. In: Cotman CW, ed. *Synaptic Plasticity* 1985. New York: Guilford Press, 1985;457-484.
3. Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson JD. Internal membranes and the synthesis of macromolecules. In: *Molecular Biology of the Cell* 1983. New York, London: Garland Publishing, Inc., 1983;319-384.
4. Appel SH. A unifying hypothesis for the cause of amyotrophic lateral sclerosis, Parkinsonism, and Alzheimer's disease. *Ann. Neurol.* 1981;10:499-505.
5. Bernd P, Greene LA. Electron microscopic radioautographic localization of iodinated nerve growth factor bound to and internalized by PC12 cells. *J. Neurosci.* 1983;3:631-643.

6. Bowen DM, Smith CB, White P, Davison AN. Neurotransmitter-related enzymes and indices of hypoxia in senile dementia and other abiotrophies. *Brain* 1976;99:459–496.
7. Butcher LL, Woolf NJ. Histochemical distribution of acetylcholinesterase in the central nervous system: Clues to the localization of cholinergic neurons. In: Björklund A, Hökfelt T, Kuhar MJ, eds. *Handbook of Chemical Neuroanatomy*. Vol. 3: *Classical Transmitters and Transmitter Receptors in the CNS, Part II*, 1984. Amsterdam: Elsevier Science Publishers B.V., 1984;1–50.
8. Chandler CE, Parsons LM, Hosang M, Shooter EM. A monoclonal antibody modulates the interaction of nerve growth factor with PC12 cells. *J. Biol. Chem.* 1984; 259:6882–6889.
9. Chang TMS. *Microencapsulation and artificial cells*. Clifton, New Jersey: Humana Press, 1984.
10. Cheresh DA, Pytela R, Pierschbacher D, Klier FG, Ruoslahti E, Reisfeld RA. An Arg-Gly-Asp-directed receptor on the surface of human melanoma cells exists in a divalent cation-dependent functional complex with the disialoganglioside GD2. *J. Cell Biol.* 1987;105:1163–1173.
11. Cremins J, Wagner JA, Halegouia S. Nerve growth factor action is mediated by cyclic AMP and  $\text{Ca}^{+2}$ /phospholipid dependent protein kinases. *J. Cell. Biol.* 1986;103:887–893.
12. Cuello AC, Garofalo L, Kenigsberg RL, Maysinger D. Gangliosides potentiate *in vivo* and *in vitro* effects of nerve growth factor on central cholinergic neurons. *Proc. Natl. Acad. Sci. USA* 1989;86:2056–2060.
13. Cuello AC, Kenigsberg RL, Maysinger D, Garofalo L. Application of gangliosides and nerve growth factor to prevent cholinergic degeneration in the central nervous system. In: Ledeen RW, Hogan EL, Tettamanti G, Yates AJ, Yu RK, eds. *New Trends in Ganglioside Research. Neurochemical and Neuroregenerative Aspects* 1988. Padova, Italy: Liviana Press, 1988;535–548.
14. Cuello AC, Maysinger D, Garofalo L, Tagari P, Stephens PH, Pioro E, Piotte M. Influence of gangliosides and nerve growth factor on plasticity of forebrain cholinergic neurons. In: Fuxe K, Agnati LF, eds. *Receptor-receptor interactions* 1987. London: McMillan Press, 1987;62–77.
15. Cuello AC, Sofroniew MV. The anatomy of the CNS cholinergic neurons. *Trends Neurosci.* 1984;7:74–78.
16. Cuello AC, Stephens PH, Tagari PC, Sofroniew MV, Pearson RCA. Retrograde changes in the nucleus basalis of the rat, caused by cortical damage, are prevented by exogenous ganglioside GM<sub>1</sub>. *Brain Res.* 1986;376:373–377.
17. Davies P, Maloney AJF. Selective loss of central cholinergic neurons in Alzheimer's disease. *Lancet* 1976;II:1403.
18. Dawbarn D, Allen SJ, Semenenko FM. Coexistence of choline acetyltransferase and nerve growth factor receptors in the rat basal forebrain. *Neurosci. Lett.* 1988;94:138–144.
19. Doherty P, Dickson JG, Flanigan TP, Walsh FS. Ganglioside GM<sub>1</sub> does not initiate but enhances neurite regeneration of nerve growth factor-dependent sensory neurones. *J. Neurochem.* 1985;44:1259–1265.
20. Eckenstein F. Transient expression of NGF-receptor-like immunoreactivity in postnatal rat brain and spinal cord. *Brain Res.* 1988;446:149–154.

21. Eckenstein G, Thoenen H. Production of specific antisera and monoclonal antibodies in choline acetyltransferase. Characterization and use for identification of cholinergic neurons. *EMBO J.* 1982;1:363–368.
22. Elliott PJ, Garofalo L, Cuello AC. Limited neocortical devascularizing lesions causing deficits in memory retention and choline acetyltransferase activity—effects of the monosialoganglioside GM<sub>1</sub>. *Neuroscience* 1989;31:63–76.
23. Ferrari G, Soranzo C, Callegaro L, Dal Toso R, Benvegnu D, Toffano G, Leon A. Characterization and purification of a striatal-derived neuronotrophic factor (SDNF). In: Biggio G, Spano PF, Toffano G, Appel SH, Gessa GL, eds. *Neuronal Plasticity and Trophic Factors* 1988. Padova, Italy: Liviana Press, 1988;87–94.
24. Fibiger HC. The organization and some projections of cholinergic neurons of the mammalian forebrain. *Brain Res. Rev.* 1982;4:327–388.
25. Fischer W, Wictorin K, Bjorklund A, Williams LR, Varon S, Gage FH. Amelioration of cholinergic neuron atrophy and spatial memory impairment in aged rats by nerve growth factor. *Nature* 1987;329:65–68.
26. Gage FH, Armstrong DM, Williams LR, Varon S. Morphological response of axotomized septal neurons to nerve growth factor. *J. Comp. Neurol.* 1988;269:147–155.
27. Gage FH, Batchelor P, Chen KS, Chin D, Deputy S, Shaw T, Rosenberg M, Fischer W, Bjorklund A. NGF-mediated NGF receptor re-expression and cholinergic neuronal hypertrophy in the damaged adult neostriatum. *Abstr. Soc. Neurosci.* 1989;14:256.
28. Gnahn H, Hefti F, Heumann R, Schwab ME, Thoenen H. NGF-mediated increase of choline acetyltransferase (ChAT) in the neonatal rat forebrain: evidence for a physiological role of NGF in the brain? *Dev. Brain Res.* 1983;9:45–52.
29. Goldstein JL, Anderson RGW, Brown MS. Coated pits, coated vesicles and receptor-mediated endocytosis. *Nature* 1979;279:679–685.
30. Gomez-Pinilla F, Cotman CW, Nieto-Sampedro M. NGF receptor immunoreactivity in rat brain: topographical distribution and response to entorhinal ablation. *Neurosci. Lett.* 1987;82:260–266.
31. Greenberg ME, Greene LA, Ziff EB. Nerve growth factor and epidermal growth factor induce rapid transient changes in proto-oncogene transcription in PC12 cells. *J. Biol. Chem.* 1985;260:14101–14110.
32. Hadjiconstantinou M, Neff NA. Treatment with GM<sub>1</sub> ganglioside restores striatal dopamine in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine treated mouse. *J. Neurochem.* 1988;51:1190–1196.
33. Hagg T, Manthorpe M, Vahlsing HL, Varon S. Delayed treatment with NGF reverses the apparent loss of cholinergic neurons after acute brain damage. *Exper. Neurol.* 1988;101:303–312.
34. Hama T, Huang D-P, Guroff G. Protein kinase C as a component of a nerve growth factor sensitive phosphorylation system in PC12 cells. *Proc. Natl. Acad. Sci. USA* 1986;83:2352–2357.
35. Hatanaka H, Tsukui H. Differential effects of nerve-growth factor and glioma-conditioned medium on neurons cultured from various regions of fetal rat central nervous system. *Dev. Brain Res.* 1986;30:47–56.
36. Hefti F. Nerve growth factor promotes survival of septal cholinergic neurons after fimbrial transections. *J. Neuroscience* 1986;6:2155–2162.
37. Hefti F. Is Alzheimer's disease caused by a lack of nerve growth factor? *Ann. Neurol.* 1983;13:109–110.

38. Hefti F, Dravid A, Hartikka J. Chronic intraventricular injections of nerve growth factor elevate hippocampal choline acetyltransferase activity in adult rats with partial septo-hippocampal lesions. *Brain Res.* 1984;293:305–311.
39. Hefti F, Hartikka J, Eckenstein F, Gnahn H, Heumann R, Schwab ME. Nerve growth factor increases choline acetyltransferase but not survival or fibre outgrowth of cultured foetal septal cholinergic neurons. *Neuroscience*. 1985;14:55–68.
40. Hefti F, Hartikka J, Salvaterra A, Weiner WJ, Mash DC. Localization of nerve growth factor receptors in cholinergic neurons of the human basal forebrain. *Neurosci. Lett.* 1986;69:37–41.
41. Heumann R, Schwab M, Merkl R, Thoenen H. Nerve growth factor-mediated induction of choline acetyltransferase in PC12 cells: evaluation of the site of action of nerve growth factor and the involvement of lysosomal degradation products of nerve growth factor. *J. Neurosci.* 1984;4:3039–3050.
42. Hosang M, Shooter EM. The internalization of nerve growth factor by high affinity receptors on pheochromocytoma PC12 cells. *EMBO J.* 1987;6:1197–1202.
43. Houser CR, Crawford GD, Barber RP, Salvaterra PM, Vaugh JE. Organization and morphological characteristics of cholinergic neurons: an immunocytochemical study with a monoclonal antibody to choline acetyltransferase. *Brain Res.* 1983;266:97–119.
44. Isacson O, Fischer W, Wictorin K, Dawbarn D, Björklund A. Astroglial response in the excitotoxically lesioned neostriatum and its projection areas in the rat. *Neuroscience* 1987;20:1043–1056.
45. Kenigsberg RL, Mazzoni IE, Collier B, Cuello AC. Effects of epidermal growth factor on cholinergic properties of dissociated septa cells in culture. *Neurosci. Lett.*; submitted.
46. Kiss J, McGovern J, Patel AJ. Immunohistochemical localization of cells containing nerve growth factor receptors in the different regions of the adult rat forebrain. *Neuroscience* 1988;27:731–748.
47. Koh S, Loy R. Age-related loss of nerve growth factor sensitivity in rat basal forebrain neurons. *Brain Res.* 1988;440:396–401.
48. Kordower JH, Bothwell MA, Schattemann G, Gash DM. Nerve growth factor receptor immunoreactivity in the nonhuman primate (*Cebus apella*): distribution, morphology and localization with cholinergic enzymes. *J. Comp. Neurol.* 1988;277:465–486.
49. Korsching S. The role of nerve growth factor in the CNS. *Trends Neurosci.* 1986;9:570–573.
50. Kromer L. Nerve growth factor treatment after brain injury prevents neuronal death. *Science* 1987;235:214–216.
51. Kruijer W, Schubert D, Verma I. Induction of the proto-oncogene *fos* by nerve growth factor. *Proc. Natl. Acad. Sci. USA* 1985;82:7330–7334.
52. Ledeen R. Gangliosides of the neuron. *Trends Neurosci.* 1985;8(4):169–174.
53. Lefebvre PP, Rogister B, Delree P, Leprince P, Selak I, Moonen G. Potassium-induced release of neuronotoxic activity by astrocytes. *Brain Res.* 1987;413:120–128.
54. Leonard DGB, Ziff EB, Green LA. Identification and characterization of mRNAs regulated by nerve growth factor in PC2 cells. *Mol. Cell. Biol.* 1987;7:3156–3167.
55. Levi-Montalcini R. The nerve growth factor 35 years later. *Science* 1987;237:1154–1162.
56. Levi-Montalcini R, Aloe R. Differentiating effects of murine nerve growth factor in the peripheral and central nervous system of *Xenopus laevis* tadpoles. *Proc. Natl. Acad. Sci. USA* 1985;82:7111–7115.

57. Levi-Montalcini R, Calissano P. Nerve growth factor as a paradigm for other polypeptide growth factors. *Trends Neurosci.* 1986;9:473-477.
58. Li SP, Kowarski CR, Feld KM, Grim WM. Recent advances in microencapsulation technology and equipment. *Drug Develop. Indust. Pharm.* 1988;14:353-376.
59. Loy R, Koh S. Developmental expression of nerve growth factor receptors in sensory systems of the rat central nervous system. *Soc. Neurosci. Abstr.* 1986;12:393.
60. Maysinger D, Garofalo L, Jalsenjak I, Cuello AC. Effects of microencapsulated monosialoganglioside GM<sub>1</sub> on cholinergic neurons. *Brain Res.* 1989;496:165-172.
61. Maysinger D, Herrera-Marschitz M, Carlsson A, Garofalo L, Cuello AC, Ungerstedt U. Striatal and cortical acetylcholine release *in vivo* in rats with unilateral decortication: effects of treatment with monosialoganglioside GM<sub>1</sub>. *Brain Res.* 1988;461:355-360.
62. Milbrandt J. Nerve growth factor rapidly induces c-fos mRNA in PC12 rat pheochromocytoma cells. *Proc. Natl. Acad. Sci. USA* 1986;83:4789-4793.
63. Mobley WC, Rutkowski JL, Tennekoon GI, Buchanan K, Johnston MV. Choline acetyltransferase activity in striatum of neonatal rats increased by nerve growth factor. *Science* 1985;229:284-287.
64. Mobley WC, Rutkowski JL, Tennekoon GI, Gemski J, Buchanan K, Johnston MV. Nerve growth factor increases choline acetyltransferase activity in developing basal forebrain neurons. *Molec. Brain Res.* 1986;1:53-62.
65. Mufson EJ, Gash DM, Bothwell MA, Hersh LB, Kordower JH. Alteration in nerve growth factor receptor immunoreactive profiles in the basal forebrain of Alzheimer's and Parkinson's patients. *Soc. Neurosci. Abstr.* 1988;14:1222.
66. Nieto-Sampedro M, Manthorpe M, Barbin G, Varon S, Cotman CW. Injury-induced neuronotrophic activity in adult rat brain: correlation with survival of delayed implants in the wound cavity. *J. Neurosci.* 1983;3:2219-2229.
67. Oderfeld-Nowak B, Skup M, Ulas J, Jezierska M, Gradknowska R, Zaremba M. Effect of GM<sub>1</sub> ganglioside treatment on post lesion responses of cholinergic neurons in rat hippocampus after various partial deafferentations. *J. Neurosci. Res.* 1984;12:409-420.
68. Paxinos G, Watson C. *The Rat Brain in Stereotaxic Coordinates*. Sydney: Academic Press, 1986.
69. Ramon y Cajal S. *Degeneration and regeneration of the nervous system*. London, New York: Hafner Pub. Co., 1968.
70. Raivich G, Kreutzberg GW. The localization and distribution of high affinity beta-nerve growth factor binding sites in the central nervous system of the adult rat. A light microscopic autoradiographic study using [<sup>125</sup>I]beta-nerve growth factor. *Neuroscience* 1987;20:23-36.
71. Richardson PM, Verge VMK, Riopelle RJ. Distribution of neuronal receptors for nerve growth factor in the rat. *J. Neurosci.* 1986;6:2312-2321.
72. Riopelle RJ, Richardson PM, Verge VMK. Distribution and characteristics of nerve growth factor binding on cholinergic neurons of rat and monkey forebrain. *Neurosci. Lett.* 1988;94:138-144.
73. Ross AH, Grob M, Bothwell M, Elder DE, Ernst CS, Marano N, Ghrist BFD, Slemp CC, Herlyn M, Atkinson B, Koprowski H. Characterization of nerve growth factor receptor in neural crest tumors using monoclonal antibodies. *Proc. Natl. Acad. Sci. USA* 1984;81:6681-6685.
74. Schatteman GC, Gibbs L, Lanahan AA, Claude P, Bothwell M. Expression of NGF

- receptor in the developing and adult primate central nervous system. *J. Neurosci.* 1988; 8:860–873.
75. Schwab ME, Otten U, Agid Y, Thoenen H. Nerve growth factor (NGF) in the rat CNS: absence of specific retrograde axonal transport and tyrosine hydroxylase induction in the locus coeruleus and substantia nigra. *Brain Res.* 1979;168:473–483.
76. Schwartz M, Spirman N. Sprouting from chicken embryo dorsal root ganglia induced by nerve growth factor is specifically inhibited by affinity purified antiganglioside antibodies. *Proc. Natl. Acad. Sci. USA* 1982;79:6080–6083.
77. Schweitzer JB. Nerve growth factor receptor-mediated transport from cerebrospinal fluid to basal forebrain neurons. *Brain Res.* 1987;423:309–317.
78. Sofroniew MV, Pearson RCA, Cuello AC, Tagari PC, Stephens PH. Parenterally administered GM<sub>1</sub> ganglioside prevents retrograde degeneration of cholinergic cells of the rat basal forebrain. *Brain Res.* 1986;398:393–396.
79. Sofroniew MV, Pearson RCA, Eckenstein F, Cuello AC, Powell TPS. Retrograde changes in cholinergic neurons in the basal forebrain of the rat following cortical damage. *Brain Res.* 1983;289:370–374.
80. Springer JE, Koh S, Tayrien MW, Loy R. Basal forebrain magnocellular neurons stain for nerve growth factor receptor: correlation with cholinergic cell bodies and effects of axotomy. *J. Neurosci. Res.* 1987;17:111–118.
81. Stach RW, Perez-Polo JR. Binding of nerve growth factor to its receptor. *J. Neurosci. Res.* 1987;17:1–10.
82. Stephens PH, Cuello AC, Sofroniew MV, Pearson RCA, Tagari P. The effects of unilateral decortication upon choline acetyltransferase and glutamate decarboxylase activities in the nucleus basalis and other areas of the rat brain. *J. Neurochem.* 1985; 45:1021–1026.
83. Stephens PH, Tagari PC, Garofalo L, Maysinger D, Piotte M, Cuello AC. Neural plasticity of basal forebrain cholinergic neurons: effects of gangliosides. *Neurosci. Lett.* 1987;80:80–84.
84. Taniuchi M, Schweitzer JB, Johnson Jr EM. Nerve growth factor receptor molecules in rat brain. *Proc. Natl. Acad. Sci. USA* 1988;83:1950–1954.
85. Thoenen H, Bandtlow C, Heumann R. The physiological function of nerve growth factor in the central nervous system: comparison with the periphery. *Rev. Physiol. Biochem. Pharmacol.* 1987;109:145–178.
86. Toffano G, Benvegnu A, Bonetti A, Facci L, Leon A, Orlando F, Ghidoni R, Tet-tamanti G. Interaction of GM<sub>1</sub> ganglioside with crude rat brain neuronal membranes. *J. Neurochem.* 1980;35:861–866.
87. Toffano G, Savoini GE, Moront F, Lombardt G, Calz L, Agnati LF. Chronic GM<sub>1</sub> ganglioside treatment reduces dopamine cell body degeneration in the substantia nigra after unilateral hemitransection in rat. *Brain Res.* 1983;296:233–239.
88. Tomozawa Y, Appel SH. Soluble striatal extracts enhance development of mesencephalic dopaminergic neurons *in vitro*. *Brain Res.* 1986;399:111–124.
89. Vantini G, Fusco M, Bigon E, Leon A. GM<sub>1</sub> ganglioside potentiates the effect of nerve growth factor in preventing vinblastine induced sympathectomy in new born rats. *Brain Res.* 1988;448:252–258.
90. Varon S, Manthorpe M, Davis GE, Williams LR, Skaper SD. Growth factors. In: Waxman SG, ed. *Advances in Neurology*, Vol. 47: *Functional Recovery in Neurological Disease* 1988. New York: Raven Press, 1988;493–521.

91. Varon S, Skaper SD, Katoh-Semba R. Neuritic responses to GM1 ganglioside in several *in vitro* systems. In: Tettamanti G, Ledeen RW, Sandhoff K, Nagui Y, Toffano G, eds. *Gangliosides and Neuronal Plasticity* 1986. Padova, Italy: Liviana Press, 1986; 215.
92. Whittemore SR, Seiger A. The expression, localization and functional significance of  $\beta$  nerve growth factor in the central nervous system. *Brain Res. Rev.* 1987;12:439–464.
93. Williams L, Varon S, Peterson G, Wictorin K, Fischer W, Björklund A, Gage F. Continuous infusion of nerve growth factor prevents basal forebrain neuronal death after timbria fornix transection. *Proc. Natl. Acad. Sci. USA* 1986;83:9231–9235.
94. Yan Q, Johnson Jr EM. An immunohistochemical study of the nerve growth factor receptor in developing rats. *J. Neurosci.* 1988;8:3481–3498.
95. Yapel AI. Albumin microspheres: heated chemical stabilization. *Methods in Enzymology* 1985;112:3–18.

# Development of Cholinergic Neurons in Septum, Nucleus Basalis, Striatum and Pons in Culture and their Response to Nerve Growth Factor

F. Hefti, J. Hartikka, E.O. Junard and B. Knusel

*Department of Neurology, University of Miami, Miami, FL 33101*

Nerve growth factor (NGF), the best studied neurotrophic factor, affects growth and differentiation of sympathetic and sensory neurons and cholinergic neurons of the basal forebrain. The action on basal forebrain cholinergic neurons has received particular interest in recent years because this population was found to degenerate in Alzheimer's disease. The cell bodies of these neurons, which are located in medial septum, diagonal band of Broca, and nucleus basalis of Meynert provide a widespread and topographically organized innervation to hippocampus and cortex (25,41,43). It has been well established that NGF affects development of septal cholinergic neurons. Intraventricular administration of NGF increases choline acetyltransferase (ChAT) activity in the basal forebrain of neonatal rats *in vivo* (8,18,27,28). Similar NGF-mediated increases in ChAT activity have been reported in cell cultures prepared from fetal rat septum (10,12,14,17,24). NGF can also promote survival and neurite growth of fetal septal neurons *in vitro* (10). In the developing rat brain, levels of NGF and mRNA encoding NGF parallel the growth of cholinergic neurons, implying a role for NGF in the establishment of the cholinergic projection from the septum to the hippocampus (1,21,26,42).

In addition to the basal forebrain cholinergic neurons, which project over long distances, there are cholinergic interneurons in the corpus striatum. The striatal interneurons form an anatomically and functionally distinct population (25,35,41,44). They have been reported to respond to NGF with a dose-dependent increase in ChAT activity, both in embryonic striatal explant cultures (23), and in neonatal rats *in vivo* (18,27,28).

Another population of central cholinergic neurons with highly collateralized and widely distributed centrally ascending projections has recently been described in the human, rat, and mouse brain (9,33,43). The cell bodies of these neurons are located in two nuclei in the pontine metencephalon, the pedunculopontine teg-

mental nucleus, and the dorsolateral tegmental nucleus. These nuclei form two contiguous cell groups, which, in the rat extend approximately from the substantia nigra to the locus coeruleus (33,43). It has been speculated that these centrally projecting cholinergic neurons of the pontine area could be an anatomical substrate of the ascending reticular activating system, as proposed by Shute and Lewis (38), and also that these neurons are likely to be involved in the control of respiration, cardiovascular function, temperature regulation, modulations of sleep cycles, motor activity, and ingestion (43). A loss of cells in the pedunculopontine nucleus has been found in progressive supranuclear palsy and in Parkinson's disease (16,46). This finding is particularly interesting in light of the anatomically and physiologically established projection of this nucleus to the substantia nigra (2,4,40).

While the effects of NGF on the development of septal cholinergic neurons have been well documented, much less is known about its role in the development of cholinergic neurons of nucleus basalis and corpus striatum. It was not known whether NGF is able to exert trophic actions on developing pontine cholinergic neurons. To address these issues, we have prepared primary cell cultures from four different areas of fetal rat brain: septum, striatum, nucleus basalis, and pons. The development of cholinergic neurons from the four areas was investigated *in vitro*, and the effects of NGF on their survival, fiber growth, and expression of transmitter-specific enzymes was characterized.

## RESULTS

In the adult rat brain, morphology and function of cholinergic neurons in septum, nucleus basalis, and striatum are different. Neurons belonging to these populations developed similar morphological characteristics *in vitro* as *in vivo*. By visual inspection, cholinergic neurons of striatum and nucleus basalis appeared to have a more extensive fiber network than cholinergic neurons of septum (Fig. 1). Morphometric analysis of individual cholinergic neurons revealed that the total neurite length of cholinergic neurons of striatum and nucleus basalis was larger and that there were more branching points when compared with septal cholinergic neurons (Table 1).

NGF treatment approximately doubled the total length of neurites and the number of branching points of septal cholinergic neurons but failed to affect neurite growth of striatal or nucleus basalis cholinergic neurons (Table 2). Interestingly, while septal cholinergic neurons had significantly fewer fibers in control cultures, there was no significant difference between the morphology of cholinergic neurons of septum, striatum, and nucleus basalis when these cells were grown in the presence of NGF.

We earlier demonstrated that NGF increases survival of septal cholinergic neurons, when the cells are grown at low densities (10). Similarly, treatment of low density striatum and nucleus basalis cultures with NGF increased the number of

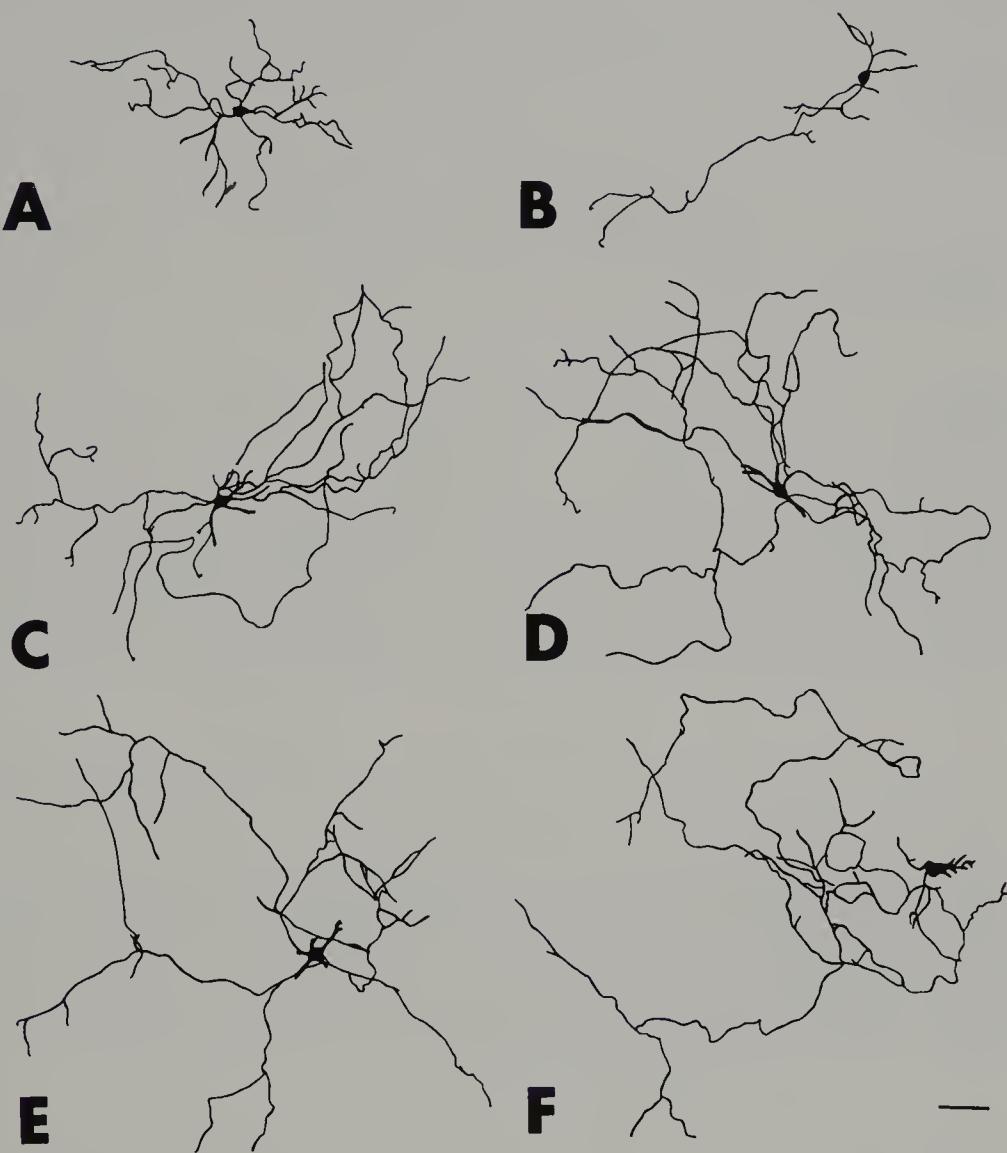


FIG. 1. Camera lucida drawings of typical cholinergic neurons of septum (A, B), striatum (C, D), and nucleus basalis (E, F). Dissociated cells from different areas of fetal rat forebrain were grown under control conditions. After three weeks *in vitro*, cultures were fixed and the cholinergic neurons were visualized using AChE cytochemistry. Bar, 100  $\mu$ m.

**TABLE 1.** Effect of NGF on survival and neurite growth of cholinergic neurons in septum, striatum and nucleus basalis

	Control	NGF
<u>No of AChE-positive neurons per cm<sup>2</sup></u>		
SEPTUM	192 ± 29	342 ± 58*
STRIATUM	7.7 ± 2.3	20.5 ± 3.1*
NUCLEUS BASALIS	10.8 ± 3.8	25.3 ± 2.3*
<u>Total fiber length of a single neuron (mm)</u>		
SEPTUM	2.21 ± 0.12	4.50 ± 0.73*
STRIATUM	6.14 ± 1.04	5.31 ± 0.97
NUCLEUS BASALIS	5.39 ± 0.49	6.31 ± 1.15
<u>Branching points per neuron</u>		
SEPTUM	17.7 ± 1.4	34.3 ± 6.1*
STRIATUM	36.7 ± 7.2	29.7 ± 5.3
NUCLEUS BASALIS	36.6 ± 3.7	43.0 ± 8.7

Septum, striatum, and nucleus basalis cultures were prepared from fetal rats of embryonic age E17 described in detail elsewhere (10, 11). Treated cultures received NGF (100 ng/ml). After two weeks *in vitro*, the number of cholinergic neurons was visualized using acetylcholinesterase (AChE) cytochemistry, a selective marker for cholinergic neurons in this culture system (14). The morphology of cholinergic neurons was analyzed using a computerized image analysis system. Values shown are means ± S.E.M.; \* significantly different from control value ( $p < 0.05$ ).

cholinergic neurons (Table 1). As found for septal cholinergic neurons, survival of striatum and nucleus basalis cholinergic neurons in high density cultures was independent of NGF (11).

ChAT and acetylcholinesterase (AChE) activities were considerably lower in striatum and nucleus basalis cultures than in septal cultures of equal plating density (Table 2). This difference reflects the fact that the percentage of cholinergic neu-

**TABLE 2.** Effect of NGF and ChAT and AChE activity of cultured cholinergic neurons in septum, striatum and nucleus basalis

	Control	NGF		
	ChAT fmol/min/μg prot	AChE pmol/min/μg prot	ChAT fmol/min/μg prot	AChE pmol/min/μg prot
SEPTUM	336 ± 56	16.8 ± 0.6	1336 ± 202*	32.1 ± 2.7*
STRIATUM	65 ± 18	6.1 ± 1.2	139 ± 17*	6.8 ± 1.3
N. BASALIS	59 ± 13	7.3 ± 1.2	91 ± 5*	8.5 ± 1.6

Cultures of dissociated cells prepared from fetal rat septum, striatum, and nucleus basalis were treated with NGF (100 ng/ml). After 10 days *in vitro*, the amount of protein and the activity of ChAT and AChE were determined. Values shown represent means ± S.E.M.; \* significantly different from corresponding control,  $p < 0.05$ .

rons was lower in striatum and nucleus basalis cultures. When NGF was added to the medium of septum, striatum, and nucleus basalis cultures, ChAT activity increased by 297%, 113%, and 55%, respectively (Table 2). NGF treatment approximately doubled AChE activity in septal cultures, but did not increase AChE activity of cholinergic neurons of striatum and nucleus basalis. The observation that NGF increases fiber growth and AChE activity of septal cholinergic neurons but fails to affect either of the two parameters in striatum and nucleus basalis cultures suggests that the AChE content of cholinergic neurons reflects the size of their fiber network. In contrast, NGF stimulated ChAT activity of all forebrain cholinergic neurons, and this effect was independent of the effects on survival, fiber growth, and AChE activity. These results suggest that NGF can specifically increase the number of ChAT molecules in cholinergic neurons without stimulating their fiber growth.

In cultures of dissociated neurons from the pons, ChAT immunostained cells were very rare and the staining intensity was weak. Addition of NGF failed to increase the number or staining intensity of labeled cells in pontine cultures (19). This finding sharply contrasts with the observations made on septal cultures, where only a small number of weakly stained neurons are visible in absence of NGF but many darkly stained, large bipolar, and multipolar cells with branched processes are visualized in cultures treated with NGF (10). In confirmation of the findings obtained with immunocytochemical methods, NGF failed to increase ChAT activity in pontine cultures (Table 3).

## DISCUSSION

The studies on cholinergic neurons *in vitro* further characterize the selectivity of action of NGF in the developing mammalian brain. While NGF trophically affects developing cholinergic neurons of basal forebrain and corpus striatum, pontine cholinergic neurons do not respond to NGF. The development of nucleus basalis cholinergic neurons *in vitro* and their response to NGF differed from those

**TABLE 3.** Effect of NGF on protein levels and choline acetyltransferase (ChAT) activity in primary cultures of dissociated pontine neurons

	Protein/Well ( $\mu$ g)	ChAT/Well (pmol/min)	ChAT/Protein (pmol/min/mg prot)
Controls	99.1 $\pm$ 3.6	10.3 $\pm$ 0.6	103.4 $\pm$ 5.0
NGF	101.6 $\pm$ 4.8	10.4 $\pm$ 0.6	101.1 $\pm$ 4.2

Cultures of dissociated pontine cells were prepared and grown as described in detail elsewhere (19). They were kept 7–8 days in presence or absence of NGF and then taken for the analysis of protein content and ChAT activity. Values shown are means  $\pm$  SEM.

of septal cholinergic neurons, suggesting that they represent functionally distinct neuronal populations. The development of cholinergic striatal interneurons *in vitro* was similar to that of nucleus basalis neurons, suggesting that determinants in the local environment influence their developmental fate *in situ*. These results support the notion that the effects of NGF on development of CNS neurons are highly selective.

The cholinergic neurons of septum, diagonal band of Broca, and nucleus basalis form a continuous, albeit irregularly shaped, column of neurons (35,36) and are often considered to represent a functionally homogenous neuronal population. However, the cholinergic neurons located in the medial septal nucleus are relatively small and mostly oval in shape and these neurons usually are bipolar and their dendrites branch only occasionally (39). In contrast, cholinergic neurons of the diagonal band are larger and more irregularly shaped than those of the medial septal nucleus. Those of the nucleus basalis are multipolar, their dendrites pass for considerable distances with little branching, and they are the largest cholinergic neurons in the forebrain. The mean cross-sectional area of the cell body of these neurons is approximately twice the area of septal cholinergic neurons (39). In the present study, we found that cholinergic neurons in nucleus basalis cultures developed a different morphology than those in cultures of septal area neurons, which contained the cholinergic neurons of the medial septal nucleus and the vertical limb of diagonal band of Broca. Our results suggest that some of the morphological differences observed *in vivo* represent intrinsic properties of these cholinergic neurons.

Nucleus basalis and septal cholinergic neurons also differed in their response to NGF. While NGF was able to promote survival and increase ChAT activity of all forebrain cholinergic neurons, it stimulated fiber growth of septal but not nucleus basalis cells. These findings again are in agreement with results obtained *in vivo*, where intraventricular injections of NGF to neonatal rats increased ChAT activity in septum to a larger extent than in the nucleus basalis region (18,27,28). They suggest that NGF is more important for the development of septal cholinergic neurons than for those of nucleus basalis, and that an early developmental decision separates the two populations. Interestingly, septal and nucleus basalis cholinergic neurons also differ in their response to a lesion in the adult rat brain. While axonal transection of septal neurons apparently results in degeneration of septal cholinergic neurons (29), removal of the terminal field of nucleus basalis neurons leads to shrinkage of the cholinergic cell bodies (39).

Striatal cholinergic neurons in the rat brain have a large oval or irregularly shaped cell body and long, infrequently branching dendrites; it is believed that they represent a single neuronal population (3,6,30,35). Our results show that these neurons develop similar morphological characteristics when grown in cell cultures. The morphology of striatal cholinergic interneurons *in vitro* resembles that of nucleus basalis cholinergic neurons which, *in vivo*, project over long distances. This is rather surprising, because in many brain regions local circuit neurons are generally considered to be small in size. However, similar to nucleus basalis neurons, striatal

neurons seem to innervate large areas and, therefore, have an extensive network of fibers supported by a large cell body (35). Furthermore, striatal cholinergic neurons may be transformed into projection neurons under special experimental conditions. This was shown in cultures of striatal slices, where cholinergic neurons were found to innervate co-cultured slices of hippocampal tissue (13). These results suggest that striatal and nucleus basalis cholinergic neurons in the fetal rat brain have a similar developmental potential and that their final morphology is influenced by local determinants.

Our finding that NGF treatment increases ChAT activity of cultured striatal neurons confirms earlier results obtained on explant cultures (23) and on neonatal rats injected with NGF (18,27,28). In addition, we showed that NGF promotes survival of striatal cholinergic neurons *in vitro* in a similar way as that of basal forebrain cholinergic neurons. Taken together, these results conclusively demonstrate that NGF is able to affect the striatal cholinergic neurons during early development. Interestingly, the responsiveness of striatal neurons to NGF declines during development. While producing a more than two-fold increase in striatal ChAT activity in two-day-old rats, a single intraventricular injection of NGF failed to be effective after the third postnatal week (18). In contrast, no such age-related decline was observed in the septal area (18).

Our observations on cultured embryonic neurons from the pons suggest that pontine cholinergic neurons do not respond to NGF. These negative findings are in line with immunohistochemical findings indicating that, during development and in the adult brain, pontine cholinergic neurons do not express NGF receptors (5,7,15,32,34). The location of a small group of NGF receptor-positive cells in the pons does not coincide with that of the ChAT-positive pedunculopontine and dorsolateral tegmental nuclei (32). Similarly, the distributions of endogenous NGF and mRNA[NGF] fail to indicate a role of NGF for the pontine cholinergic neurons. Levels of NGF and mRNA[NGF] are high in hippocampus and cortex, i.e., in target areas of the basal forebrain cholinergic cells, and low or very moderate in thalamus, septum, hypothalamus and caudate-putamen, i.e., in target areas of the pontine cholinergic neurons (20,37,42).

We believe that our findings represent further evidence for a high degree of selectivity of the action of NGF in the developing brain. While clearly affecting cholinergic neurons of the basal forebrain, NGF is not a central "cholinergic neurotrophic factor", since cholinergic pontine neurons do not respond to NGF. However, NGF probably affects some populations other than the forebrain cholinergic neurons. Detectable but low levels of NGF protein were found in diencephalon and cerebellum, despite the lack of major cholinergic innervation to these areas (21,37). During the first postnatal weeks, NGF receptors were found to be transiently expressed in olive nucleus, brain stem reticular formation, amygdala, and in the cerebellum. There was transitory staining in the retina and on the fibers of somatosensory and visual pathways in various sensory system of the CNS including olfactory bulb, retina, optic nerve, solitary tract and nucleus, cuneate and gracile nucleus, and the dorsal horn of the spinal cord (5,34,45). This somewhat broader

spectrum of NGF actions during ontogeny may have a parallel during phylogeny, since NGF seems to affect a larger number of neuronal populations in the brain of lower vertebrates (22).

## ACKNOWLEDGMENTS

The studies were supported by NIH grant NS22933, NSF grant BNS-8708049, and by grants from the Alzheimer's Disease and Related Disorders Association, Chicago, IL (P-85-004B), and the National Parkinson Foundation, Miami, FL.

## REFERENCES

1. Auburger GR, Heumann R, Korschning S, Thoenen H. Developmental changes of nerve growth factor and its mRNA in the rat hippocampus: comparison with choline acetyltransferase. *Dev. Biol.* 1987;120:322-328.
2. Beninato M, Spencer RF. A cholinergic projection to the rat substantia nigra from the pedunculopontine tegmental nucleus. *Brain Res.* 1987;412:169-174.
3. Bolam JP, Wainer BH, Smith AD. Characterization of cholinergic neurons in the rat neostriatum. A combination of choline acetyltransferase immunocytochemistry, Golgi-impregnation and electron microscopy. *Neuroscience* 1984;12:711-718.
4. Clarke PBS, Hommer DW, Pert A, Skirboll LR. Innervation of substantia nigra neurons by cholinergic afferents from pedunculopontine nucleus in the rat: neuroanatomical and electrophysiological evidence. *Neuroscience* 1987;23:1011-1019.
5. Eckenstein F. Transient expression of NGF-receptor-like immunoreactivity in postnatal rat brain and spinal cord. *Brain Res.* 1988;446:149-154.
6. Eckenstein F, Sofroniew MW. Identification of central cholinergic neurons containing both choline acetyltransferase and acetylcholinesterase and of central neurons containing only acetylcholinesterase. *J. Neurosci.* 1983;3:2286-2291.
7. Gage FH. Effects of NGF on cholinergic neurons in the central nervous system. In: Davis P, Finch CB, eds. *Molecular Biology of Alzheimer's Disease*, Banbury Report; in press.
8. Gnahn H, Hefti F, Heumann R, Schwab M, Thoenen H. NGF-mediated increase of choline acetyltransferase (ChAT) in the neonatal forebrain: evidence for a physiological role of NGF in the brain? *Dev. Brain Res.* 1983;9:45-52.
9. Goldsmith M, van der Kooy D. Separate non-cholinergic descending and cholinergic ascending projections from the nucleus tegmenti pedunculopontinus. *Brain Res.* 1988;445:386-391.
10. Hartikka J, Hefti F. Development of septal cholinergic neurons in culture: plating density and glial cells modulate effects of NGF on survival, fiber growth, and expression of transmitter-specific enzymes. *J. Neurosci.* 1988;8:2967-2985.
11. Hartikka J, Hefti F. Comparison of nerve growth factor's effects on development of septum, striatum, and nucleus basalis cholinergic neurons *in vitro*. *J. Neurosci. Res.* 1988;21:352-364.
12. Hatanaka H, Tsukui H. Differential effects of nerve growth factor and glioma-con-

- ditioned medium on neurons cultured from various regions of fetal rat central nervous system. *Dev. Brain Res.* 1986;30:47–56.
13. Hefti F, Gahwiler BH. Cholinergic neurons of the rat forebrain in slice cultures: interactions with target tissue and effects of nerve growth factor. In: Haber B, Perez-Polo JR, Gorio A, eds. *Cellular and Molecular Aspects of Neuronal Development and Regeneration*. New York: Springer-Verlag, 1988;81–92.
  14. Hefti F, Hartikka J, Eckenstein F, Gnahn H, Heumann R, Schwab M. Nerve growth factor (NGF) increases choline acetyltransferase but not survival or fiber outgrowth of cultured fetal septal cholinergic neurons. *Neuroscience* 1985;14:55–68.
  15. Hefti F, Hartikka J, Salvatierra A, Weiner WJ, Mash DC. Localization of nerve growth factor receptors in cholinergic neurons of the human basal forebrain. *Neurosci. Lett.* 1986;69:37–41.
  16. Hirsch EC, Graybiel AM, Duyckaerts C, Javoy-Agid F. Neuronal loss in the pedunculopontine tegmental nucleus in Parkinson disease and in progressive supranuclear palsy. *Proc. Natl. Acad. Sci. USA* 1987;84:5976–5980.
  17. Honegger P, Lenoir D. Nerve growth factor (NGF) stimulation of cholinergic telencephalic neurons in aggregating cell cultures. *Dev. Brain Res.* 1982;3:229–239.
  18. Johnston MV, Rutkowski JL, Wainer BH, Long JB, Mobley WC. NGF effects on developing forebrain cholinergic neurons are regionally specific. *Neurochem. Res.* 1987;12:985–994.
  19. Knusel B, Hefti F. Development of cholinergic pedunculopontine neurons *in vitro*: comparison with cholinergic septal cells and response to nerve growth factor, ciliary neuronotrophic factor, and retinoic acid. *J. Neurosci. Res.* 1988;21:365–375.
  20. Korschning S, Auburger G, Heumann R, Scott J, Thoenen H. Levels of nerve growth factor and its mRNA in the central nervous system of the rat correlate with cholinergic innervation. *EMBO J.* 1985;4:1389–1393.
  21. Large TH, Bodary SC, Clegg DO, Weskamp G, Otten U, Reichardt LF. Nerve growth factor gene expression in the developing rat brain. *Science* 1986;234:352–355.
  22. Levi-Montalcini R, Aloe L. Differentiation effects of murine nerve growth factor in the peripheral and central nervous systems of *Xenopus laevis* tadpoles. *Proc. Natl. Acad. Sci. USA* 1985;82:7111–7115.
  23. Martinez HJ, Dreyfus CF, Jonakait GM, Black IB. Nerve growth factor promotes cholinergic development in brain striatal cultures. *Proc. Natl. Acad. Sci. USA* 1985;82:7777–7781.
  24. Martinez HJ, Dreyfus CF, Jonakait GM, Black IB. Nerve growth factor selectively increases cholinergic markers but not neuropeptides in rat basal forebrain in culture. *Brain Res.* 1987;412:295–301.
  25. Mesulam MM, Mufson BH, Wainer BH, Levey AI. Central cholinergic pathways in the rat: an overview based on an alternative nomenclature (Ch1-Ch6). *Neuroscience* 1983;10:1185–1201.
  26. Milner TA, Loy R, Amaral DG. An anatomical study of the development of the septohippocampal projection in the rat. *Dev. Brain Res.* 1983;8:343–371.
  27. Mobley WC, Rutkowski JL, Tennekoon GI, Buchanan K, Johnston MV. Choline acetyltransferase in striatum of neonatal rats increased by nerve growth factor. *Science* 1985;239:284–287.
  28. Mobley WC, Rutkowski JL, Tennekoon GI, Gemski J, Buchanan K, Johnston MV. Nerve growth factor increases choline acetyltransferase activity in developing basal forebrain neurons. *Mol. Brain Res.* 1986;1:53–62.

29. Montero CN, Hefti F. Rescue of lesioned septal cholinergic neurons by nerve growth factor: specificity and requirement for chronic treatment. *J. Neurosci.* 1988;8:2985–2994.
30. Phelps PE, Houser CR, Vaughn JN. Immunocytochemical localization of choline acetyltransferase within the rat neostriatum: a correlated light and electron microscopic study of cholinergic neurons and synapses. *J. Comp. Neurol.* 1985;238:286–307.
31. Raivich G, Kreutzberg GW. The localization and distribution of high affinity  $\beta$ NGF binding sites in the central nervous system of the adult rat. A light microscopic autoradiographic study using ( $^{125}$ I) $\beta$ NGF. *Neuroscience* 1987;20:23–36.
32. Richardson PM, Verge Issa VMK, Riopelle RJ. Distribution of neuronal receptors for nerve growth factor in the rat. *J. Neurosci.* 1986;6:2312–2321.
33. Rye DB, Saper CB, Lee JH, Wainer BH. Pedunculopontine tegmental nucleus of the rat: cytoarchitecture, cytochemistry, and some extrapyramidal connections of the mesopontine tegmentum. *J. Comp. Neurol.* 1987;259:483–528.
34. Schatteman GC, Gibbs L, Lanahan AA, Claude P, Bothwell M. Expression of NGF receptor in the developing and adult primate central nervous system. *J. Neurosci.* 1988;8:860–873.
35. Schwaber JS, Rogers WT, Satoh K, Fibiger HC. Distribution and organization of cholinergic neurons in the rat forebrain demonstrated by computer-aided data acquisition and three-dimensional reconstruction. *J. Comp. Neurol.* 1987;263:309–325.
36. Sembra K, Fibiger HC. Time of origin of cholinergic neurons in the rat basal forebrain. *J. Comp. Neurol.* 1988;269:87–95.
37. Shelton DL, Reichardt LF. Studies on the expression of beta NGF gene in the central nervous system: level and regional distribution of NGF mRNA suggest that NGF functions as a trophic factor for several neuronal populations. *Proc. Natl. Acad. Sci. USA* 1986;83:2714–2718.
38. Shute CCD, Lewis PR. The ascending cholinergic reticular system: neocortical, olfactory and subcortical projections. *Brain* 1967;90:497–520.
39. Sofroniew MV, Pearson RCA, Powell TPS. The cholinergic nuclei of the basal forebrain of the rat: normal structure, development and experimentally induced degeneration. *Brain Res.* 1987;411:310–331.
40. Tokuno H, Moriizumi T, Kudo M, Nakamura Y. A morphological evidence for monosynaptic projections from the nucleus tegmenti pedunculopontinus pars compacta (TPC) to nigrostriatal projection neurons. *Neurosci. Lett.* 1988;85:1–4.
41. Wainer BH, Levey AI, Mufson EF, Mesulam MM. Cholinergic systems in mammalian brain identified with antibodies against choline acetyltransferase. *Neurosci. Int.* 1984;6:163–182.
42. Whittemore SR, Ebendal T, Larkfors L, Olson L, Seiger A, Stromberg I, Persson H. Developmental and regional expression of beta nerve growth factor messenger RNA and protein in the rat central nervous system. *Proc. Natl. Acad. Sci. USA* 1986;83:817–821.
43. Woolf NJ, Butcher LL. Cholinergic systems in the rat brain: III. Projections from the pontomesencephalic tegmentum to the thalamus, tectum, basal ganglia, and basal forebrain. *Brain Res. Bull.* 1986;16:603–637.
44. Woolf NJ, Eckenstein F, Butcher LL. Cholinergic systems in the rat brain. I. Projections to the limbic telecephalon. *Brain Res. Bull.* 1984;13:751–784.

45. Yan Q, Johnson EM. An immunohistochemical study of the nerve growth factor receptor in developing rats. *J. Neurosci.* 1988;8:3481–3498.
46. Zweig RM, Whitehouse PJ, Casanova MF, Walker LC, Jankel WR, Price DL. Loss of pedunculopontine neurons in progressive supranuclear palsy. *Ann. Neurol.* 1987; 22:18–25.



# Monosialoganglioside Effects Following Cerebral Ischemia: Relationship with Anti-neuronotoxic and Pro-neuronotrophic Properties

M.S. Seren, M. Lipartiti, A. Lazzaro, R. Rubini, S. Mazzari,  
L. Facci, G. Vantini, R. Zanoni, A. Zanotti, G. Bonvento and  
A. Leon

FIDIA Research Laboratories  
Via Ponte della Fabbrica 3/A  
35031-Abano Terme (PD) Italy

**A**lthough the sequelae of neurodegenerative events observed after an acute brain insult depend on the characteristics of the etio-pathogenetic event, a common feature is the occurrence of neuronal cell death. Groups of neurons primarily affected by an anoxic or traumatic episode die during or immediately after the insult. In addition, many more neurons, in the vicinity of the damaged area and, at times, in areas not directly affected by the insult, die at later times. However, the fact that some neurons undergo delayed (secondary) death indicate that these neuronal populations represent areas of viable but metabolically disturbed cells whose salvage may be pharmacologically feasible, provided the underlying causes are understood. In addition, after the destructive events leading to secondary neuronal death have subsided, a new dynamic state (involving processes such as collateral sprouting, reactive synaptogenesis, etc.) is reached in which the neurons attempt to restore lost function(s). As the innate expression of such neuroplastic response ultimately depends on the number of surviving neurons, significant efforts have evolved to understand the mechanisms underlying the pathophysiology of secondary neuronal damage/death and neuronal plasticity, in the attempt to define novel therapeutical strategies aiming to improve neurological outcome after various central nervous system (CNS) insults, e.g., cerebrovascular insufficiencies and brain trauma.

This article will briefly focus on some current concepts concerning the pathophysiology of secondary brain damage and expression of neuroplastic behaviors applicable to therapeutic strategies aiming to maintain and restore function after acute brain injury. This in turn will be exemplified by experimental results supporting the use of monosialoganglioside ( $GM_1$ ) treatment in both the early and late stages after acute CNS injury, in particular cerebrovascular insufficiencies.

## ROLE OF NEURONOTOXIC AND NEURONOTROPHIC FACTORS AFTER BRAIN INJURY: DEFINITION OF A THERAPEUTIC STRATEGY

The secondary neuronal damage/death which occurs after acute CNS insults is now known to be mediated, at least in part, by endogenous excitotoxins such as glutamate and aspartate. Elevation in the extracellular concentrations of these excitatory amino acids have been demonstrated after cerebral ischemia (2,17,18), and ablation of excitatory amino acid transmitter inputs reduces ischemia-induced neuronal death (20,35,53). Furthermore, these amino acids are known to be neurotoxic *in vitro* (6,37) where they have been shown to cause, via specific postsynaptic receptors, neuronal overactivation and consequent triggering of a cascade of cellular events ultimately leading to neuronal cell death.

In addition, a growing number of experimental data indicate that the maintenance of neuronal survival as well as expression of their plasticity depends on the presence and availability of neuronotrophic factors (47). These proteins (e.g., nerve growth factor, [NGF]), originating from the innervation territories and/or surrounding non-neuronal cells, have been known for some time to regulate neuronal numbers and degree of target innervation in early life (8,52). More recent evidence indicates that trophic signals, although less apparent, persist in maturity. Furthermore, numerous types of brain insults have been found to be associated with increases in neuronotrophic activity, especially at a time subsequent to most secondary neuronal cell death and in the tissue immediately surrounding the lesion (31). This and other data have led to the suggestions that: 1) a trophic deficit, due to an increased trophic need of the neurons at early post-injury times, is another of the underlying causes of progressive secondary neuronal cell damage/death (25); and 2) neuronotrophic factors are involved in regulating recovery after CNS injury. It is noteworthy that recent studies concerning NGF effects in CNS further support such possibilities (19,23,54).

Thus, it can be deduced that, after a cerebral insult, neuronal survival and neuroplasticity are controlled by a delicate balance between neuronotoxic and neuronotrophic activities. Pharmacological approaches aiming to ameliorate neurological outcome after acute brain injury should be targeted towards antagonizing the neuronotoxic activity and/or potentiating the action of neuronotrophic factors present in the brain. These two, not mutually exclusive, approaches should result in neuronal sparing when applied in the early phases after CNS injury. In addition, the second type of therapeutic strategy may facilitate long-term repair processes aiming to restore lost function(s).

## GM<sub>1</sub> EFFECTS AFTER CEREBRAL ISCHEMIA: AN ANTI-NEURONOTOXIC AND A PRO-NEURONOTROPHIC PHARMACOLOGICAL APPROACH

The past and present research efforts focused on the study of the effects of systemic GM<sub>1</sub> treatment after CNS injury, in particular cerebral ischemia, derive essentially

from evidence indicating that the endogenously occurring ganglioside on the neuronal cell surface is involved in the process of neuritogenesis in the embryo and neurite repair in the adult (41). In addition, these experiments have gained impetus from studies demonstrating that the ganglioside, when added *in vitro*, is capable of both limiting excitatory amino acid-related neurotoxicity (13) and facilitating neuronal cell responsiveness to neuronotrophic factors (14,24,26). Likewise, when systemically administered *in vivo*, the ganglioside was demonstrated to be efficacious in reducing secondary neuronal damage/death as well as in facilitating occurrence of long-term recovery processes after a variety of CNS insults, including cerebral ischemia (29). Each of these aspects, i.e., anti-neuronotoxic and pro-neuronotrophic effects of the ganglioside *in vitro* and its relationship with GM<sub>1</sub> effects *in vivo*, especially after cerebral ischemia, is briefly exemplified and discussed below.

### GM<sub>1</sub> Anti-neuronotoxic Effects *in vitro* and *in vivo*: Relationship with GM<sub>1</sub> Capability to Reduce Secondary Neuronal Death after Cerebral Ischemia

Glutamate or related compounds are known to be neuronotoxic both when added to cultured neuronal cells (e.g., cerebellar granule cells in normoxic conditions, [13]), or after exposure of the cells to anoxia. Likewise, intracerebral injection of N-methyl-D-aspartate (NMDA) induces, in neonatal rats, extensive brain lesions, positively correlated with reduction in brain weight and similar to those occurring after a cerebral hypoxic/ischemic insult (28). Furthermore, in adult rats, transient, near-complete cerebral ischemia induces a relatively selective pattern of secondary neuronal degeneration (e.g., pyramidal neurons in the hippocampal CA1 region) while focal cerebral ischemia results in a localized but diffuse area of neuronal necrosis. In both of these cases, investigations into the underlying mechanisms have indicated that excitatory amino acid neurotransmission is, at least in part, involved (see also Costa, *this symposium*).

Results obtained in the above-mentioned experimental model systems show that the monosialoganglioside (as well as its internal ester derivative, siagosome) is able to reduce, in a dose-dependent manner, glutamate neurotoxicity when cerebellar granule cells are maintained in normoxic or anoxic conditions (Fig. 1). When systemically administered *in vivo*, the monosialoganglioside is also efficacious in reducing the loss of brain weight induced by intraventricular NMDA administration in neonatal rats (Table 1). This indicates that the ganglioside is able to reduce excitatory amino acid-related neuronotoxicity not only *in vitro* but also *in vivo*. In addition, treatment with the ganglioside has been shown to limit (a) hippocampal CA1 damage (Table 2) as well as the entity of cortical electroencephalographic deterioration that occurs with time after near-complete transitory ischemia (4 vessel occlusion model, [34]) in adult rats; (b) brain edema, Na<sup>+</sup> and Ca<sup>++</sup> intracellular loading and behavioral deficits after focal cortical ischemia in adult rats (21); (c) morphological damage and neurological deficits subsequent to transient middle artery occlusion in cats (22,44); (d) neurological impairment after global ischemia in monkeys (4). GM<sub>1</sub> treatment has also recently been shown to ameliorate neu-

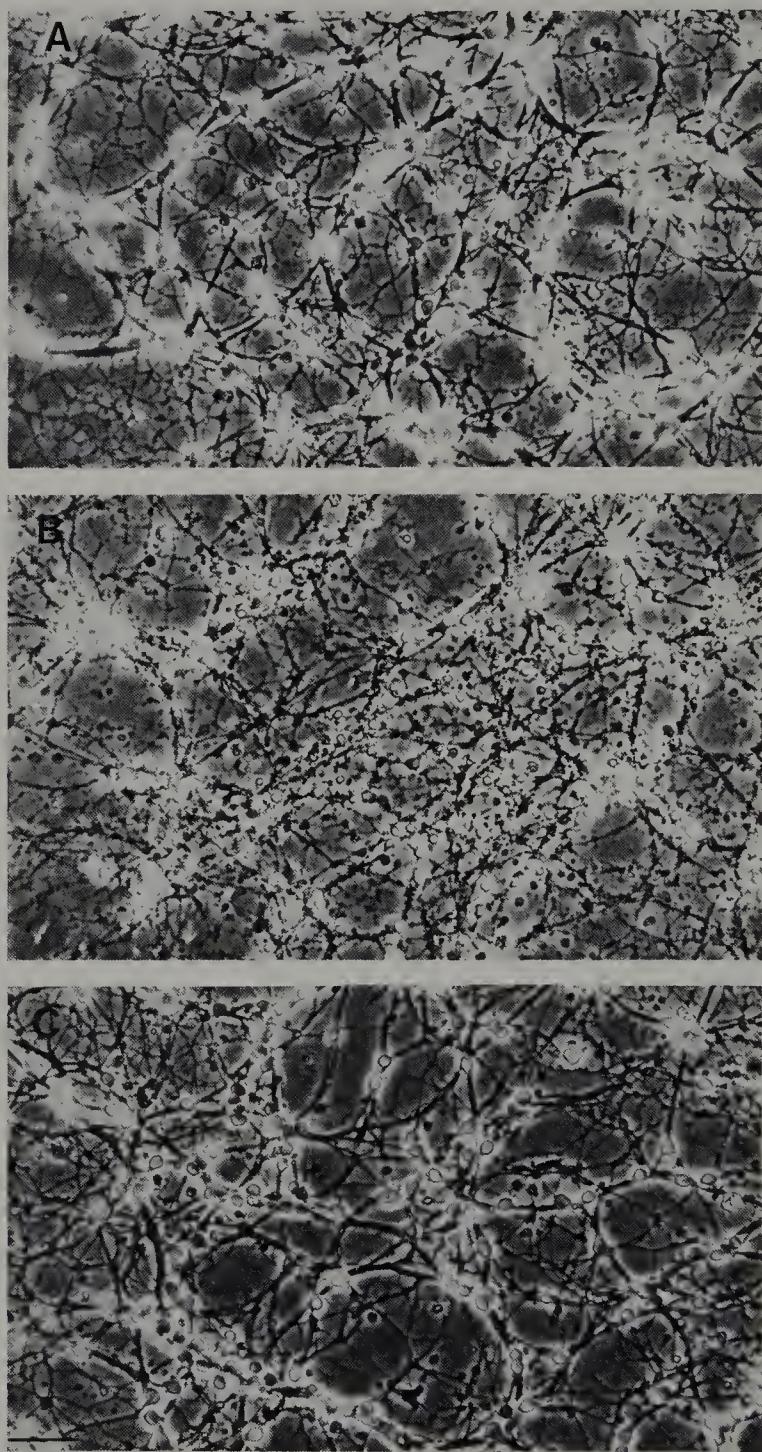


FIG. 1. GM<sub>1</sub> prevents morphologic evidence of anoxic injury. Phase-contrast photomicrographs of 11 day *in vitro* neonatal rat cerebellar granule cells before (A) and 24 hr after (B) a 5-hr anoxia exposure. In (C), a matched culture was pretreated with 100  $\mu$ M GM<sub>1</sub> for 2 hr prior to oxygen deprivation. Most cell bodies and processes remain intact 24 hr later. Bar, 25  $\mu$ m.

**TABLE 1.** Reduction of NMDA neurotoxicity in neonatal rat brain after monosialoganglioside treatment

Treatment		Brain Weight (mg) $\pm$ SEM
SAL + SAL	(n = 20)	1.010 $\pm$ 12
NMDA + SAL	(n = 18)	880 $\pm$ 24*
NMDA + MONOSIAL	(n = 19)	950 $\pm$ 17†

Seven-day-old rats were injected with 25 nmoles (1  $\mu$ l-buffered saline) or NMDA into the lateral ventricle. Monosialoganglioside (20 mg/kg) or saline were injected subcutaneously 1 hr before and immediately after NMDA injection. Animals were sacrificed five days later and their brains (forebrain + cerebellum) weighted on a high precision balance.

\*  $p < 0.01$  vs SAL + SAL: Duncan's test (one-tailed comparison).

†  $p < 0.05$  vs SAL + SAL and vs NMDA + SAL: Duncan's test (one-tailed comparison).

iological outcome in patients afflicted by acute cerebrovascular insufficiency (1). Consequently, due to the largely recognized involvement of excitatory amino acids in ischemia-induced neuronal damage/death (2,17,18), one feasible interpretation is that most probably the above-mentioned ganglioside effects reflect its capability to reduce excitatory amino acid related neurotoxicity after cerebral ischemia.

The molecular bases of the monosialoganglioside action are currently under investigation. In cultured cerebellar granule cells, it has been reported that the ganglioside decreases glutamate-induced translocation (from the cytosol to the cellular

**TABLE 2.** Morphological classification of CA1 Pyramidal neurons

Class of damage	% of Animals	
	Saline-treated	Ganglioside-treated
1	0	11%
2	12%	31%
3	88%	58%

After 30 min of four-vessel occlusion, only animals showing flattening of electrocorticographic activity for the whole occlusion period were selected and systemically treated with saline ( $n = 17$ ) or monosialoganglioside ( $n = 19$ ) at the dose of 20 mg/kg. The first injection was intravenous just after carotid-unclipping; the animals were then treated intramuscularly 3 hr after release, twice a day for the following two days and once a day until sacrifice (day 21). After perfusion-fixation with BOUIN's solution, paraffin sections of 8  $\mu$ m were taken from dorsal hippocampus and stained with Cresyl violet-Luxol Fast Blue. Hippocampal damage was then graded by light microscopy and classified as follows: 1, damage up to 25%; 2, up to 50%; and 3, over 50%. Statistical analysis of group distribution by means of Fischer's exact test revealed a significant difference ( $p < 0.05$ ) between saline- and ganglioside-treated animals.

membrane) of protein kinase C (PKC) (49), an effect which may account for the observed capability of the ganglioside to antagonize glutamate toxicity. Interestingly, these latter effects involve stable ganglioside association with the neuronal cell surface (5,12,36) and occur in the absence of any interference with glutamate binding to its recognition sites or receptor-operated  $\text{Ca}^{2+}$  influx, phosphoinositide hydrolysis, or *c-fos* induction (13). These aspects clearly differentiate gangliosides from glutamate antagonists which, although able to reduce post-ischemic neuronal death (16,33,42), also cause, at effective doses, widespread CNS depression and learning impairment (7,10,30).

### **GM<sub>1</sub> Pro-neuronotrophic Effects *in vitro* and *in vivo*: Relationship with GM<sub>1</sub> Capability to Ameliorate Neurological Outcome after Cerebral Ischemia**

Among various neuronotrophic factors, NGF is still today the best characterized one. NGF is known to be essential for the survival, neurite outgrowth, proper development, and, presumably, maintenance and regeneration of its target sympathetic and sensory ganglionic neurons *in vivo* and *in vitro* (27,45). Furthermore, recent studies in the developing and adult CNS have shown that NGF is also a neuronotrophic factor for forebrain cholinergic neurons. These neurons possess NGF receptors and their terminal targets produce NGF which is transported retrogradely to the cell bodies of these same cholinergic neurons. In addition, among several other findings, intracerebrally administered NGF has been shown to limit the occurrence of biochemical, immunocytochemical, and behavioral alterations after damage to forebrain cholinergic neurons in adult rodents. Also of interest is the observation that NGF levels in the brain increase after injury to the forebrain cholinergic neurons (46,52).

To date, many laboratories, including our own, have provided evidence indicating that GM<sub>1</sub> is able to potentiate NGF effects *in vitro*, namely in pheochromocytoma (PC12) cells (14), fetal chick dorsal root (24) or sympathetic ganglionic neurons (40), as well as septal neurons in culture (9). Likewise, when administered *in vivo*, the ganglioside was shown to potentiate the effects of exogenously supplied NGF after damage to sympathetic ganglia in neonatal rats (50) and to forebrain cholinergic neurons in adult rats (9,11). Furthermore, the ganglioside, *per se*, was reported to be efficacious in limiting biochemical and morphological alterations that occur after injury to the forebrain cholinergic neurons (32,43). This latter result most probably reflects the capability of the ganglioside to also potentiate the effects of endogenously occurring NGF. In addition, GM<sub>1</sub> has been shown *in vitro* to potentiate the effects of neuronotrophic factors other than NGF (26,40). This, in turn, is consistent with the capability of the systemically administered ganglioside to maintain and promote recovery of function after injury of a variety of neuronal populations, other than the forebrain cholinergic neurons (15,29,38,48,51).

Recent evidence indicates that NGF can also play a role in cerebral ischemia.

It has been reported that NGF can protect hippocampal CA1 pyramidal neurons against ischemic injury in rodents (3,39). As such, we cannot exclude the possibility that the ganglioside potentiation of endogenously occurring neuronotrophic factors may contribute, together with its anti-neuronotoxic effects, to the ganglioside capability to reduce neuronal damage after cerebral ischemia. In addition, in view of the role of neuronotrophic factors in regulating recovery processes, such a possibility is most probably related to the ganglioside capability to improve long-term neurological outcome after cerebrovascular insufficiencies, even in clinical situations.

## SUMMARY

Knowledge of the implication of excitatory amino acid-related neurotoxicity in the definition of secondary brain damage, and of neuronotrophic factors in the maintenance of neuronal survival and regulation of neuronal plasticity, has provided insights with regard to the identification of novel therapeutic strategies aiming to maintain and restore function after acute brain injury (e.g., cerebrovascular insufficiencies). In this context, the applicability of drugs targeted towards antagonizing the neurotoxic activity and potentiating the neuronotrophic activity present in brain is exemplified by the use of monosialoganglioside. The latter ganglioside has been shown to limit excitatory amino acid-related neurotoxic effects and to facilitate neuronotrophic efficacy both *in vivo* and *in vitro*. In addition, the systemically administered ganglioside has been shown to decrease secondary brain damage as well as to facilitate occurrence of long-term repair processes after various types of brain insults, including cerebral ischemia.

## REFERENCES

1. Argentino C, Sacchetti ML, Toni D, Savoini G, D'Arcangelo E, Erminio F, Federico F, Ferro Milone F, Gallai V, Gambi D, Mamoli A, Ottonello GA, Ponari O, Rebucci G, Senin U, Fieschi C. GM<sub>1</sub> ganglioside therapy in acute ischemic stroke. *Stroke* 1989;20:1143–1149.
2. Benveniste H, Drejer J, Schousboe A, Diemer NH. Elevation of the extracellular concentrations of glutamate and aspartate in rat hippocampus during transient cerebral ischemia monitored by intracerebral microdialysis. *J. Neurochem.* 1984;43:1369–1374.
3. Buchan AM, Pulsinelli WA. Fimbria-fornix lesions: the temporal profile for protection of CA1 hippocampus against ischemic injury. *J. Cereb. Blood Flow Metab.* 1989; 9:(suppl 1)S749.
4. Cahn R, Borzeix M, Aldinio C, Toffano G, Cahn J. Influence of monosialoganglioside inner ester on neurologic recovery after global cerebral ischemia in monkeys. *Stroke* 1989;20:652–656.

5. Callies R, Schwarzmann G, Radzak K, Seigert R, Wiegandt H. Characterization of the cellular binding of exogenous gangliosides. *Eur. J. Biochem.* 1977;80:425–432.
6. Choi DW, Maulucci-Gedde MA, Kriegstein AR. Glutamate neurotoxicity in cortical cell culture. *J. Neurosci.* 1987;7:357–368.
7. Collingridge GL, Bliss TV. NMDA receptors—their role in long-term potentiation. *Trends Neurosci.* 1987;10:288–293.
8. Cowan WM. Neuronal death as a regulative mechanism in the control of cell number in the nervous system. In: Rockstein M, ed. *Development and Aging in the Nervous System*. New York: Academic Press, 1973;19–41.
9. Cuello AC, Garofalo L, Kenigsberg RL, Maysinger D. Gangliosides potentiate *in vivo* and *in vitro* effects of nerve growth factor on central cholinergic neurons. *Proc. Natl. Acad. Sci. USA* 1989;86:2056–2060.
10. Danysz W, Wroblewski JT, Costa E. Learning impairment in rats by N-methyl-D-aspartate receptor antagonists. *Neuropharmacology* 1988;27:653–656.
11. Di Patre PL, Casamenti F, Cenni A, Pepeu G. Interaction between nerve growth factor and GM<sub>1</sub> monosialoganglioside in preventing cortical choline acetyltransferase and high affinity choline uptake decrease after lesion of the nucleus basalis. *Brain Res.* 1989;480:219–224.
12. Facci L, Leon A, Toffano G, Sonnino S, Ghidoni R, Tettamanti G. Promotion of neuritogenesis in mouse neuroblastoma cells by exogenous gangliosides. Relationship between the effect and the cell association of ganglioside GM<sub>1</sub>. *J. Neurochem.* 1984; 42:299–305.
13. Favaron M, Manev H, Alho H, Bertolino M, Ferret B, Guidotti A, Costa E. Gangliosides prevent glutamate and kainate neurotoxicity in primary neuronal cultures of neonatal rat cerebellum and cortex. *Proc. Natl. Acad. Sci. USA* 1988;85:7351–7355.
14. Ferrari G, Fabris M, Gorio A. Gangliosides enhance neurite outgrowth in PC12 cells. *Dev. Brain. Res.* 1983;8:215–221.
15. Fusco M, Figliomeni B, Gorio A, Vantini G. Postnatal development of bulbospinal serotonergic system. Effects of GM<sub>1</sub> ganglioside following neonatal 5,7-dihydroxy-tryptamine treatment. *Neurochem. Int.* 1988;13:251–259.
16. Gill R, Foster AC, Woodruff GN. Systemic administration of MK-801 protects against ischemia-induced hippocampal neurodegeneration in the gerbil. *J. Neurosci.* 1987; 7:3343–3349.
17. Globus MYT, Busto R, Dietrich WD, Martinez E, Valdes I, Ginsberg MD. Effect of ischemia on the *in vivo* release of striatal dopamine, glutamate, and γ-aminobutyric acid studied by intracerebral microdialysis. *J. Neurochem.* 1988;51:1455–1464.
18. Hagberg H, Lehmann A, Sandberg M, Nystrom B, Jacobson I, Hamberger A. Ischemia-induced shift of inhibitory and excitatory amino acids from intra- to extracellular compartments. *J. Cereb. Blood Flow. Metab.* 1985;5:413–419.
19. Hefti F. Nerve growth factor promotes survival of septal cholinergic neurons after fimbrial transection. *J. Neurosci.* 1986;6:2155–2162.
20. Jorgensen MB, Johansen FF, Diemer NH. Removal of the entorhinal cortex protects hippocampal CA1 neurons from ischemic damage. *Acta Neuropathol. (Berl.)* 1987; 73:189–194.
21. Karpik SE, Li YS, Mahadik SP. Ischemic injury reduced by GM<sub>1</sub> ganglioside. In: Ledeen RW, Hogan EL, Tettamanti G, Yates AJ, Yu RK, eds. *New Trends in Ganglioside Research: Neurochemical and Neurodegenerative Aspects*, Fidia Research Series, vol. 14. Padova, Italy: Liviana Press, 1988;549–556.

22. Komatsu S, Greenberg JH, Hickey WF, Reivich M. Effect of the ganglioside GM<sub>1</sub> on neurologic function, electroencephalogram amplitude, and histology in chronic middle cerebral artery occlusion in cats. *Stroke* 1988;19:1027–1035.
23. Kromer LF. Nerve growth factor treatment after brain injury prevents neuronal death. *Science* 1987;235:214–216.
24. Leon A, Benvegnù D, Dal Toso R, Presti D, Facci L, Giorgi O, Toffano G. Dorsal root ganglia and nerve growth factor: a model for understanding the mechanism of GM<sub>1</sub> effects on neuronal repair. *J. Neurosci. Res.* 1984;12:277–287.
25. Leon A, Dal Toso R, Vantini G, Fusco M, Toffano G. The neuronotrophic hypothesis applied to GM<sub>1</sub> effects on CNS repair. In: Masland RL, Portera-Sanchez A, Toffano G, eds. *Neuroplasticity: A New Therapeutic Tool in the CNS Pathology*, Fidia Research Series, vol. 12. Padova, Italy: Liviana Press, 1987;125–140.
26. Leon A, Dal Toso R, Presti D, Benvegnù D, Facci L, Kirschner G, Tettamanti G, Toffano G. Development and survival of neurons in dissociated fetal mesencephalic serum-free cell cultures: II. Modulatory effects of gangliosides. *J. Neurosci.* 1988;8:746–753.
27. Levi-Montalcini R. The nerve growth factor 35 years later. *Science* 1987;237:1154–1162.
28. McDonald JW, Silverstein FS, Johnston MV. Neurotoxicity of N-methyl-D-aspartate is markedly enhanced in developing rat central nervous system. *Brain Res.* 1988;459:200–203.
29. Mahadik SP, Karpiak SK. Ganglioside in treatment of neural injury and disease. *Drug Develop. Res.* 1988;15:337–360.
30. Morris RGM, Anderson E, Lynch GS, Baudry M. Selective impairment of learning and blockade of long-term potentiation by an N-methyl-D-aspartate receptor antagonist, AP5. *Nature* 1976;319:774–776.
31. Nieto-Sampedro M, Lewis ER, Cotman CW, Manthorpe M, Skaper SD, Barbin G, Longo FM, Varon S. Brain injury causes a time-dependent increase in neuronotrophic activity at the lesion site. *Science* 1982;217:860–861.
32. Oderfeld-Nowak B, Skup M, Ulas J, Jezierska M, Gradowska M, Zaremba M. Effect of GM<sub>1</sub> ganglioside treatment on postlesion responses of cholinergic enzymes in rat hippocampus after various partial deafferentations. *J. Neurosci. Res.* 1984;12:409–420.
33. Ozyurt E, Graham DI, Woodruff GN, McCullough J. Protective effect of the glutamate antagonist, MK801 in focal cerebral ischemia in the cat. *J. Cereb. Blood Flow Metab.* 1988;8:138–143.
34. Pulsinelli WA, Brierley JB. A new model of bilateral hemispheric ischemia in unanesthetized rat. *Stroke* 1979;10:267–272.
35. Pulsinelli WA. Selective neuronal vulnerability: morphological and molecular characteristics. *Prog. Brain Res.* 1985;63:29–37.
36. Radzak K, Schwarzmann G, Wiegandt H. Studies on the cell association of exogenously added sialoglycolipids. *Hoppe Seylers Z. Physiol. Chem.* 1982;263:243–272.
37. Rothman SM, Olney JW. Excitotoxicity and the NMDA receptor. *Trends Neurosci.* 1987;10:299–302.
38. Sabel BA, Dunbar GL, Butler WM, Stein DG. GM<sub>1</sub> gangliosides stimulate neuronal reorganization and reduce rotational asymmetry after hemitransection of the nigrostriatal pathway. *Exp. Brain Res.* 1985;60:27–37.
39. Shigeno T, Mima T. Prevention of hippocampal cell death after cerebral ischemia by

- intraventricular administration of nerve growth factor. *Proceedings of the International Symposium on Alzheimer's Disease, Kuopio, Finland (abstr.)* 1988;97.
40. Skaper SD, Katoh-Semba R, Varon S. GM<sub>1</sub> ganglioside accelerates neurite outgrowth from primary peripheral and central neurons under selective culture conditions. *Dev. Brain. Res.* 1985;23:19–26.
  41. Skaper SD, Leon A, Toffano G. Factors promoting the growth and regeneration of neuronal cells. In: Müller EE, Macleod RM, eds. *Neuroendocrine Perspectives*, vol. 7. New York: Springer-Verlag, 1989.
  42. Simon RP, Swan JH, Griffiths T, Meldrum BS. Blockade of N-methyl-D-aspartate receptors may protect against ischemic damage in the brain. *Science* 1984;226:850–852.
  43. Sofroniew MV, Pearson RCA, Cuello AC, Tagari PC, Stephens PH. Parenterally administered GM<sub>1</sub> ganglioside prevents retrograde degeneration of cholinergic cells of the rat basal forebrain. *Brain Res.* 1986;398:393–396.
  44. Tanaka K, Dora E, Urbanics R, Greenberg JH, Toffano G, Reivich M. Effects of the ganglioside GM<sub>1</sub> on cerebral metabolism, microcirculation, recovery kinetics of ECoG and histology during the recovery period following focal ischemia in cats. *Stroke* 1986; 17:1170–1178.
  45. Thoenen H, Barde YA. Physiology of nerve growth factor. *Physiol Rev.* 1980;60:1284–1335.
  46. Thoenen H, Edgar D. Neurotrophic factors. *Science* 1985;229:238–242.
  47. Thoenen H, Bandtlow C, Heumann R. The physiological function of nerve growth factor in central nervous system: comparison with the periphery. *Rev. Physiol. Biochem. Pharmacol.* 1987;109:145–178.
  48. Toffano G, Savoini GE, Moroni F, Lombardi G, Calzà L, Agnati LF. Chronic GM<sub>1</sub> ganglioside treatment reduces dopamine cell body degeneration in the substantia nigra after unilateral hemitransection in rat. *Brain Res.* 1984;296:233–239.
  49. Vaccarino F, Guidotti A, Costa E. Ganglioside inhibition of glutamate-mediated protein kinase C translocation in primary cultures of cerebellar neurons. *Proc. Natl. Acad. Sci. USA* 1987;84:8707–8711.
  50. Vantini G, Fusco M, Bigon E, Leon A. GM<sub>1</sub> ganglioside potentiates the effects of nerve growth factor in preventing vinblastine-induced sympathectomy in newborn rats. *Brain Res.* 1988;448:252–258.
  51. Weihmuller FB, Hadjiconstantinou M, Bruno JP, Neff NH. Administration of GM<sub>1</sub> ganglioside eliminates neuroleptic-induced sensorimotor deficits in MPTP-treated mice. *Neurosci. Lett.* 1988;92:207–212.
  52. Whittemore SR, Seiger A. The expression, localization and functional significance of β-nerve growth factor in the central nervous system. *Brain Res. Rev.* 1987;12:439–464.
  53. Wieloch T, Lindvall O, Blomqvist P, Gage FH. Evidence for amelioration of ischaemic neuronal damage in the hippocampal formation by lesions of the perforant path. *Neurol. Res.* 1985;7:24–26.
  54. Williams LR, Varon S, Peterson G, Wictorian K, Fischer W, Björklund A, Gage FH. Continuous infusion of nerve growth factor prevents basal forebrain neuronal death after fimbria-fornix transection. *Proc. Natl. Acad. Sci. USA* 1986;83:9231–9235.

# Subject Index

## A

- Abnormal behavior, nigrostriatal neuron, 298–299  
Acetylcholine, 3–4  
Acrylamide, peripheral neuropathy, 167  
Adenylate cyclase, nerve growth factor, 89–90  
Adrenal cell, mitogen, 107  
Adrenal medulla  
    nerve growth factor, 195  
    tyrosine hydroxylase, induction, 7, 9  
Aging  
    nerve growth factor, 107–108  
    oxidative stress, 111  
Akinesia, ganglioside, 299–300  
Alzheimer's disease, nerve growth factor, 75, 327  
Amygdala damage, melanocortin, 164  
Angiogenesis  
    diabetic retinopathy, 57  
    immune reaction, 57  
    inflammation, 57  
Apolipoprotein, growth-associated triggering factor, 51  
Apolipoprotein-A-I, lipid degradation, 53  
Astrocyte  
    ciliary neuronotrophic factor, 247  
    gliogenesis, 40  
    platelet-derived growth factor, 247  
Axonal injury, regenerative capability, 53

## B

- Basal forebrain  
    cell-specific expression, nerve growth factor receptor, 95–103

- cholinergic neuron, 327  
Basal forebrain projection neuron  
    high-affinity receptor site, 99  
    low-affinity receptor, 99  
    nerve growth factor, 95  
Binding protein, ganglioside, rat brain membrane, 123–126  
Binding site, nerve growth factor, 97–98  
Biochemical measurement, nerve terminal sprouting, 296  
Biochemistry, heparin-binding growth factor, 57–61  
Biological assay, recombinant human nerve growth factor, 76–77  
Biological property, recombinant human nerve growth factor, 75–81  
Biology, heparin-binding growth factor, 57–61  
Blood cell, nerve growth factor, 195  
Bovine brain ganglioside, calcium, 18–19  
Brain, ganglioside, 119–131  
    bovine, 18–19  
    protein kinase, 119  
Brain injury  
    monosialoganglioside, 339–345  
    neuronotoxic factor, 340  
    neurotrophic factor, 340
- C
- c-fos-jun*, multigene expression, 10–14  
*c-jun*, 10–14  
C6–2B rat glioma cell, nerve growth factor, 86–87  
Calcium  
    confocal microscopy, 287–288  
    cytoskeleton, neuron, 286–289

- Calcium (*contd.*)  
 energy depletion, 283–286  
 fluorescent probe, 282–286  
 ganglioside, functional connection, 29  
 growth cone, 238–240  
 intracellular, neuronal growth, 231–244  
 intracellular dynamic, 282–286  
 N2A cell, 18–20  
 neuraminidase, 27–29  
 neurite outgrowth, 18–19  
 neuritogenesis, blockage, 27–28  
 neuron, 287  
   cell injury, 280–282  
   trophic intervention, 279–290
- Calcium homeostasis, 241  
 cAMP, 3–4  
   tyrosine hydroxylase function, 4–7  
   tyrosine hydroxylase increase, 5–7
- Cancer. *See specific type*
- Carbohydrate specificity, ganglioside receptor, 124–128
- Catalase, nerve growth factor, 107–108
- Cell death, nerve growth factor, 108–109
- Cell injury, energy metabolism, 279–280
- Cell proliferation  
   ganglioside, 135–154  
   glycolipid, 135–136
- Cell shape, nerve growth factor, 203–215
- Cell-specific expression, basal forebrain, nerve growth factor receptor, 95–103
- Central nervous system  
   cholinergic neuron, 219–226  
   nerve growth factor, 219–226  
   nerve growth factor, 112–113  
   neurodegenerative disease,  
     neurotrophic factors, 293  
   plasticity, melanocortin, 163–164  
   regeneration, 47–54  
     He-Ne laser, 51–53
- Cerebral ischemia  
   monosialoganglioside, 340–341  
   neuronotoxic properties, 339–345  
   neurotrophic properties, 339–345
- neurological outcome, GM<sub>1</sub>, 344–345  
 secondary neuronal death, GM<sub>1</sub>, 341–344
- Cerebrovascular insufficiency  
   neuron damage, 339  
   neuron plasticity, 339
- Chartin, 205–206  
   cholera toxin, 205–206  
   forskolin, 205–206  
   kinase A activator, 205–206  
   lithium chloride, 205–206
- Chloramphenicol acetyltransferase, 7
- Cholera B, neurite inhibition, 23, 26
- Cholera toxin, chartin, 205–206
- Choline acetyltransferase, nerve growth factor, 95, 100, 107, 219–226, 327, 331
- Cholinergic neuron  
   basal forebrain, 307–320  
   central nervous system, 219–226  
   nerve growth factor, somal size, 222–226  
   nucleus basalis, 328–329  
   septum, 328–329  
   striatum, 328–329
- Cholinergic population, nerve growth factor, 100
- Chromaffin cell, protein kinase, 9–10
- Ciliary neurotrophic factor, astrocyte, 247
- Cisplatin  
   ovarian cancer, 168–169  
   testicular cancer, 168–169
- Cognitive deficit, nerve growth factor, 107
- Cognitive function, melanocortin, 163–164
- Colchicine, tyrosine hydroxylase, 7–8
- Collagen, growth cone, 232
- Corpus callosum, ganglioside, 130
- Corpus striatum  
   cholinergic neuron, 327  
   nucleus basalis, 328
- Corticospinal tract, ganglioside, 130
- Corticosteroid receptor, hippocampus, 164
- Cycloheximide, 108

Cytoskeletal phosphoprotein, nerve growth factor-regulated, 203–215  
 Cytoskeletal phosphorylation, function, 211–215  
 Cytoskeleton  
   calcium, neuron, 286–289  
   neuron, 286  
   trophic intervention, 279–290  
 Cytosolic protein molecule, neuronal membrane regulation, 4–7

**D**

Defecation reflex, synaptic plasticity, 264  
 Diabetes, streptozotocin, 168  
 Diabetic neuropathy, melanocortin, 167–168  
 Diabetic retinopathy, angiogenesis, 57  
 Dimethylsulfoxide, ganglioside, 175  
 Dopamine  
   ganglioside, striatum recovery, 295  
   GM<sub>1</sub> ganglioside, 293–302  
     treatment parameters, 297–298  
   1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine, 293–302

**E**

Electrical activity, neurite, 233–234  
 Embryonic development, neuronal growth cone, 231  
 Endogenous ganglioside  
   neurite outgrowth, 23–27  
   neuronal differentiation, 23–27  
 Endorphin, proopiomelanocortin, 167  
 Energy depletion  
   calcium, 283–286  
   microscopy, 288–289  
   trophic intervention, 279–290  
 Energy metabolism, cell injury, 279–280  
 Exogenous calcium, neurite outgrowth, 18–19  
 Exogenous ganglioside  
   calcium, 18–23  
   neuritogenesis, 17–18  
 Exon-intron organization, gene coding, heparin-binding growth factor, 61–63

Extracellular matrix protein, growth-associated triggering factor, 49–50

**F**

Fibroblast, ganglioside, 136–139  
 Fibroblast growth factor, heparin-binding growth factor, 66–69  
 Filopodia, neurite, 231  
 Fimbria-fornix transection, nerve growth factor, 220–221  
 Fluorescent probe, calcium, 282–286  
 Forskolin, chartin, 205–206  
 Function recovery  
   neuron, 277–345  
   sacral spinal cord, 271–274

**G**

Galactolipid, myelinogenesis, 254–257  
 Ganglioside. *See also* GM<sub>1</sub> ganglioside  
   akinesia, 299–300  
   binding protein, rat brain membrane, 123–126  
   bioactive, 136–137  
   bovine brain, 18–19  
   brain, 119–131  
   <sup>14</sup>C-labeled, 177  
   calcium, functional connection, 29  
   cell proliferation, 135–154  
   corpus callosum, 130  
   corticospinal tract, 130  
   dimethylsulfoxide, 175  
   dopamine, striatum recovery, 295  
   endogenous  
     neurite outgrowth, 23–27  
     neuronal differentiation, 23–27  
   exogenous  
     calcium, 18–23  
     neuritogenesis, 17–18  
     neuronal differentiation, 17–23  
   fibroblast, 136–139  
   haloperidol, 299–301  
   immunoadsorption, 146–147  
   insulin receptor, 150–152  
   lactosylceramide sialytransferase, 176, 179

- Ganglioside (contd.)  
 leukemia cell, 175–190  
 melanoma cell, 175  
 microencapsulated, cholinergic neuron, 315–317  
 mitogenesis, 142–147  
 N2A cell, 19, 20, 21, 25  
 nerve growth factor  
     cholinergic septal neuron, 312–314  
     damaged cholinergic neuron, 310–312  
 neurodegeneration, 294–295  
     dopamine, 293–302  
     phosphorylation assay, 146  
     protein kinase C, 178–179  
     protein phosphorylation, 175  
     rat brain membrane, 119–131  
     tetradecanoylphorbol-13-acetate, 175  
     transmembrane signal, 135–154  
     tyrosine kinase, 139
- Ganglioside receptor  
 assay, 123  
 carbohydrate specificity, 124–128  
 neoganglioprotein, 119–123  
 phosphorylation, 142–147  
 regional distribution, 129–130  
 subcellular distribution, 129–130  
 tissue distribution, 129–130
- Gene coding, exon-intron organization, heparin-binding growth factor, 61–63
- Gene expression, nerve growth factor, 85–92  
*in vitro* study, 85–91  
 regulation, 85–92
- Gene structure, heparin-binding growth factor, 61–63
- Glia cell, neuron survival, 86
- Glia cell modulator, growth-associated triggering factor, 49–50
- Glia fibrillary acidic protein, growth-associated triggering factor, 51–53
- Glioblastoma  
 platelet-derived growth factor, 35–40  
 v-sis, 39
- Gliogenesis, platelet-derived growth factor, 35–40
- Glutamate, growth cone, 233
- Glutathione transferase, nerve growth factor, 107–108
- Glycoconjugate, neuraminidase, cell surface, 23, 27
- Glycolipid, cell proliferation, 135–136
- Glycosphingolipid  
     <sup>14</sup>C-labeled, 177  
     extraction, 177  
     kinase, 135  
     purification, 177  
     tyrosine kinase, 135
- GM<sub>1</sub>  
     anti-neuronotoxic effects, 341–344  
     pro-neuronotrophic effect, 344–345
- GM<sub>1</sub> ganglioside, dopamine, 293–302  
 treatment parameters, 297–298
- Gray Type I synapse, 17
- Growth-associated triggering factor, 53  
 apolipoprotein, 51  
 extracellular matrix protein, 49–50  
 glial cell modulator, 49–50  
 glial fibrillary acidic protein, 51–53  
 laser irradiation, 51–53  
 regeneration, 48–49  
 remyelination, 53
- Growth cone  
 calcium concentration  
     determination, 241, 243  
     heterogeneity, 241, 243  
 degeneration, 231  
 embryonic, 231  
 glutamate, 233  
 intracellular calcium, 238–240  
 mature life, 231  
 neurite outgrowth, 229–276  
 neuronal form, 231  
 neuronal integration, 236–238  
 neurotransmitter, 233  
 regulation, 232–236  
 serotonin, 233  
 stimulus, 232–236  
 trophic factor, 233
- Growth factor. See specific type
- H
- Haloperidol  
 ganglioside, 299–301

somatosensory orientation, 299–301  
**H**e-Ne laser, central nervous system  
 regeneration, 51–53  
**H**eparin-binding growth factor  
 biochemistry, 57–61  
 biology, 57–61  
 class 1, 67–68  
   gene structure, 61–63  
 class 2, 67–68  
   exon-intron organization, 61–63  
 fibroblast growth factor, 66–69  
 Kaposi's sarcoma, 63  
 keratinocyte growth factor, 66–67  
 oncogene, 57–69  
 protein sequence, 63–64  
 RNA, 63–64  
 stomach tumor, 63  
   transforming potential, 67–68  
**H**igh-affinity receptor site, basal  
   forebrain projection neuron, 99  
**H**ippocampus, corticosteroid receptor,  
 164  
**H**omodimerization, tyrosine hydroxylase,  
 7–10  
**H**ormonal change, synaptic plasticity,  
 270–271  
**H**uman placenta, nerve growth factor,  
 76  
**H**yperemotionality, melanocortin, 163

**I**

**I**mmune reaction, angiogenesis, 57  
**I**mmunoadsorption, ganglioside, 146–147  
**I**mmunoblot  
   nerve growth factor, 77–80  
   recombinant human nerve growth  
     factor, 76  
**I**mmunochemical property, recombinant  
   human nerve growth factor,  
 75–81  
**I**mmunocytochemistry, nerve growth  
   factor, 96  
**I**mmunoreactivity, nerve growth factor,  
 78–81  
**I**nflammation, angiogenesis, 57  
**I**nsulin receptor, ganglioside, 150–152  
**i**nt-2, oncogene, 63–66  
**I**ntron. *See* Exon-intron organization

**J**

**K**  
**K**-252a  
   nerve growth factor-sensitive  
     phosphorylation, 195–200  
   ornithine decarboxylase, 198–199  
   PC12 cell, interaction, 199  
     structure, 198  
 Keratinocyte growth factor, heparin-  
   binding growth factor, 66–67  
**K**inase, glycosphingolipid, 135  
**K**inase A activator, chartin, 205–206

**L**

Lactosylceramide sialytransferase,  
 ganglioside, 176, 179  
**L**amellipodia, neurite, 231  
**L**aser  
   growth-associated triggering factor  
     irradiation, 51–53  
   He-Ne, 51–53  
**L**earning  
   nerve growth factor, 107  
   neuron, structural modification, 3–4  
**L**eukemia cell  
   ganglioside, 175–190  
   replication, ganglioside, 175–190  
**L**igand, platelet-derived growth factor,  
 35–37  
**L**ipid degradation, apolipoprotein-A-I,  
 53  
**L**ithium chloride, chartin, 205–206  
**L**ow-affinity receptor, basal forebrain  
   projection neuron, 99  
**L**ymphoid cell  
   mitogen, 107  
   nerve growth factor, 109

**M**

**M**elanocortin  
   amygdala damage, 164  
   central nervous system plasticity,  
 163–164  
   cognitive function, 163–164

- Melanocortin (*contd.*)  
 diabetic neuropathy, 167–168  
 hyperemotionality, 163  
 mechanism of action, 166–167  
 motoneuron, 165  
 motor nerve conduction velocity, 165  
 neurofilament protein, 166  
 neuronal development, 161–162  
 neuronal plasticity, 161–169  
 neuropeptide, 161  
 neurotrophic effect, 162  
 neurotrophic effect, 167  
 parafascicular nucleus, 163–164  
 peptide hormone, 161  
 peripheral neuropathy, 167–169  
 proopiomelanocortin, 167  
 working memory, 164
- Melanoma cell, ganglioside, 175
- Memory, DNA transcription, 3–4
- $\beta$ -Mercaptoethanol, nerve growth factor, 77–79
- 1-Methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine, dopamine, 293–302
- N-Methyl D-aspartic acid, 4
- Microscopy, energy depletion, 288–289
- Microtubule-associated protein, 204–208  
 chartin, 205–206
- Micturition reflex, 264
- Mitogen  
 adrenal cell, 107  
 lymphoid cell, 107
- Mitogenesis, ganglioside, 142–147
- Monoclonal antibody  
 myelinogenesis, 254–257  
 nerve growth factor, 98
- Monosialoganglioside. *See also* GM<sub>1</sub>  
 ganglioside  
 cerebral ischemia, 340–341  
 secondary brain damage treatment, 339–345
- Motoneuron, melanocortin, 165
- Motor nerve conduction velocity, melanocortin, 165
- Mouse neuroblastoma cell, 17
- mRNA  
*c-fos*, 13–14  
*c-jun*, 13–14
- code, language, 13
- nerve growth factor  
 C6–2B rat glioma cell, 87–89  
 cerebellum, 90  
 hippocampus, 90  
 hypothalamus, 90
- Multigene expression  
*c-fos-jun*, 10–14  
 third nuclear messenger, 10–14
- Multigene transcriptional activation, 3–15
- Myelin sheath, myelinogenesis, 247–249
- Myelinogenesis  
 galactolipid, 254–257  
 monoclonal antibody, 254–257  
 myelin sheath, 247–249  
 oligodendrocyte, 247–254  
 telencephalon, 249–254  
 trophic regulation, 247–258
- N
- N2A cell  
 calcium efflux, 19–20  
 calcium influx, 18–19  
 ganglioside, 19, 20, 21, 25  
 neuritogenesis, sodium, 20, 22
- Neoganglioprotein, 120  
 characterization, 122–123  
 ganglioside receptor, 119–123  
 synthesis, 120–122
- Nerve damage, peripheral, regeneration, 164–166
- Nerve growth factor  
 adenylate cyclase, 89–90  
 adrenal medulla, 195  
 aging, 107–108  
 Alzheimer's disease, 75, 327  
 basal forebrain projection neuron, 95  
 binding site, 97–98  
 blood cell, 195  
 C6–2B rat glioma cell, 86–87  
 catalase, 107–108  
 cell death, 108–109  
 cell shape, 203–215  
 central nervous system, 112–113  
 characterization, 111–112  
 chemical characterization, 77–79

- choline acetyltransferase, 95, 100, 107, 219–226, 327, 331  
cholinergic neuron, 327–334  
  somal size, 222–226  
cholinergic population, 100  
cognitive deficit, 107  
cognitive impairment, reversal, 221  
fimbria-fornix transection, 220–221  
gamma-aminobutyric acid, 96–103  
ganglioside  
  cholinergic septal neuron, 312–314  
  damaged cholinergic neuron, 310–312  
gene expression, 85–92  
glutathione transferase, 107–108  
human placenta, 76  
immunoblot, 77–80  
immunocytochemistry, 96  
immunoreactivity, 78–81  
learning, 107  
lymphoid cell, 109  
 $\beta$ -mercaptoethanol, 77–79  
monoclonal antibody, 98  
mRNA  
  brain stem, 90  
  C6–2B rat glioma cell, 87–89  
  cerebellum, 90  
  hippocampus, 90  
  hypothalamus, 90  
neurite growth, cholinergic neurons, 328–330  
neurodegenerative disorder, 85  
neuronal sprouting, 107  
non-cholinergic population, 100–101  
PC12 cell, 203  
peripheral nervous system, 107  
protein level, 331  
recombinant DNA, 77  
recombinant human  
  biological assay, 76–77  
  biological property, 75–81  
  immunoblotting, 76  
  immunochemical property, 75–81  
suicide gene, 108–109  
tumor, 195  
Nerve growth factor gene expression  
  cAMP, 90  
  *in vitro* study, 90–91  
Nerve growth factor receptor, 107–114  
  immunoreactivity, forebrain  
  cholinergic neuron, 308–310  
Nerve growth factor-regulated  
  phosphoprotein, 203–215  
  polyclonal antiserum, 209  
Nerve growth factor-sensitive  
  phosphorylation  
  K-252a, 195–200  
  PC12 cell, 195–200  
Nerve terminal sprouting, biochemical  
  measurement, 296  
Neuraminidase  
  calcium, 27–29  
  glycoconjugate, cell surface, 23, 27  
  neurite outgrowth, 23–27  
Neurite  
  cholera B inhibition, 23, 26  
  electrical activity, 233–234  
  elongation, 203–204  
  intermediate filament, 214  
  microtubule, 214  
Neurite outgrowth  
  endogenous ganglioside, 23–27  
  exogenous calcium, 18–19  
  growth cone, 229–276  
  neuraminidase, 23–27  
Neuritogenesis  
  exogenous ganglioside, 17–18  
  N2A cell, sodium, 20, 22  
  neuraminidase-stimulated, blockage, 27–28  
Neuroblastoma cell  
  GM<sub>1</sub> ganglioside, 27  
  mouse, 17  
Neurodegeneration  
  dopamine recovery, 293–302  
  ganglioside, 294–295  
Neurodegenerative disease, 75  
  central nervous system, neurotrophic  
  factors, 293  
  nerve growth factor, 85  
Neurofilament protein, melanocortin, 166  
Neurological outcome, cerebral ischemia, GM<sub>1</sub>, 344–345

- Neuron  
 calcium, 231–244, 286–289  
 cell injury, 280–282  
 cholinergic  
   basal forebrain, 307–320  
   central nervous system, 219–226  
   nerve growth factor, somal size, 222–226  
   nucleus basalis, 328–329  
   septum, 328–329  
   striatum, 328–329  
 confocal microscopy, 287–288  
 cytoskeleton, 286  
 development  
   melanocortin, 161–162  
   nerve growth factor, 107  
 function recovery, 277–345  
 nucleus basalis cholinergic, nerve growth factor, 328–329  
 regeneration, 277–345  
 septal cholinergic, nerve growth factor, 328–329  
 Neuronal death, cerebral ischemia, 341–344  
 Neuronal form, origin, 231  
 Neuronal growth cone  
   calcium concentration determination, 241, 243  
   heterogeneity, 241, 243  
 degeneration, 231  
 embryonic growth cone, 231  
 glutamate, 233  
 intracellular calcium, 238–240  
 mature life, 231  
 neurite outgrowth, 229–276  
 neuronal form, 231  
 neuronal integration, 236–238  
 neurotransmitter, 233  
 regulation, 232–236  
 serotonin, 233  
 stimulus, 232–236  
 trophic factor, 233  
 Neuronal membrane regulation, cytosolic protein molecule, 4–7  
 Neuronal plasticity  
   cerebrovascular insufficiency, 339  
   melanocortin, 161–169  
   neuronal growth cone, 231  
 Neuronal sprouting, nerve growth factor, 107  
 Neuronal third messenger, 3–15  
 Neuronotoxic factor, brain injury, 340  
 Neuronotrophic factor, brain injury, 340  
 Neuropathy, peripheral. *See* Peripheral neuropathy  
 Neuropeptide, melanocortin, 161  
 Neurotransmitter, 3–15  
   growth cone, 233  
 Neurotrophic effect, melanocortin, 162  
 Neutrotrophic effect, melanocortin, 167  
 Nigrostriatal neuron, abnormal behavior, 298–299  
 Non-cholinergic population, nerve growth factor, 100–101  
 Nucleus basalis  
   cholinergic neuron, 327–334  
   corpus striatum, 328
- O**
- Oligodendrocyte, 247–248  
 gliogenesis, 40  
 myelinogenesis, 247–254  
 Oncogene  
   *int-2*, 63–66  
   retroviral, 3  
   simian sarcoma, 37–38  
 Onuf's nucleus, 264–265  
 Onuf's nucleus motoneuron, 269  
   synaptic input, 266–267  
 Ornithine decarboxylase, K-252a, 198–199  
 Ovarian cancer, cisplatin, 168–169  
 Oxidative stress, aging, 111
- P**
- Parafascicular nucleus, melanocortin, 163–164  
 Parasympathetic nucleus, sacral, 264–265  
 Parasympathetic preganglionic neuron, 264–265  
 Parkinson's disease, 293  
 PC12 cell

- K-252a, interaction, 199  
nerve growth factor, 203  
nerve growth factor-sensitive phosphorylation, 195–200  
Peptide, brain, aging, 164  
Peptide hormone, melanocortin, 161  
Peripheral nervous system  
  nerve damage, 164–166  
  nerve growth factor, 107  
Peripheral neuropathy,  
  acrylamide, 167  
  melanocortin, 167–169  
Peripherin, 208–211  
Phosphoprotein  
  cytoskeletal, 203–215  
  nerve growth factor-regulated, 203–215  
    polyclonal antiserum, 209  
Phosphorylation  
  cytoskeletal, 211–215  
  ganglioside assay, 146  
  K-252a, 195–200  
  nerve growth factor-sensitive, 195–200  
  PC12 cell, 195–200  
  protein, ganglioside, 175  
  receptor, 142–147  
  slow migrating protein, 196  
  tyrosine hydroxylase, 7–10  
Phosphorylation assay, ganglioside, 146  
Platelet-derived growth factor  
  astrocyte, 247  
  glioblastoma, 35–40  
  gliogenesis, 35–40  
  glioma, malignant, 39–40  
  ligand, 35–37  
  receptor, 35–37  
Polyclonal antiserum, nerve growth factor-regulated phosphoprotein, 209  
Polylysine, growth cone, 232  
Pons, cholinergic neuron, 327–334  
Post-receptor phenomena, 159–228  
Proopiomelanocortin  
  endorphin, 167  
  melanocortin, 167  
Protein, microtubule-associated, 204–208  
Protein kinase, 175  
brain, ganglioside, 119  
chromaffin cell, 9–10  
Protein kinase A, 3–10  
Protein kinase C, ganglioside, 178–179  
Protein level, nerve growth factor, 331  
Protein phosphorylation, ganglioside, 175  
Protein sequence, heparin-binding growth factor, 63–64  
Proto-oncogene, 3
- Q**
- R**
- Radiation, growth-associated triggering factor, 51–53  
Rat brain membrane  
  binding protein, 123–126  
  ganglioside, 119–131  
Rat glioma cell C6–2B, 86–87  
Receptor  
  basal forebrain projection neuron  
    high-affinity site, 99  
    low-affinity site, 99  
  corticosteroid, 164  
Receptor-binding protein, platelet-derived growth factor, 123–126  
Receptor phosphorylation, ganglioside, 142–147  
Recombinant DNA, nerve growth factor, 77  
Recombinant human nerve growth factor  
  biological assay, 76–77  
  biological property, 75–81  
  immunoblotting, 76  
  immunochemical property, 75–81  
Reflex plasticity, sacral spinal cord, 271–274  
Regeneration  
  central nervous system, 47–54  
  growth-associated triggering factor, 48–49  
  neuron, 277–345  
Regenerative capability, axonal injury, 53

Regulation, nerve growth factor gene expression, 85–92  
 Remyelination, growth-associated triggering factor, 53  
 Retroviral oncogene, 3  
 RNA, heparin-binding growth factor, 63–64

**S**

Sacral micturition reflex, synaptic plasticity, 264  
 Sacral parasympathetic nucleus, 264–265  
 Sarcoma. See Simian sarcoma  
 Secondary brain damage, monosialoganglioside treatment, 339–345  
 Secondary neuronal death, cerebral ischemia, GM<sub>1</sub>, 341–344  
 Septum, cholinergic neuron, 327–334  
 Serotonic, growth cone, 233  
 Simian sarcoma oncogene, 37–38  
*v-sis*, 37–38  
 Slow migrating protein, phosphorylation, 196  
 Somatosensory orientation, haloperidol, 299–301  
 Spinal cord function recovery, 271–274  
 sacral, 263–274  
 degenerating fiber, 267–268  
 Onuf's nucleus motoneuron, 267–268  
 preganglionic neuron, 269–270  
 synaptic plasticity, 263–274  
 transection efferent neuron, 264–270  
 elimination reflex, 264–270

Stomach tumor, heparin-binding growth factor, 63

Streptozotocin, diabetes, 168  
 Striatum, cholinergic neuron, 327–334  
 Substantia nigra, tyrosine hydroxylase immunoreactive cell, 296–297  
 Suicide gene, nerve growth factor, 108–109  
 Synaptic input, Onuf's nucleus motoneuron, 266–267  
 Synaptic plasticity

defecation reflex, 264  
 defined, 263  
 hormonal change, 270–271  
 sacral micturition reflex, 264  
 sacral spinal cord, 271–274  
 spinal cord, 263–274

**T**

Telencephalon, myelinogenesis, 249–254  
 Testicular cancer, cisplatin, 168–169  
 Tetradecanoylphorbol-13-acetate, ganglioside, 175  
 Third messenger neuronal, 3–15  
 nuclear, multigene expression, 10–14  
 Transcriptional activation, DNA, 3–4  
 Transforming potential, heparin-binding growth factor, 67–68  
 Transmembrane signal, ganglioside, 135–154  
 Trophic factor receptor, 83–158  
 Trophic regulation, myelinogenesis, 247–258  
 β-Tubulin, 204  
 Tumor, nerve growth factor, 195  
 Tyrosine hydroxylase, 3–4  
 adrenal medulla, induction, 7, 9  
 cAMP increase, 5–7  
 colchicine, 7–8  
 homodimerization, 7–10  
 phosphorylation, 7–10  
 Tyrosine hydroxylase immunoreactive cell, substantia nigra, 296–297  
 Tyrosine kinase ganglioside, 139  
 glycosphingolipid, 135

**U****V**

*v-sis* glioblastoma, 39  
 simian sarcoma, 37–38  
 Virus, simian sarcoma, 37–38

**W**

Working memory, melanocortin, 164

**X, Y, Z**





















NIH Library, Building 10  
National Institutes of Health  
Bethesda, Md. 20892



<http://nihlibrary.nih.gov>

---

10 Center Drive  
Bethesda, MD 20892-1150  
301-496-1080

3 1496 00451 3340

**N**~~DEC 01 1990~~~~DEC 20 1990~~~~FEB 05 1991~~~~MAR 01 1991~~~~FEB 20 1991~~~~MAY 23 1991~~