INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

U·M·I

University Microfilms International
A Bell & Howell Information Company
300 North Zeeb Road. Ann Arbor. MI 48106-1346 USA
313/761-4700 800/521-0600

Order Number 9405571

A study of the effects of epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) on mesencephalic dopaminergic neurons in culture: Glia-mediated protection against neurotoxins and stimulation of regrowth

Park, Tina Hairyung, Ph.D.

City University of New York, 1993

U·M·I
300 N. Zeeb Rd.
Ann Arbor, MI 48106

A STUDY OF THE EFFECTS OF EPIDERMAL GROWTH FACTOR (EGF) AND BASIC FIBROBLAST GROWTH FACTOR (bFGF) ON MESENCEPHALIC DOPAMINERGIC NEURONS IN CULTURE: GLIA-MEDIATED PROTECTION AGAINST NEUROTOXINS AND STIMULATION OF REGROWTH

by

TINA H. PARK

A dissertation submitted to the Graduate Faculty in
Biomedical Sciences in partial fulfillment of the requirements
for the degree of Doctor of Philosophy,
The City University of New York

1993

This manuscript has been read and accepted for the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirements for the degree of Doctor of Philosophy.

8/30/93
Date Signature

James L. Roberts, Ph.D.
Chair of Examining Committee

Aug 30, 1993
Date Signature

Terry Krulwich, Ph.D.
Executive Officer

Catherine Mytilineou, Ph.D.

Pedro Pasik, M.D.

Mariann Blum, Ph.D.

Cheryl Dreyfus, Ph.D.

Supervisory Committee

THE CITY UNIVERSITY OF NEW YORK

ABSTRACT

A STUDY OF THE EFFECTS OF EPIDERMAL GROWTH FACTOR (EGF) AND BASIC FIBROBLAST GROWTH FACTOR (bFGF) ON MESENCEPHALIC DOPAMINERGIC NEURONS IN CULTURE: GLIA-MEDIATED PROTECTION AGAINST NEUROTOXINS AND STIMULATION OF REGROWTH

by

Tina H. Park

Advisor: Dr. Catherine Mytilineou

Many peptide trophic factors have been shown to be able to maintain the survival and promote the recovery of injured central nervous system neurons. We investigated the effects of two trophic factors, epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF), on rat embryonic mesencephalic cultures in order to test whether or not they could (a) modify the damage caused by the specific dopaminergic neurontoxins 1-methyl-4-phenylpyridinium (MPP^+) and 6-hydroxydopamine (6-OHDA), or (b) affect the ability of the dopaminergic neurons to recover following toxin-induced damage.

Our results demonstrate that both EGF and bFGF protect dopaminergic neurons against the neurotoxic effects of MPP^+ and 6-OHDA, and also promote regenerative processes of injured dopaminergic neurons. We provide evidence that these effects are mediated by astrocytic glial cells. Therefore, our studies suggest that astrocytic glial cells synthesize and secrete factors which enhance the biochemical and morphological

development of dopaminergic neurons *in vitro* and increase the resistance of these cells to specific neurotoxins.

ACKNOWLEDGEMENTS

I wish to express my sincere appreciation to my advisor, Dr. Catherine Mytilineou, for her constant support, guidance and encouragement as my mentor and a true friend. She has been my source of inspiration to persevere and to enjoy scientific research.

I am grateful to the members of my dissertation committee for their invaluable advice and criticism during the formulation of this research and its completion.

I would like to acknowledge Julia Shen, who passed away during the last year of my dissertation work. She provided me with technical assistance on timeless occasions and became a caring friend. She is missed greatly.

I thank my parents for their confidence and pride in all my accomplishments, however big or small. It is through their support, financially and emotionally, that I have been able to continue my education this far.

Last, but most importantly, I wish to express my gratitude to my husband, Il-Pyung, for giving me all the patience, love and understanding that was needed for me to finish this work. He sacrificed much more than I asked for, so that I may achieve my goal.

TABLE OF CONTENTS

Approval Page ii

Abstract iii

Acknowledgments v

Table of Contents vi

List of Tables x

List of Figures xi

I. Introduction 1

A. Parkinson's Disease 2

B. Dopaminergic Neurotoxins 3

1. MPTP and MPP^+ 3

2. 6-OHDA 5

C. Neurotrophic Factors 6

1. Relevance to Injury and Regeneration 6

2. Astroglial-Derived Factors 7

3. EGF 8

4. bFGF 10

II. Experimental Procedures 12

A. Preparation and Maintenance of Dissociated Cell Cultures 12

1. Neuronal Cultures 12

2. Glial Cultures 13

3. Neuronal-Glial Cocultures 14

B. Experimental Treatment of Cultures 14

1. Growth Factors 14

2. Inhibitor of Cell Proliferation 15

3. Glial Conditioned Medium 15

4. Neurotoxins 15

C. Analytical Procedures 16

1. High Affinity DA Uptake 16

2. Immunocytochemistry 16

3. Cell Counts and Morphometry 17

4. HPLC 17

5. Protein Assay 18

D. Statistical Analysis 18

III. Results 19

A. Development of Dopaminergic Neurons in Primary Mesencephalic Culture 19

B. Effects of Growth Factors on Mesencephalic Culture 24

1. Survival and Neurite Outgrowth of Dopaminergic Neurons 24

2. Glial Cell Proliferation 28

C. Development of Dopaminergic Neurons in Neuronal-Glial Coculture 35

D. Effects of Glial Conditioned Media on Mesencephalic Culture 43

1. Survival and Neurite Outgrowth of Dopaminergic Neurons 43

2. Glial Cell Proliferation 45

E. Growth Factor Effects on the Neurotoxicity of MPP^+ and 6-OHDA on Dopaminergic Neurons 45

1. Short-term Pretreatment with Growth Factors 50

2. Long-term Pretreatment with Growth Factors 57

3. Inhibition of Growth Factor-Stimulated Glial Cell Proliferation 65

F. Effects of Glial Monolayers on the Neurotoxicity of MPP^+ and 6-OHDA on Dopaminergic Neurons ... 65

G. Effects of Glial Conditioned Media on the Neurotoxicity of MPP^+ and 6-OHDA on Dopaminergic Neurons ... 79

H. Growth Factor Effects on the Regrowth of Damaged Dopaminergic Neurons ... 83

IV. Discussion ... 90

A. Enhanced Development of Dopaminergic Neurons ... 90

1. Effects of Growth Factors ... 90
2. Effects of Glial Monolayers ... 91
3. Effects of Glial Conditioned Media ... 91
4. Summary of Results ... 92

B. Mechanisms of Glial Neurotrophic Effects ... 93

1. Extracellular Matrix Molecules ... 93
2. Secreted Factors ... 93

C. Neurotrophic Factors in Glial Conditioned Medium ... 94

D. Region-Specific Differences in Neuron-Glia Interactions ... 95

E. Protection Against MPP^+ and 6-OHDA Neurotoxicity ... 96

1. Effects of Growth Factors ... 96
2. Effects of Glial Monolayers and Glial Conditioned Media ... 97
3. Summary of Results ... 98

F. Neuroprotective Mechanisms ... 99

1. Glial Cells ... 99
2. Glial Secretion of Trophic Factors ... 100
3. Defense System Against Oxygen Radicals ... 101

G MPP$^+$ Neurotoxicity 102
H. Neuronal Recovery from Injury 103
I. Neurotrophic Factors and Degenerative Disease 105
J. Conclusion 106
V. References 107

LIST OF TABLES

Table 1: EGF and bFGF increase the protein content in mesencephalic cultures 34

Table 2: Effects of glial cell substrate on the dopaminergic cell survival 42

Table 3: Effects of glial conditioned media on the dopaminergic cell survival 44

Table 4: Effects of glial conditioned media on the stimulation of astrocytic cell proliferation 49

Table 5: Effect of MPP^+ treatment on TH+ cell survival 52

Table 6: Effects of bFGF treatment against 6-OHDA neurotoxicity 60

Table 7: Effects of glial substrate on MPP^+ and 6-OHDA neurotoxicity 74

Table 8: Concentration of MPP^+ in the culture medium 76

Table 9: Concentration of 6-OHDA in the culture medium 77

Table 10: Lack of potentiation of glial protective effects against MPP^+ and 6-OHDA by growth factors 78

LIST OF FIGURES

Figure 1: The in vitro development of [^{3}H]DA uptake 20

Figure 2: The developmental growth of dopaminergic neurons over time in vitro visualized with TH immunocytochemistry 21

Figure 3: The in vitro survival of dopaminergic neurons 23

Figure 4: Effects of EGF and bFGF on the in vitro development of [^{3}H]DA uptake 25

Figure 5: EGF and bFGF stimulation of dopaminergic neuritic outgrowth visualized with TH immunocytochemistry 26

Figure 6: Long-term survival of dopaminergic neurons with EGF or bFGF treatment ... 29

Figure 7: EGF stimulation of serotonergic neuritic outgrowth visualized with 5-HT immunocytochemistry 30

Figure 8: EGF and bFGF stimulation of cell proliferation visualized with phase contrast microscopy 32

Figure 9: GFAP-immunoreactive astrocytes respond to EGF and bFGF 36

Figure 10: Effects of glial substrates on the [^{3}H]DA uptake 39

Figure 11: Glial stimulation of dopaminergic neuronal development visualized with TH immunocytochemistry 40

Figure 12: Effects of glial conditioned media on the [^{3}H]DA uptake 46

Figure 13: Stimulation of dopaminergic neuronal development by glial conditioned media visualized with TH immunocytochemistry 47

Figure 14: Effect of MPP^+ treatment on [^{3}H]DA uptake 51

Figure 15: Effect of MPP^+ treatment on dopaminergic neurons visualized with TH immunocytochemistry, and EGF and bFGF stimulation of the regrowth of dopaminergic processes following MPP^+ treatment 53

Figure 16: Effect of MPP^+ treatment on dopaminergic neuritic length 55

Figure 17: Generalized toxicity produced by 6-OHDA visualized with phase contrast microscopy 58

Figure 18: Protective effects of bFGF against 6-OHDA neurotoxicty on dopaminergic neurons visualized with TH immunocytochemistry 61

Figure 19: Protective effects of EGF and bFGF against MPP^+-induced reduction in $[^3H]$DA uptake 64

Figure 20: Protective effects of EGF and bFGF against MPP^+-induced TH+ cell loss 66

Figure 21: Inhibition of glial cell proliferation by FUDR treatment visualized with phase contrast microscopy 67

Figure 22: Inhibition of glial cell proliferation abolishes the protective effects of EGF and bFGF against MPP^+-induced reduction in $[^3H]$DA uptake 69

Figure 23: Protective effects of glial substrates against MPP^+- and 6-OHDA-induced reduction of $[^3H]$DA uptake 71

Figure 24: Protective effects of glial substrate against MPP^+ and 6-OHDA toxicity on dopaminergic neurons visualized with TH immunocytochemistry 72

Figure 25: Protective effects of glial conditioned media against MPP^+- and 6-OHDA-induced reduction of $[^3H]$DA uptake 80

Figure 26: Protective effects of glial conditioned media against MPP^+ and 6-OHDA toxicity on dopaminergic neurons visualized with TH immunocytochemistry. 81

Figure 27: EGF and bFGF stimulation of the recovery of $[^3H]$DA uptake following MPP+ treatment 84

Figure 28: Effect of MPP^+ treatment on dopaminergic neurons visualized with TH immunocytochemistry, and EGF and bFGF stimulation of the regrowth of dopaminergic processes following MPP^+ treatment 85

Figure 29: EGF and bFGF increase dopaminergic neuritic length following MPP^+ treatment 88

I. INTRODUCTION

Many peptide growth factors have been shown to be able to maintain the survival and promote the recovery of injured central nervous system (CNS) neurons. Since epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) have been demonstrated to enhance the survival and differentiation of dopaminergic neurons in mesencephalic cell cultures (Ferrari et al., 1989; Casper et al., 1991; Engele and Bohn, 1991), we investigated in this thesis the possible neuroprotective effects of EGF and bFGF against the neurotoxicity of 1-methyl-4-phenylpyridinium (MPP^+) and 6-hydroxydopamine (6-OHDA) on cultured dopaminergic neurons. More specifically, we examined whether treatment of rat embryonic mesencephalic cultures with these growth factors could (a) modify the damage caused by the specific dopaminergic neurotoxins MPP^+ and 6-OHDA, or (b) affect the ability of the dopaminergic neurons to recover following toxin-induced damage.

EGF and bFGF were found to stimulate the proliferation of astrocytes in our cultures, and thus we hypothesized that astrocytes mediate the neurotrophic effects of these growth factors on dopaminergic neurons. Therefore, we examined whether astrocytic glial cells alone, derived from the mesencephalon and from the striatum, could mimic the effects produced by EGF and bFGF on mesencephalic cultures. These two anatomical regions, containing the dopaminergic neuronal somata and axon terminals, were selected to determine the presence of region-specific neuron-glia interactions. Mesencephalic and striatal glial monolayers in neuron-glia cocultures, and conditioned media obtained from these glia were tested for their effects against the neurotoxicity produced by MPP^+ and 6-OHDA on dopaminergic neurons.

Our results demonstrate the essential role of glial cells in mediating the trophic effects on dopaminergic neurons and protective effects against neurotoxins produced by EGF and bFGF. It is proposed in this thesis that astrocytic glial cells synthesize and secrete factors which enhance the biochemical and morphological development of dopaminergic neurons *in vitro* and increase the resistance of these cells to specific neurotoxins.

A. Parkinson's Disease

Parkinson's Disease is a neurodegenerative disorder which increases in incidence with age (see review by Marsden, 1990). Degeneration of the pigmented dopaminergic neurons in the substantia nigra pars compacta, resulting in striatal dopamine (DA) deficiency, and the presence of eosinophilic Lewy body inclusions in the cytoplasm of surviving neurons are pathological hallmarks of Parkinson's disease. A reduction in the striatal DA content by 80% or greater leads to manifestation of the clinical symptoms. The major parkinsonian symptoms include akinesia, rigidity and tremor. Although the cause of idiopathic Parkinson's disease is unknown, it is hypothesized that either heredity or environmental neurotoxins may have important roles in the etiology of the disease (Agid, 1991).

Degeneration of dopaminergic neurons appears to continue during the process of the disease (McGeer et al., 1988), which is probably the reason for the progressive deterioration of motor functions in parkinsonian patients. The possibility to prevent or delay the progression of Parkinson's disease has recently been the subject of intensive experimental and clinical research (Cohen et al., 1984; Langston et al., 1984; The Parkinson Study Group, 1989; Tetrud and Langston, 1989). Therapeutic approaches

which have been tested include neurotransmitter replacement with levodopa--a precursor of DA biosynthesis, antioxidative interventions and neural implantation.

B. Dopaminergic Neurotoxins

1. MPTP and MPP+

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) was discovered as a contaminant in illicit preparations of the meperidine-like narcotic which caused clinical effects very closely resembling Parkinson's disease (Davis et al., 1979; Langston et al., 1983). MPTP has since been widely used to produce experimental models of Parkinson's disease in various animals, including monkeys (Burns et al., 1983), dogs (Johannessen et al., 1989), cats (Schneider et al., 1986) and mice (Heikkila et al., 1984). Although neurotoxic effects have been demonstrated in these animals, species differences in sensitivity to MPTP was found. For example, while primates are very sensitive to the toxic effects of MPTP, mice require high doses (Heikkila et al., 1984) and rats appear to be resistant (Chiueh et al., 1984). It is suggested that differences in MPTP metabolism and metabolite clearance rates may be responsible for species differences in sensitivity to this toxin (Johannessen et al., 1985). MPTP neurotoxicity is also found to be age-dependent, so that older animals are much more susceptible to its toxic effects than younger ones (Gupta et al., 1986; Langston et al., 1987). This is possibly due to increased activity of the MPTP metabolizing enzyme, monoamine oxidase-B (MAO-B), with age (Strolin-Benedetti and Dostert, 1989).

Metabolism of MPTP is essential for its neurotoxic activity. MPTP is metabolized via a two-step oxidation to the intermediate, 1-methyl-4-phenyl-2,3,-dihydropyridinium ($MPDP^+$), and subsequently to 1-methyl-4-phenylpyridinium (MPP^+;

Chiba et al., 1984). The initial conversion to $MPDP^+$ is mediated by the enzyme MAO-B and further oxidation to MPP^+ occurs spontaneously. *In vivo* evidence that MPP^+ is the toxic compound comes from studies where intracerebrally administered MPP^+ caused depletion of DA and also parkinsonian behavioral deficits (Heikkila et al., 1985; Bradbury et al., 1986). The effects of both MPTP and MPP^+ *in vitro* have been studied in our laboratory on explant and dissociated cell cultures of rat embryonic mesencephalon, and it was found that they caused reduction of DA uptake levels and destruction of dopaminergic neurons (Mytilineou and Cohen, 1984; Mytilineou et al., 1985; Mytilineou and Friedman, 1988). Similar findings on selective neurotoxicity of MPP^+ for dopaminergic neurons in culture have been reported by others (Sanchez-Ramos et al., 1986, 1988; Michel et al., 1990).

MPTP is a specific neurotoxin for dopaminergic neurons because its metabolite, MPP^+, is selectively accumulated within these neurons via the high affinity DA reuptake system (Chiba et al., 1985; Javitch et al., 1985; Schinelli et al., 1988). In fact, kinetic properties of the uptake of MPP^+ and DA by rat striatal synaptosomes, as well as inhibition by DA reuptake system blockers , were found to be very similar (Javitch et al., 1985). MPTP conversion to MPP^+ takes place extraneuronally since MAO-B is most prevalent within astroglial cells (Westlund et al., 1985). It has been reported that astrocytes can convert MPTP to MPP^+ *in vitro* (Ransom et al., 1987). Inhibitors of MAO-B such as deprenyl and pargyline are able to block the neurotoxicity of MPTP *in vivo* (Cohen et al., 1984; Heikkila et al., 1984; Langston et al., 1984).

There are two major hypotheses for the mechanism of MPTP neurotoxicity (see reviews by Sayre, 1989; Gerlach et al., 1991). The first hypothesis proposes that as a result of MPP^+ redox cycling, intraneuronal generation of cytotoxic free radical species such as superoxide and hydrogen peroxide may cause oxidative stress, thus leading to cellular damage. In support of this hypothesis, Hasegawa et al. (1990) found MPP^+-

induced formation of superoxide radicals in heart submitochondrial particles. Although there is evidence that MPTP and MPP^+ can induce oxidative stress (Johannessen et al., 1986; Cleeter et al., 1992), it is not the mechanism of action that is currently accepted. According to the second hypothesis, MPP^+ is actively accumulated within the mitochondria (Ramsay and Singer, 1986), where it is believed to cause cell destruction by inhibiting NADH-linked mitochondrial respiration at the level of Complex I (Nicklas et al., 1985; Krueger et al., 1990). A significant reduction of Complex I activity has been found in the substantia nigra of patients with idiopathic Parkinson's disease (Parker et al., 1989; Schapira et al., 1990), thereby justifying the use of MPTP and MPP^+ to produce experimental models of Parkinson's disease in animals and *in vitro*.

2. 6-OHDA

6-Hydroxydopamine (6-OHDA) is a well known neurotoxin specific for catecholaminergic neurons (see review by Kostrzewa and Jacobowitz, 1974). As a structural analogue of catecholamines, 6-OHDA is selectively taken up and accumulated within catecholaminergic neurons by the high-affinity transport system. The degenerative effect of 6-OHDA in the CNS was first described by Ungerstedt (1968). It was found that 6-OHDA caused degeneration of dopaminergic cell bodies and terminal processes (Breese and Traylor, 1970; Uretsky and Iversen, 1970).

6-OHDA is unstable at physiological pH and it rapidly autooxidizes to form cytotoxic free radical species such as quinones, hydrogen peroxide, superoxide and hydroxyl radicals (Heikkila and Cohen, 1972; Sachs and Jonsson, 1975). These reactive oxidation products may cause damage to neuronal structures as a result of oxidation of sulfhydryl groups of enzymes and membrane lipid peroxidation. Loss of neuronal function, such as neurotransmitter uptake mechanisms, follows and eventually leads to cell death. Cellular defenses against these reactive oxygen species include various

antioxidant enzymatic systems such as superoxide dismutase, glutathione peroxidase, glutathione reductase and catalase.

6-OHDA has been reported to produce degeneration of dopaminergic neurons in experimental animals (Jonsson, 1976) and in mesencephalic cell cultures (Mytilineou and Danias, 1989). Although it is highly selective for catecholaminergic neurons, the effects of 6-OHDA are dose-dependent, so that at higher concentrations it can cause unspecific damage to both noncatecholaminergic neurons and glial cells.

C. Neurotrophic Factors

Neuronal survival and differentiation during development, and the maintenance of its function in the adult nervous system is regulated by peptides called neurotrophic factors. Molecules directed to the regulation of neuronal growth and survival have been distinguished from those directed specifically to the regulation of growth or regrowth of neuronal processes. All of these agents, together, will be referred to as neurotrophic factors. They appear to be involved in the repair of damaged neuronal systems and lack of these factors has been implicated as one of the possible causes in several neurodegenerative diseases (Appel, 1981).

1. Relevance to Injury and Regeneration

Neurotrophic activity is reported to increase following injury to the brain. It has been shown *in vivo* that injury to the central nervous tissue induces a time-dependent accumulation of substances at the lesion site, which are neurotrophic in test cultures for a variety of neuronal types (Lundborg et al., 1982; Nieto-Sampedro et al., 1982, 1983;

Manthorpe et al., 1983). Such a response to injury by the central nervous tissue may be interpreted as an attempt at regeneration.

Neurotrophic factors are believed to be involved in neuronal regeneration, and thus the goal of many current investigations is to search for factors which are capable of promoting neuronal survival and stimulating the neurite growth response following injury. Since regeneration of neurons in the adult CNS requires some type of trophic support, it is plausible that application of exogenous supply of trophic factors could maintain survival and enhance regrowth of damaged neurons. There have been several recent reports of neurotrophic activity on central degenerating neurons. Nerve growth factor (NGF) was found to promote the survival of septal cholinergic neurons *in vivo* following axonal injury caused by fimbrial transections (Hefti, 1986; Williams et al., 1986). Basic fibroblast growth factor (bFGF) was also found to prevent the death of lesioned septal cholinergic neurons *in vivo* (Anderson et al., 1988) and to cause morphological and biochemical recovery in MPTP-lesioned nigrostriatal system of mice (Otto and Unsicker, 1990). Recently, Hyman et al. (1991) and Beck et al. (1992) reported that brain-derived neurotrophic factor (BDNF) promotes the survival of mesencephalic dopaminergic neurons and reduces the toxicity of MPP^+ *in vitro*. In addition, BDNF was also found to be protective against 6-OHDA-induced dopaminergic cell death in culture (Spina et al., 1992). Identification of a neurotrophic factor specific for dopaminergic neurons is clearly one of the most important goals, since it is the degeneration of these neurons which is characteristic for Parkinson's disease.

2. Astroglial-Derived Factors

Purified cultures of astroglial cells produce and release neurotrophic factors into their culture medium. These soluble factors have been tested in experiments with various CNS neurons and were found to display neuronotrophic and/or neurite-promoting

activities (Banker, 1980; Manthorpe et al., 1982; Muller and Seifert, 1982; Barbin et al., 1984; Patel and Hunt, 1989; Giulian et al., 1993). Although the nature of these factors in glial conditioned medium (CM) is unknown, several investigators reported that cultured astrocytes synthesize NGF (Lindsay, 1979; Manthorpe et al., 1986; Houlgatte et al., 1989; Ono et al., 1991; Yoshida and Gage, 1991), while others (Hatten et al., 1988; Gray and Patel, 1992) found bFGF in their astroglial CM. Mattson and Rychlik (1990) also reported that FGF is synthesized by glial cells and that it can protect neurons against excitatory amino acid-induced cell death.

Implantation of neural tissue into the damaged CNS has been investigated as a way to promote functional recovery. Survival of both the grafted tissue and injured host neurons is suggested to be promoted by glial-derived neurotrophic factors. Bohn et al. (1987) grafted adrenal medullary tissue into MPTP-lesioned mouse brain and obtained recovery of dopaminergic neurons in the host. They hypothesized that one of the mechanisms for this recovery may be the synthesis of neurotrophic factors by glia, as a result of graft-stimulated gliosis. Reactive glial cells surrounding a wound site are also implicated as the source of injury-induced neurotrophic activity found in the brain (Nieto-Sampedro et al., 1983; Finkelstein et al., 1988). These findings offer new insight into the role of glia in CNS regeneration, where it has been generally believed that the formation of glial scar acts as a physical barrier to interfere with regenerative processes (Liuzzi and Lasek, 1987).

3. EGF

EGF is a polypeptide of 53 amino acids, first isolated from adult male mouse submaxillary gland (Cohen, 1962). It is a potent mitogen and a differentiation factor for a variety of cells both *in vivo* and *in vitro* (see review by Carpenter, 1979). EGF immunoreactivity (Fallon et al., 1984; Lakshmanan et al., 1986) and precursor mRNA

(Rall et al., 1985) were detected in the rodent brain, suggesting that EGF is synthesized in the brain. Although the concentration of EGF as measured by enzyme immunoassay in brain homogenates was shown to be extremely low (Probstmeier and Schachner, 1986), a recent study has provided evidence of EGF synthesis in both the developing and adult brain by the detection of EGF mRNA expression in the mouse (Lazar and Blum, 1992).

One of the earliest signals of EGF action is the activation of protein tyrosine kinase integrated in the receptor molecule (Pandiella et al., 1989). The existence of specific EGF receptor (EGFR) has been demonstrated in the mouse brain (Adamson and Meek, 1984). More recent reports have also demonstrated the presence of EGFR in the brain of rats (Hiramatsu et al., 1988) and found them to be associated with both glia (Wang et al., 1989) and neurons (Gomez-Pinilla et al., 1988). EGFR was also detected by immunocytochemistry in the human nervous system (Werner et al., 1988).

Evidence has accumulated to support the role of EGF as a neurotrophic agent in the CNS. It has been shown that EGF binds to and stimulates DNA synthesis and cell division in astrocytic glial cells (Leutz and Schachner, 1981; Simpson et al., 1982). Furthermore, EGF enhanced the survival and process outgrowth of cortical neurons and cerebellar neurons in culture (Morrison et al., 1987, 1988). Similar effects of EGF was found in mesencephalic cultures by our group, specifically for dopaminergic neurons (Casper et al., 1991). We also found that EGF increased the survival and proliferation of mesencephalic neuronal precursors in culture (Mytilineou et al., 1992). In experimental models of brain injury, EGF was shown to modify anoxic neuronal injury in cortical cultures (Kinoshita et al., 1990). In addition to its neurotrophic effects demonstrated *in vitro* , EGF was found to exert *in vivo* trophic effects on MPTP-lesioned mice to enhance injured dopaminergic parameters in the striatum (Hadjiconstantinou et al., 1991).

4. bFGF

bFGF is a 146 amino acid polypeptide, belonging to the family of heparin-binding growth factors (see reviews by Westermann et al., 1990; Wagner, 1991). Members of this family exhibit structural homology and bind to heparin with high affinity. bFGF was first isolated from bovine pituitary gland and brain (Gospodarowicz et al., 1984), and has been shown to demonstrate mitogenic and differentiation activity on a wide variety of cell types (see review by Gospodarowicz et al., 1987). Numerous reports are available on bFGF-mediated mitogenic responses in astrocytes (Pettmann et al., 1985) and oligodendrocytes (Eccleston and Silberberg, 1985), and neurotrophic effects on neurons from various brain areas (Walicke, 1988; Matsuda et al., 1990). bFGF can also stimulate the proliferation of neuroblasts and neuroepithelial cells *in vitro* (Gensburger et al., 1987; Murphy et al., 1990).

bFGF is found in relatively high concentrations in embryonic and adult mammalian CNS (Gospodarowicz et al., 1987; Caday et al., 1990). bFGF has been purified from adult human brain homogenate (Bohlen et al., 1985) and it was shown that cultured human fetal brain neurons contain and synthesize bFGF (Torelli et al., 1990). bFGF mRNA appears to be widespread and abundant in the rat brain (Ernfors et al., 1990). Immunohistochemical studies have yielded conflicting results concerning the cellular distribution of bFGF. Several investigators found bFGF localization primarily in neurons (Pettmann et al., 1986; Janet et al., 1988), while others found bFGF immunoreactivity primarily in astrocytes (Gomez-Pinilla et al., 1992; Woodward et al., 1992) and demonstrated that cultured astroglia can synthesize bFGF (Ferrara et al., 1988; Hatten et al., 1988). It is possible that bFGF is expressed at different times during CNS development in these two cell types. Evidence shows that bFGF is released from both astroglial and neuronal cells *in vitro* (Araujo and Cotman, 1992); however, it is not yet

known by what mechanism bFGF, which lacks a signal peptide sequence (Esch et al., 1985; Abraham et al., 1986), is released from these cells.

Like EGF, bFGF initiates its action by interacting with a cell surface receptor. In agreement with findings of bFGF activity on both neurons and glia, high affinity receptor sites were located on both cell types (Araujo and Cotman, 1992). Specific receptors for bFGF were demonstrated on neurons *in vitro* (Walicke, 1988) and *in vivo* (Ferguson et al., 1990). *In situ* hybridization revealed a broad distribution of bFGF receptor mRNA in the adult CNS, primarily localized in neurons (Wanaka et al., 1990).

Evidence has been accumulating which suggests that bFGF functions as a neurotrophic agent in the CNS. bFGF promotes neuronal survival in hypothalamic cultures (Torres-Aleman et al., 1990), cerebellar granule cells (Hatten et al., 1988), septal neurons (Grothe et al., 1989), and also chick spinal cord neurons (Unsicker et al., 1987). In embryonic cultures of cortex, hippocampus, and mesencephalon, bFGF promotes survival of neurons and stimulates neurite outgrowth (Morrison et al., 1986; Walicke et al., 1986; Rousselet et al., 1988; Ferrari et al., 1989). Effect of bFGF on neuronal survival has also been demonstrated in experimental lesion paradigms. For example, septal cholinergic neurons can be rescued by bFGF following fimbria-fornix lesion in the adult rat (Anderson et al., 1988). An increased production of bFGF was found in response to brain injury (Finkelstein et al., 1988; Frautschy et al., 1991). Also, bFGF was shown to be effective against glutamate excitotoxicity in cultured hippocampal neurons (Mattson et al., 1989) and striatal neurons (Freese et al., 1992). All these observations suggest that bFGF may play an important role in the development and maintenance of the CNS. In addition, as has been demonstrated in the nigrostriatal system of MPTP-treated mice (Otto and Unsicker, 1990), bFGF may stimulate regrowth of neuronal fibers and aid in the recovery of injured neuronal systems.

II. EXPERIMENTAL PROCEDURES

A. Preparation and Maintenance of Dissociated Cell Cultures

1. Neuronal Cultures

Neuronal cultures were established from rat embryonic tissues on either the 14th or the 16th day of gestation. Timed female pregnant Sprague-Dawley rats were purchased from Zivic-Miller Laboratories (Allison Park, PA). Animals were sacrificed with CO_2 vapor and cesaerian section was performed to quickly remove the uterus containing the embryos, which was then placed in a petri dish containing minimal essential medium (MEM; Gibco). Under sterile conditions, embryos were taken out of the uterus one by one and placed in Earle's balanced salt solution (BSS; Gibco) with gentamycin. After a 10 min waiting period, the embryos were rinsed with BSS and the mesencephalon was disssected out using the dissection microscope. Tissues were dissected out without their membrane coverings and collected in Ca^{++} and Mg^{++} free phosphate buffered saline (PBS; Gibco) at 4°C. Tissue fragments were minced into small pieces and then mechanically dissociated in chemically defined medium by mild trituration with a small bore Pasteur pipette. The number of viable cells was determined by exclusion of trypan blue dye (1:1 dilution with 4% solution) and counted with a hemocytometer. 0.5 ml of cell suspension into each polyornithine-coated (0.1 mg/ml, Sigma) 35 mm Falcon plastic dish was plated at a density of 5 X 10^5 cells/dish. Final volume in each dish was always kept at 1.5 ml. Cultures were maintained at 37°C in an atmosphere of 10% CO_2/90% air and 100% relative humidity, and were fed twice weekly with chemically defined medium (Bottenstein and Sato, 1979; DiPorzio et al., 1980), consisting of MEM/F12 (1:1, Gibco), glucose (33 mM), HEPES (15 mM), $NaHCO_3$

(44.6 mM), transferrin (100 μg/ml), insulin (25 μg/ml), putrescine (60 nM), sodium selenite (30 nM), progesterone (20 nM), and glutamine (2 mM).

2. Glial Cultures

Glial cultures were prepared from tissues of newborn rats aged 3 to 5 days. Animals were sacrificed with CO_2 vapor and placed in 70% ethanol for a few seconds. Under sterile conditions, brains were removed intact and incubated in MEM containing gentamycin. After rinsing with BSS, the whole mesencephalon or striatum was dissected out without their membrane coverings and collected in Ca^{++} and Mg^{++} free PBS on ice. Tissue fragments were chopped gently into small pieces with #11 blades and then placed in the above buffer containing 0.25% trypsin plus 1% DNAse I (10 μg/ml) for a 15 min incubation in a shaking water bath at 37°C. An equal volume of horse serum (Gibco) was added to inactivate the trypsin and the solution containing the tissue fragments was centrifuged for 10 min at 1000 rpm. The supernatant was discarded and the tissues were rinsed with BSS before dissociation. Tissues were mechanically triturated and the cell suspension was prepared in the medium consisting of MEM, heat-inactivated fetal calf serum (10%; Gibco), glucose (0.4%), plus glutamine (2 mM) and plated into noncoated 25 cm^2 culture fasks (Falcon) at a density of 1 X 10^6 cells/ml. Final volume was maintained at 5 ml. To remove the debris in the cultures, feeding medium was replaced 24 h after initial plating, and twice weekly thereafter. Cultures were maintained at 37°C in an atmosphere of 10% CO_2/90% air and 100% relative humidity. Once the cultures had grown to confluency, usually by 7 days *in vitro* , these cultures were used to collect mesencephalic and striatal CM.

3. Neuronal-Glial Cocultures

To prepare glial cell monolayer for use as substrate for neuronal cells, confluent glial cultures grown in 25 cm^2 tissue culture flasks were treated for 10 min with a buffer solution containing 0.04% trypsin, or until the cells were observed to separate. Cells collected from several flasks in this way were pooled together and 10% horse serum was added to inactivate the trypsin. The cells were then centrifuged for 10 min at 1000 rpm and the supernatant was discarded. After rinsing with BSS, the cells were gently triturated in an appropriate volume of the growth medium (not containing serum) to yield a density of 1×10^6 cells/dish. The dissociated cells were plated directly into 35 mm Falcon plastic dishes that were not coated. After the cultures reached confluency, mesencephalic cells from E14 embryonic brains were plated on these preformed glial cell monolayers. These neuronal-glial cocultures were maintained with the same chemically defined medium described above for neuronal cultures, and at 37°C in an atmosphere of 10% CO_2/90% air and 100% relative humidity. The feeding medium was replaced twice a week.

B. Experimental Treatment of Cultures

1. Growth Factors

In most experiments, cultures were refed 3 h after plating (day 0) with fresh medium in control cultures and with medium containing 10 ng/ml EGF (Collaborative Research) or 10 ng/ml bFGF (recombinant human bFGF; gift from Synergen) in growth factor-treated cultures. Fresh growth factors were replenished at each subsequent feeding and were present throughout the experiment. In some experiments, the growth factors were introduced to control cultures 10 days following initial plating.

2. Inhibitor of Cell Proliferation

The mitotic inhibitor 5-fluoro-2'-deoxyuridine (FUDR) and uridine (Sigma) at 8 μM and 20 μM, respectively, were added to the cultures in the feeding medium 3 h after plating in bFGF-treated cultures and 24 h after plating in control and EGF-treated cultures. In the control and EGF-treated cultures, addition of FUDR within the first 24 h after plating resulted in a large amount of cell loss. FUDR was added again at each refeeding and was present throughout the experiment.

3. Glial Conditioned Medium

Mesencephalic CM and striatal CM were obtained from their respective glial cell cultures grown as described above in 25 cm^2 tissue culture flasks. Once the glial cultures had reached confluency, the serum-containing glial growth medium was washed out with BSS and replaced with chemically defined medium. After a 24 h conditioning, the medium containing the glial secreted products was collected and centrifuged for 10 min at 1000 rpm to get rid of debris. The CM was then sterilized with a 0.22 μm filter (Millipore) and stored at -76°C until use.

4. Neurotoxins

Cultures were exposed to freshly prepared 10 μM MPP^+ (Research Biochemicals) in the feeding medium at 4 days *in vitro* (DIV) for 24 h or at 10 DIV for 48 h in the experiments with growth factors and with 100 μM at 6 DIV for 60 min in the experiments with glial cells or with glial CM. At the end of the exposure period, cultures were washed 3 times with BSS. Damage to dopaminergic neurons was determined by tyrosine hydroxylase (TH) immunocytochemistry and by measuring the [^{3}H]DA uptake 24 h after incubation in toxin-free feeding medium. Flow charts illustrating the various experimental conditions are as follows:

(1) Day 0	----->>	Day 4	----->>	Day 5	----->>	Day 6
Control, EGF, bFGF		MPP^+ (10 μM)		Wash 24 h later		Analyze
(2) Day 0	----->>	Day 10	----->>	Day 12	----->>	Day 13
Control, EGF, bFGF		MPP^+ (10 μM)		Wash 48 h later		Analyze
(3) Day 0	----->>	Day 6	----->>	Day 6	----->>	Day 7
Control, Glia, Glial CM		MPP^+ (100 μM)		Wash 60 min later		Analyze

Cultures were exposed to 100 μM 6-OHDA for 45 min (50 μM in the experiments with glial CM) at 6 DIV and analyzed 24 h later for neurotoxic damage to the dopaminergic neurons. Since 6-OHDA is known to undergo rapid autooxidation, it was prepared in the solution containing an antioxidant, ascorbic acid (10 μM final concentration in the cultures), immediately prior to use and kept on ice.

C. Analytical Procedures

1. High Affinity DA Uptake

Cultures were thoroughly washed with buffer and incubated with $[^3H]$DA (0.5 Ci/ml; 37 Ci/mmol; New England Nuclear) for 10 min in the presence of ascorbic acid (0.2 mg/ml) in PBS (pH 7.3), supplemented with 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$ at 37°C. After two rinses and a 5 min incubation with fresh buffer, $[^3H]$DA accumulated within the cells was released by incubating the cultures with 95% ethanol for 30 min at 37°C, then added to 10 ml Liquiscint (National Diagnostics) and counted in a scintillation spectrometer. Nonspecific uptake values were obtained by blocking neuronal uptake with 10 μM mazindol (Sandoz Pharmaceuticals).

2. Immunocytochemistry

Cultures were fixed in 10% formalin/0.03% glutaraldehyde in 0.1 M sodium acetate buffer (pH 6.5) for 10 min, followed by 4% paraformaldehyde/0.03% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 8.5) for 1 h at room temperature (from O'Malley et al., 1991) and stained with TH antibodies (1:1000; Eugene Tech); or fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 30 min and stained with antibodies against glial fibrillary acidic protein (GFAP; 1:1000; Accurate Chemical) or neuron specific enolase (NSE; 1:1000; Polysciences), using the Vectastain ABC kit (Vector Labs) with 3', 3'-diaminobenzidine as a chromogen. Cultures were permeabilized with 0.2% Triton X-100 for 30 min and incubated with the primary antibodies for 24 h at 4°C.

3. Cell Counts and Morphometry

The number of dopaminergic neurons in cultures was determined by counting the cells positively immunostained with TH antibodies. 200 fields (0.5 mm X 0.5 mm) in four transverse strips across the diameter of the dish, representing 5% of the total area, were counted using a Nikon inverted microscope at 200X magnification. The number of astrocytes was determined in the same way by counting GFAP-immunoreactive cells in 50 fields across one transverse strip. The length of the longest neurite of a TH immunopositive (TH+) neuron was estimated at 400X magnification by counting the number of its intersections with a reticule grid (0.25 mm X 0.25 mm, subdivided into 100 squares). A total of 60 cells (3 dishes, 20 cells in each) which were completely within the size of the field while moving the field from one end of the dish to the other were selected for measurements in each treatment group.

4. HPLC

High performance liquid chromatography (HPLC) with electrochemical detection (Bioanalytic Systems, West Lafayette, Ind.) was utilized to determine the concentration of MPP^+ and 6-OHDA in the extracellular medium of control cultures and mixed neuron-glia cultures. For the analysis of 6-OHDA content, samples were injected over a C-18 reverse phase column (5 µm beads, 25 cm length). The mobile phase consisted of 0.15 M monochloroacetic acid (pH 3.0), 0.7 mM EDTA, 2.0 mM sodium octylsulfate, and 6.0% acetonitrile. The flow rate was 1.0 ml/min. 6-OHDA concentration was calculated from the comparison of the peak heights and retention times of samples and standard solutions. The analysis of MPP^+ concentration in the medium was performed by the laboratory of Dr. Castagnoli , Jr., as described in their publication (Chiba et al., 1984).

5. Protein Assay

500 µl of 0.2 N NaOH was added to each culture dish to solubilize the protein and samples were collected by scraping off all cells. Protein determination was made by the method of Lowry et al. (1951), with bovine serum albumin used for standards.

D. Statistical Analysis

Data are expressed as the means ± standard error of the mean (SEM). All experiments were repeated 2 to 5 times. Significance between two groups was tested by independent t-test and by one-way analysis of variance (ANOVA) for multiple comparisons, followed by Tukey's HSD post hoc test. The computer program SYSTAT (SYSTAT Inc., Evanston, IL) was used for all statistical analyses.

III. RESULTS

A. Development of Dopaminergic Neurons in Primary Mesencephalic Culture

In order to characterize the development of dopaminergic neurons in dissociated fetal rat mesencephalic cultures, the high-affinity [^{3}H]DA uptake levels were measured and immunocytochemistry was performed with antibodies against TH, the rate-limiting enzyme in DA biosynthesis. TH immunocytochemistry is widely used as a specific marker for dopaminergic neurons, and the DA uptake activity which is sensitive to inhibition by mazindol is used as an index of normal function in these cells.

The development of dopaminergic neurons was followed for 18 days *in vitro* . As shown in Fig. 1, the DA uptake levels increased steadily with time. It should be noted that during the third week, the growth varied between experiments so that DA uptake could remain constant, increase slightly or be somewhat reduced. TH immunocytochemistry revealed that the morphological development of dopaminergic neurons parallel the gradual increase in DA uptake (Fig. 2). At Day 5, the TH+ cells are small and have very short processes. At Day 14, the cell body size has increased while processes, where most of the high affinity DA uptake sites are located, are much longer. By Day 18 the processes have outgrown the size of the field and the cell morphology is much more complex.

Cell counts of TH+ neurons indicated that there was a gradual decline in cell survival over time, which resulted in a 67% reduction during the two weeks between Day 4 and Day 18 (Fig. 3). However, notice that the DA uptake progressively increased during this period while cell loss is occurring, indicating that surviving dopaminergic

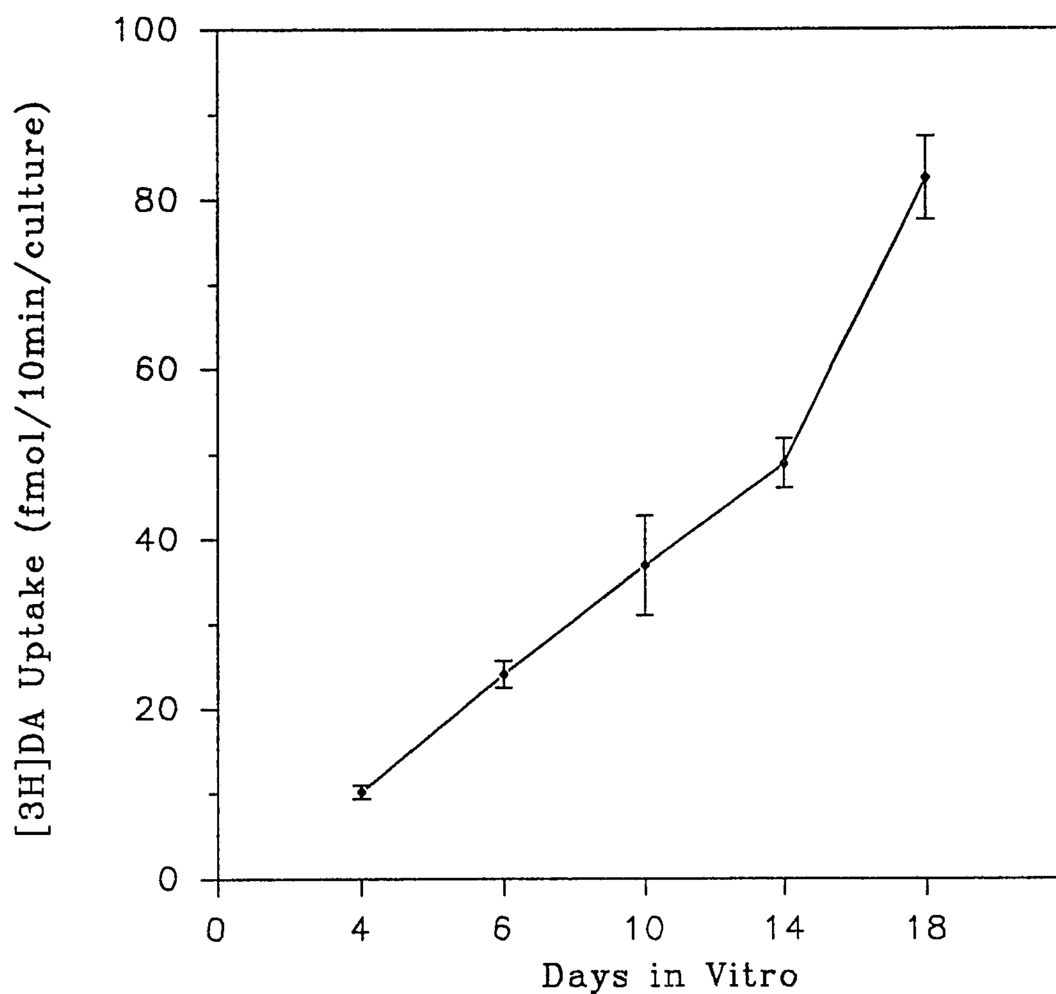
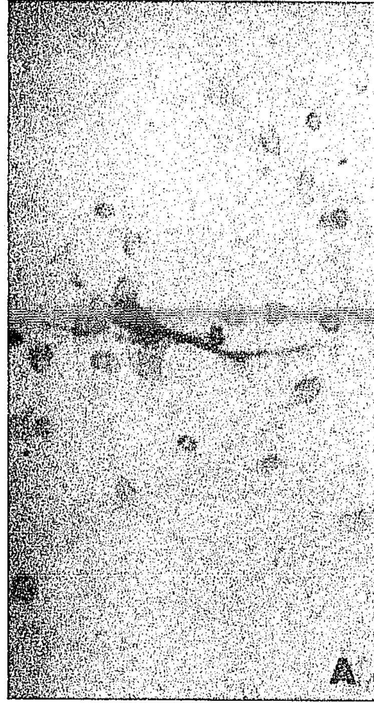
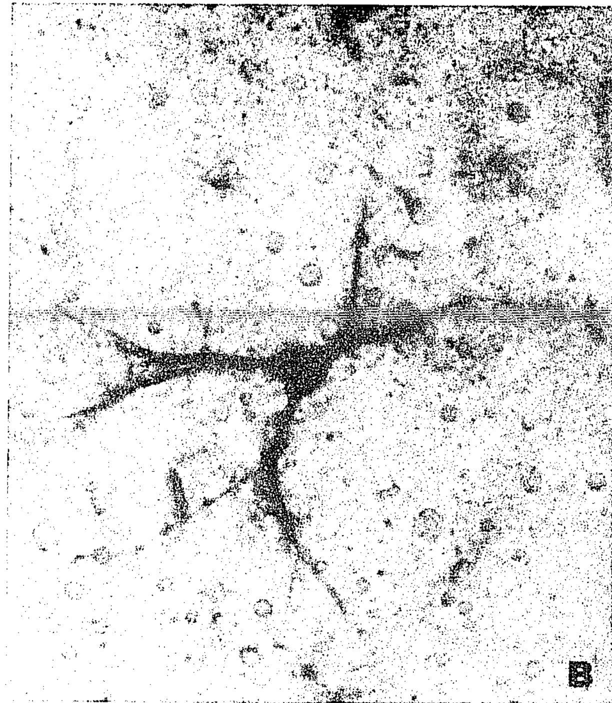
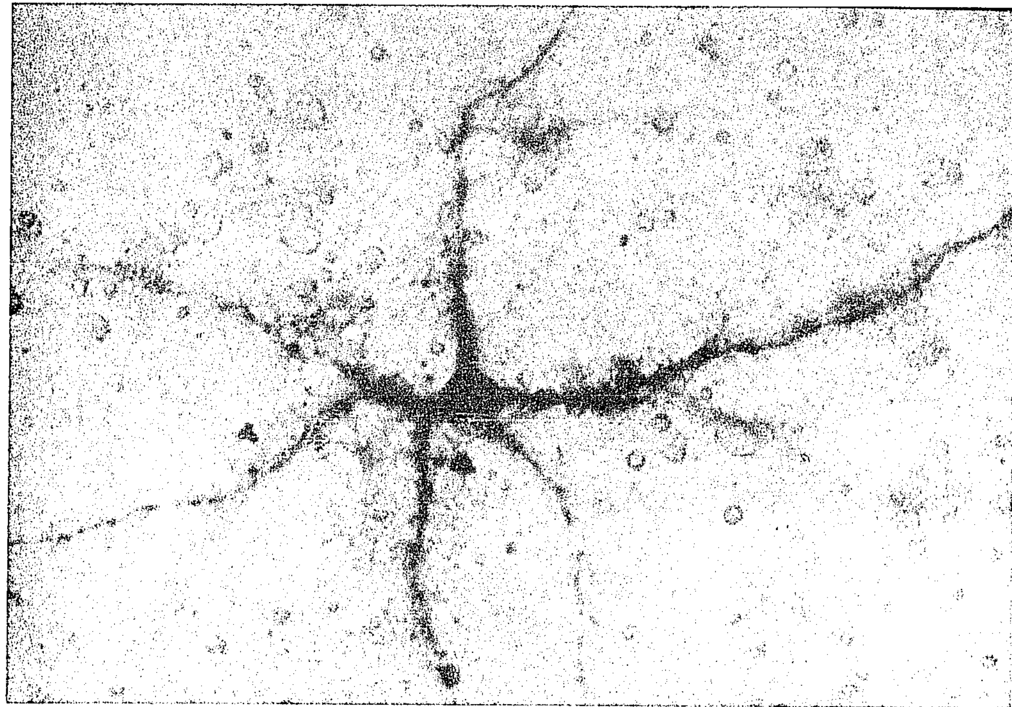


Figure 1. The in vitro development of [^{3}H]DA uptake. E16 embyonic mesencephalic cultures were plated in polyornithine-coated 35 mm dishes at a density of 5 X 10^5 cells/dish, and maintained in a serum-free, chemically defined medium for 18 days. The DA uptake levels increased steadily with time. Each point represents the mean of 5 samples ± SEM.

Figure 2. The developmental growth of dopaminergic neurons over time in vitro visualized with TH immunocytochemistry. Immunocytochemistry performed at 5 DIV (A), 14 DIV (B) and 18 DIV (C) reveals the extensive outgrowth and maturation of dopaminergic neurons with time.


A


B



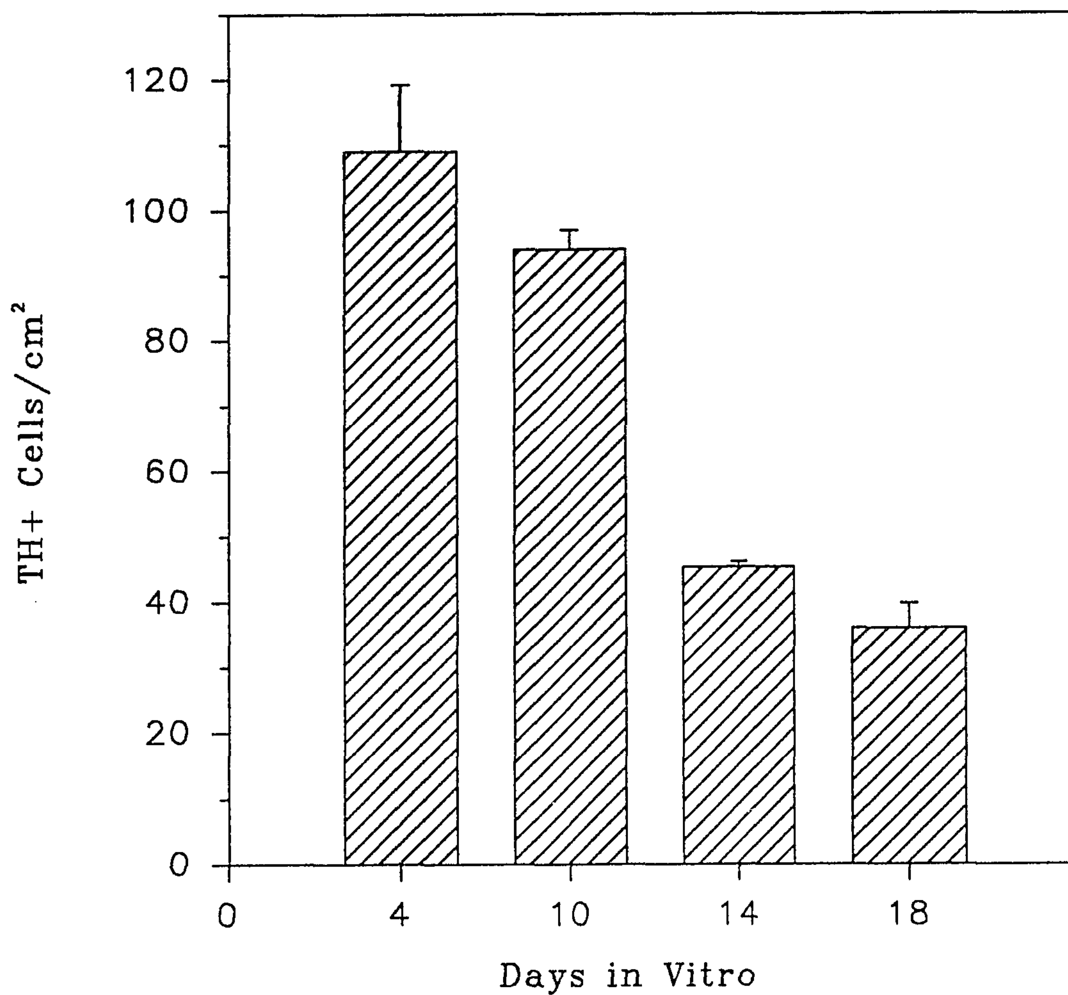


Figure 3. The in vitro survival of dopaminergic neurons. The number of TH immunopositive neurons was counted at different days of development in vitro during the 18 day growth period. A gradual decline in cell survival occurred with time. All values are the mean of 4 samples ± SEM.

neurons mature further and that neurite outgrowth continues with time, as supported by morphological observations.

B. Effects of Growth Factors on Mesencephalic Culture

1. Survival and Neurite Outgrowth of Dopaminergic Neurons

We tested whether the development of dopaminergic neurons in mesencephalic cultures is sensitive to the effects of growth factors by treating with either EGF (10 ng/ml) or bFGF (10 ng/ml) beginning at the day of plating (0 DIV) and throughout the duration of the experiments. These concentrations of the growth factors have been shown to induce a maximal trophic response in primary neuronal cultures (Morrison et al., 1987; Ferrari et al., 1989; Knusel et al., 1990; Engele and Bohn, 1991). *In vitro* development of dopaminergic neurons was monitored by measuring the high affinity [^{3}H]DA uptake levels and by immunostaining for TH. In EGF- and bFGF-treated cultures (hereafter referred to as EGF cultures and bFGF cultures, respectively), the DA uptake was approximately 2- to 3-fold higher than control levels after one week, but during the second and third weeks it increased at a much faster rate to reach levels between 10- and 15-fold higher than controls (Fig. 4). These results are in agreement with previous reports where an increase in DA uptake was observed after treatment of mesencephalic cultures with EGF (Casper et al., 1991) and bFGF (Ferrari et al., 1989; Engele and Bohn, 1991).

Dopaminergic neurons visualized with TH immunocytochemistry had longer and more elaborate processes in EGF and bFGF cultures than those in controls (Fig. 5). The time course of this enhanced outgrowth of neurites paralleled the increase in DA uptake over time, so that differences in the length and arborization of dopaminergic neuronal

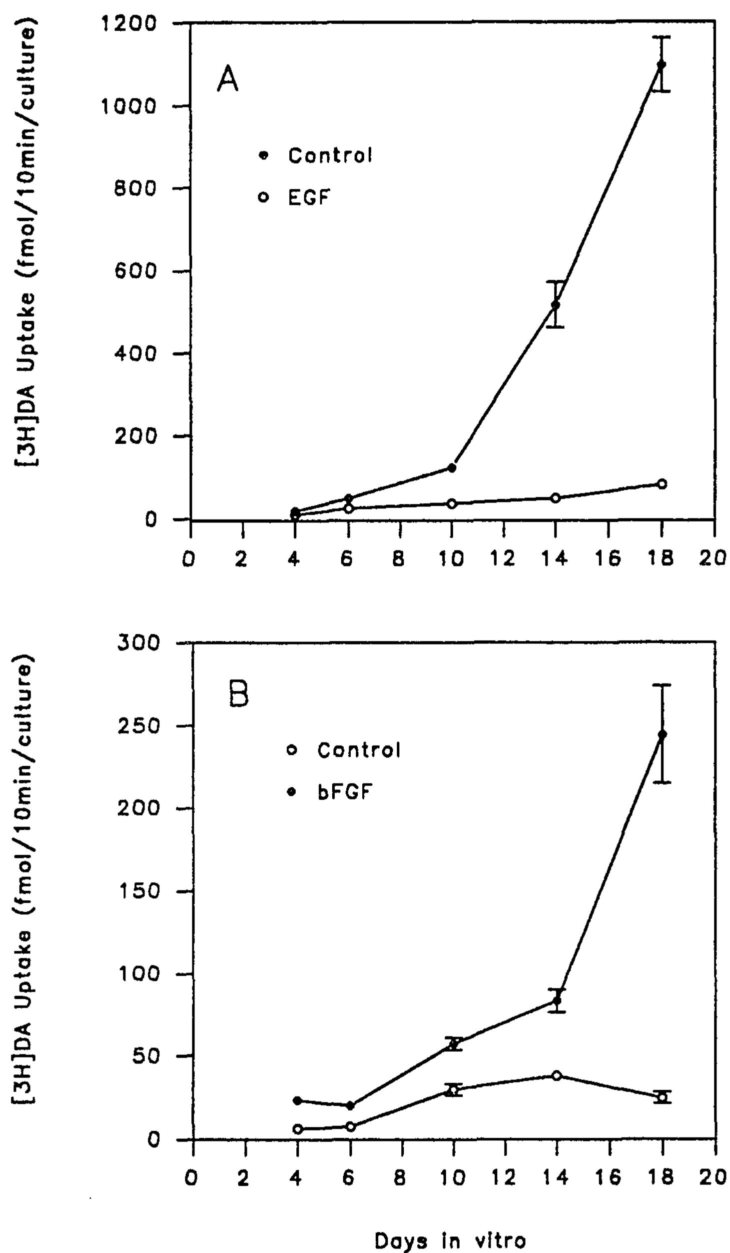
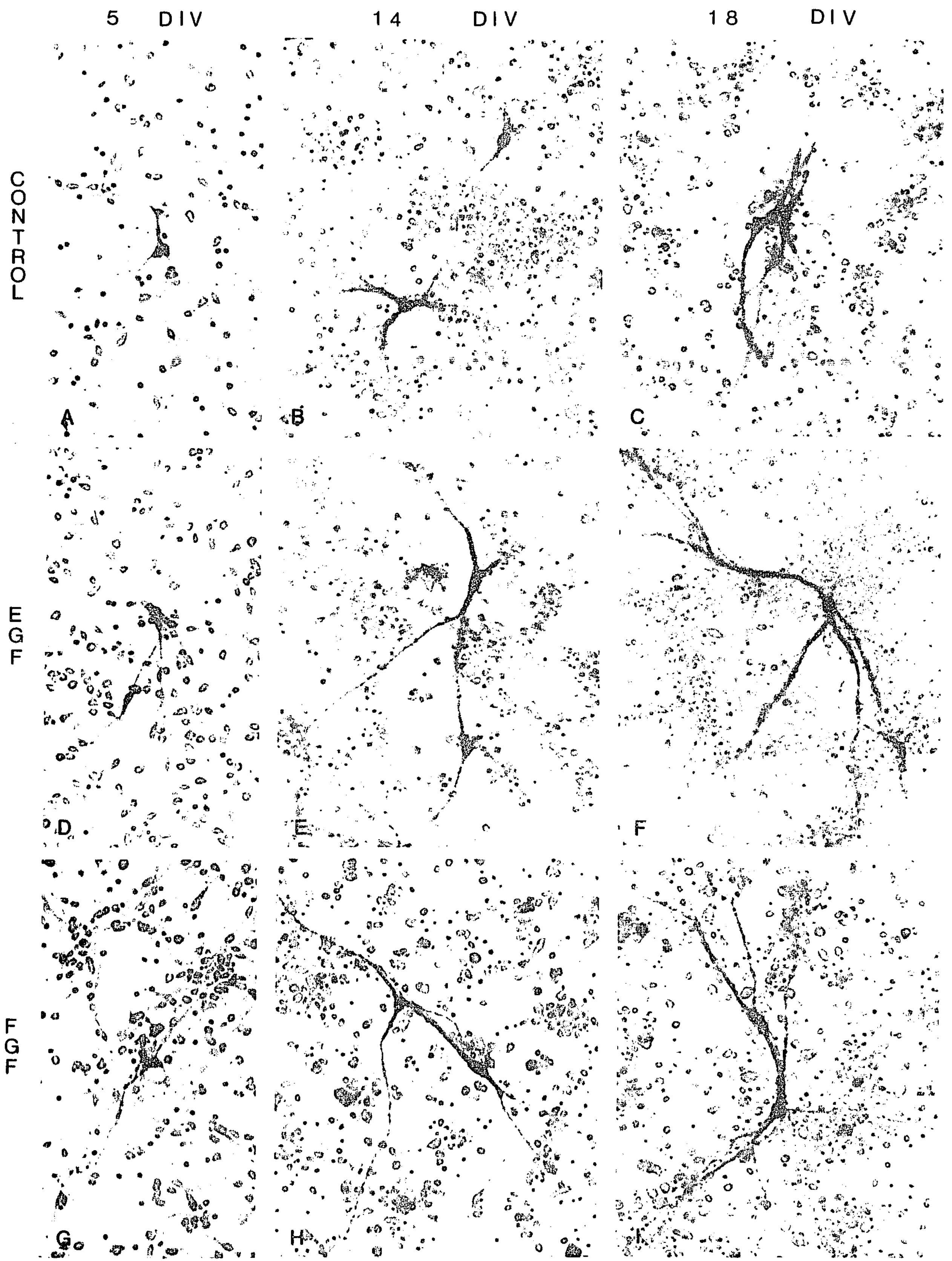


Figure 4. Effects of EGF and bFGF on the in vitro development of $[^3H]$ DA uptake. Mesencephalic cultures were maintained in a serum-free, chemically defined medium in the absence or continuous presence of EGF (10 ng/ml; A) or bFGF (10 ng/ml; B) for 18 days. Both growth factors stimulated the DA uptake to yield significantly higher values over controls at all time points ($P < 0.05$; Independent t-test). Each point represents the mean of 5 samples ± SEM.

Figure 5. EGF and bFGF stimulation of dopaminergic neuritic outgrowth visualized with TH immunocytochemistry. The development of dopaminergic neurons under different treatment conditions was followed by immunostaining for TH at 5, 14 and 18 DIV. A, B, C; No growth factors. D, E, F; EGF (10 ng/ml from 0 to 18 DIV). G, H, I; bFGF (10 ng/ml from 0 to 18 DIV). Extensive outgrowth of neurites is evident in EGF-treated (E, F) and bFGF-treated (H, I) cultures compared to controls (B, C).


5 DIV
14 DIV
18 DIV
CONTROL
EGF
FGF
A
B
C
D
E
F
G
H
I

processes became evident after one week of incubation with growth factors, while marked differences were observed after two weeks (Fig. 5E, F, H, I).

The addition of EGF and bFGF to the cultures also produced a significant increase in the long-term survival of dopaminergic neurons. In contrast to the gradual decline in cell survival in control cultures, in EGF and bFGF cultures the decrease in the number of TH+ neurons occurred at a much slower rate and there was a reduction of only about 30-40%, compared to 67% in controls, during the same two week period between Day 4 and Day 18 (Fig. 6). The number of surviving TH+ cells in EGF and bFGF cultures was significantly higher than in controls at both 14 and 18 DIV ($P < 0.05$).

The growth factor effects were not limited to dopaminergic neurons. The overall neuronal survival was also increased, as visualized with neuron specific enolase immunostaining (NSE; not shown). Also, EGF treatment in these cultures greatly enhanced the neurite outgrowth of serotonergic neurons, as shown by immunocytochemical staining with antibodies against 5-tryptophan hydroxylase which is a regulatory enzyme in serotonin biosynthesis (Fig. 7).

2. Glial Cell Proliferation

Gliogenesis *in vivo* is reported to occur mostly during late gestation and postnatally (Varon and Somjen, 1979). Therefore, our cultures which are derived from embryonic tissues at gestational days 14 or 16 presumably contain a very small number of glial cells initially. However, by about 4 days after incubation with EGF or bFGF, cell proliferation in the cultures became evident from the increase in the total number of cells observed by phase contrast microscopy (Fig. 8). Consistent with these observations, protein content in growth factor-treated cultures was significantly higher than in controls, sometimes by as much as 6- to 7-fold at 18 DIV (Table 1). Immunocytochemistry to stain for glial cells showed that the number of GFAP+ astrocytes increased slightly in

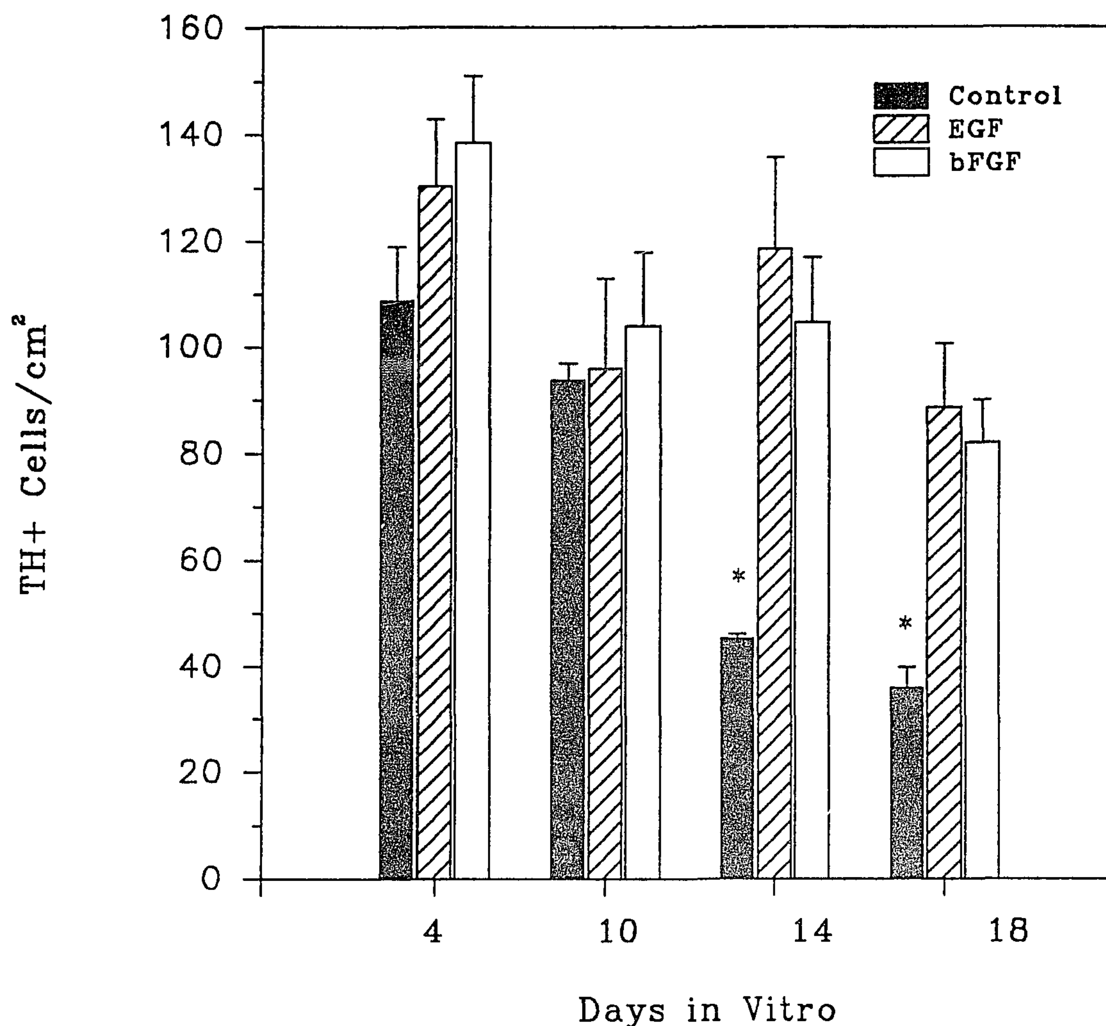
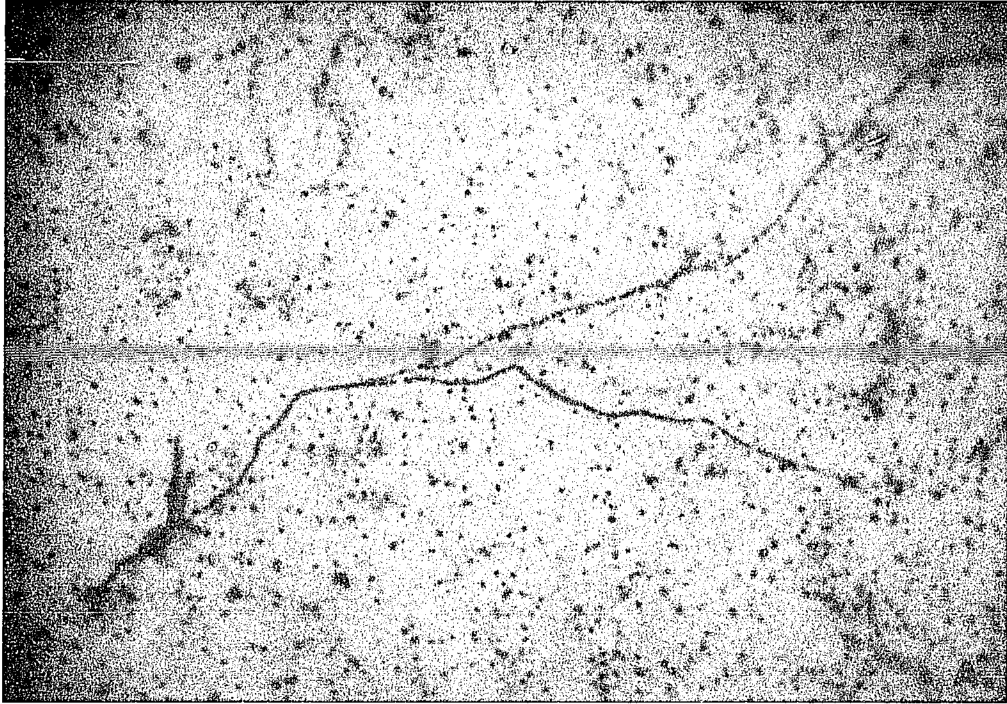


Figure 6. Long-term survival of dopaminergic neurons with EGF or bFGF treatment. The number of TH immunopositive neurons was counted at different days of development in vitro in cultures with or without EGF (10 ng/ml) or bFGF (10 ng/ml) treatment for 18 days. While the number of TH+ cells did not differ significantly between the three treatment groups at 4 and 10 DIV, the TH+ cell number of control cultures declined at 14 and 18 DIV and was significantly lower than those of growth factor-treated cultures (* $P < 0.05$; ANOVA followed by Tukey's HSD post hoc test). All values are the mean of 4 samples ± SEM.

Figure 7. EGF stimulation of serotonergic neuritic outgrowth visualized with 5-HT immunocytochemistry. Immunocytochemistry performed at 22 DIV reveals that serotonergic neurons in cultures treated with EGF (10 ng/ml from 0 to 22 DIV; B) have much more extensive outgrowth of neurites than those in control cultures (A).



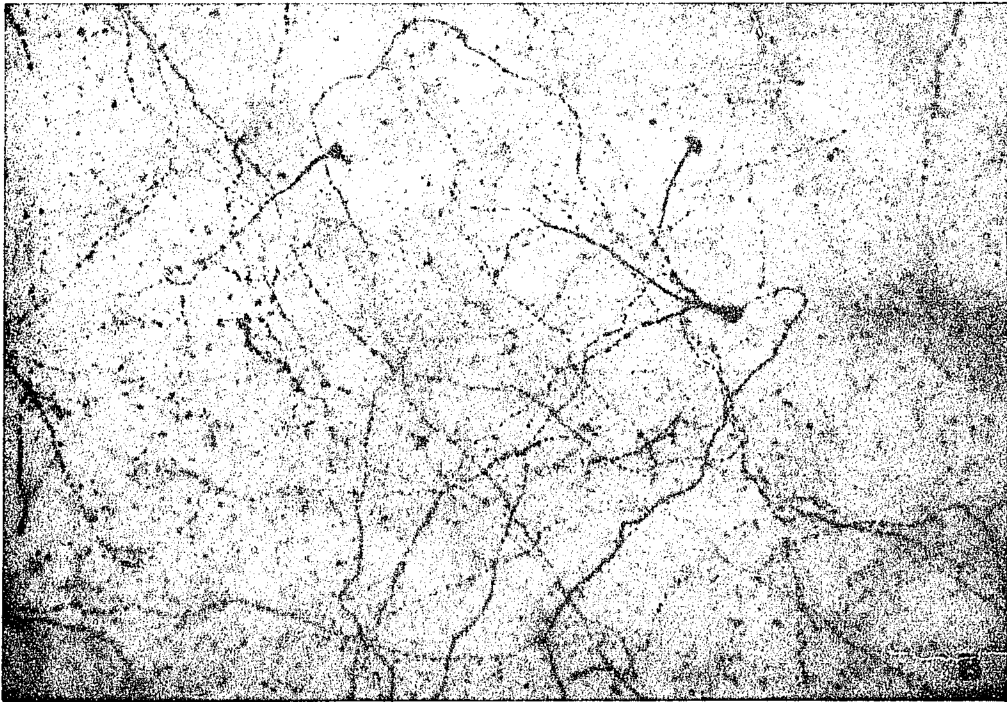
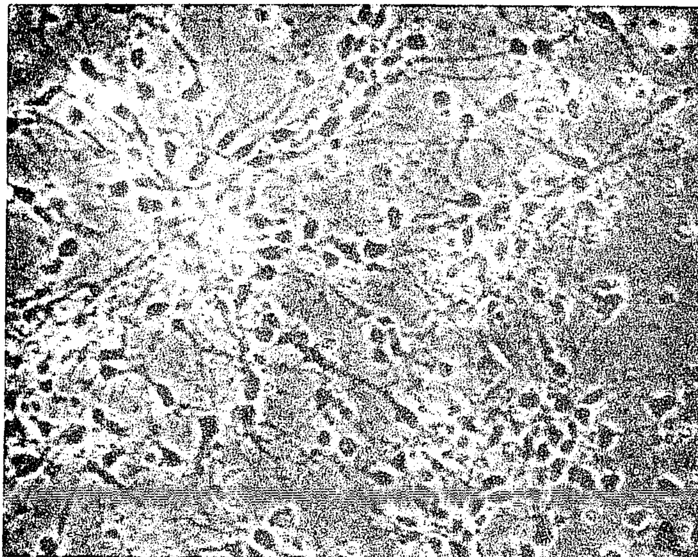
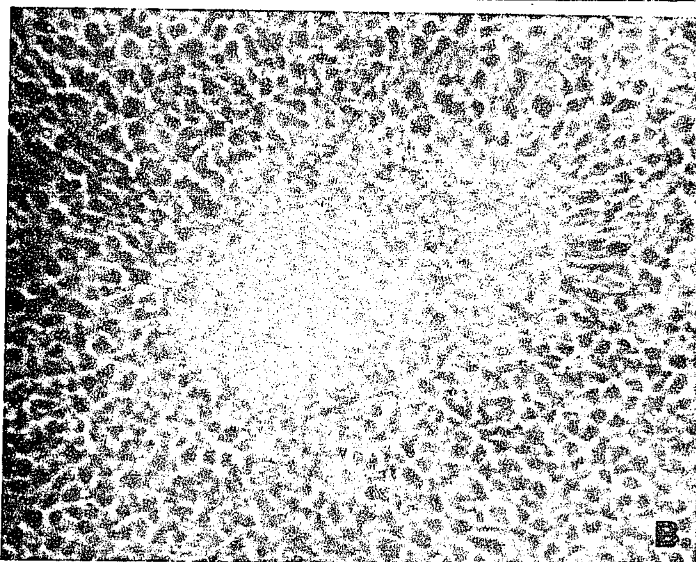


Figure 8. EGF and bFGF stimulation of cell proliferation visualized with phase contrast microscopy. At 6 DIV, the cell density in cultures treated with EGF (10 ng/ml from 0 DIV; B) or bFGF (10 ng/ml from 0 DIV; C) is visibly higher than the density of cells in control cultures (A).




B.

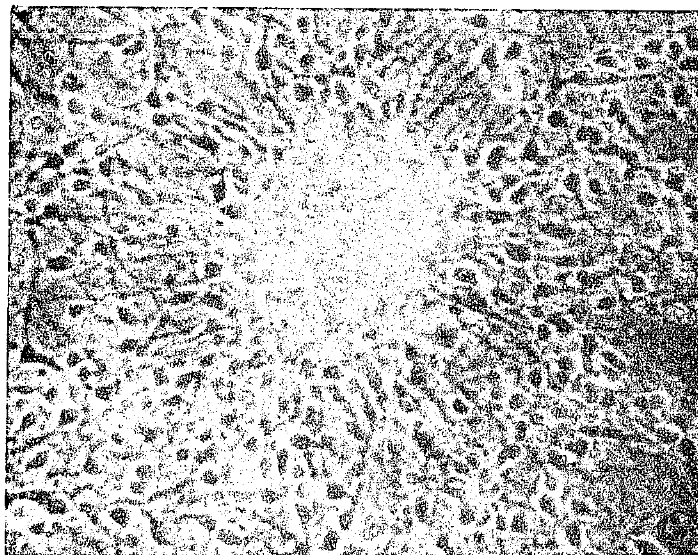


Table 1. EGF and bFGF increase the protein content in mesencephalic cultures. EGF and bFGF was added to the cultures at 0 DIV and was present throughout the experiment. Protein content was significantly higher from the respective controls at all points in the EGF-treated cultures and after 10, 14 and 18 DIV in the bFGF-treated cultures. $P < 0.05$; Independent t-test. All values represent the means of 3-5 samples ± SEM.

Treatment	Protein (μg/ culture)			
	4 DIV	10 DIV	14 DIV	18 DIV
Control	129±15	278±19	218± 8	241±19
EGF (10 ng/ml)	304±19	772±35	776±68	1012±89
Control	76± 9	49± 3	44± 4	45± 4
bFGF (10 ng/ml)	100± 9	224±15	223± 9	267± 6

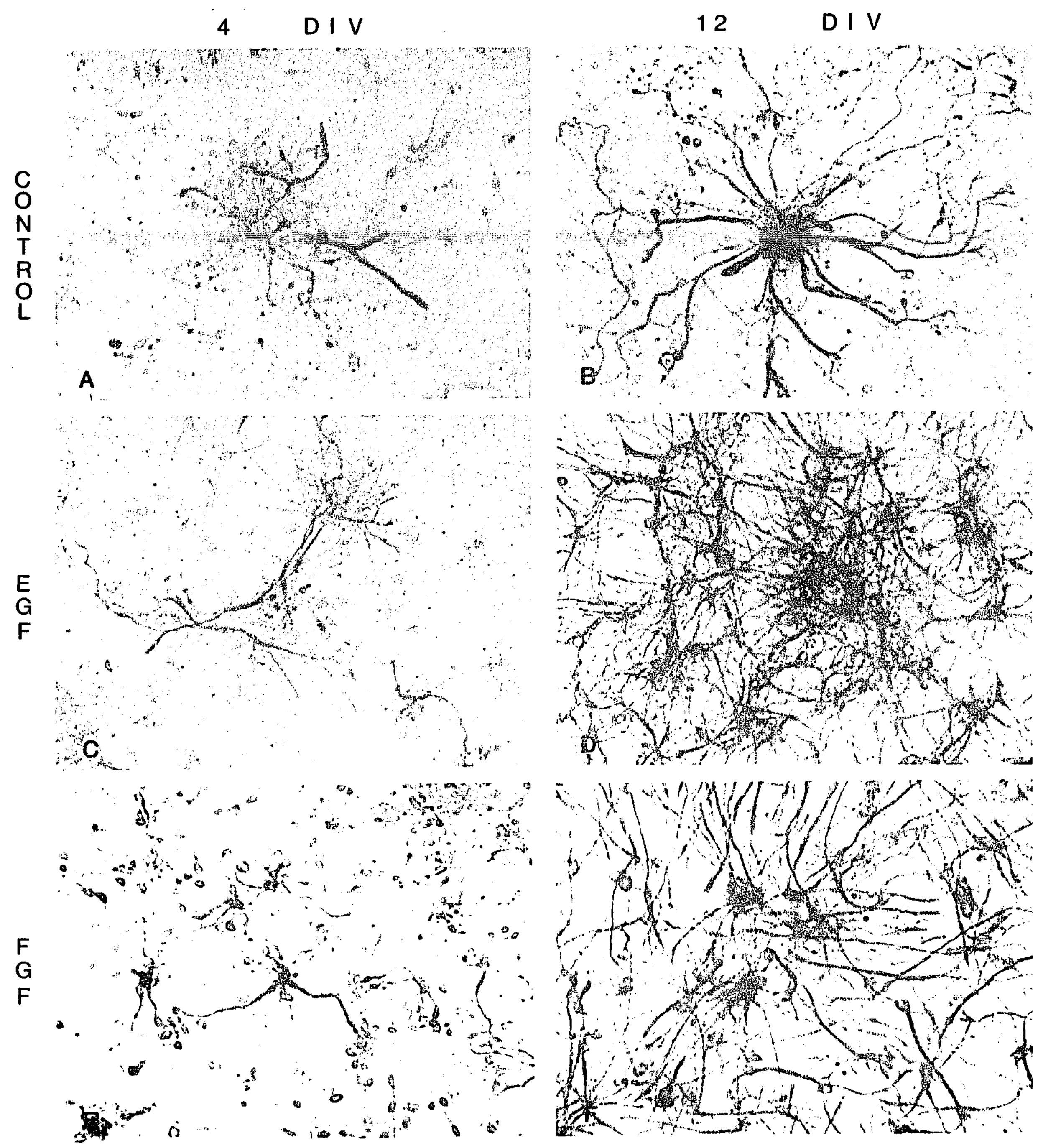
controls over time (Fig. 9A, B), whereas after growth factor treatments the density of GFAP+ astrocytes became so high that the cultures were confluent by 10 DIV (Fig. 9D, F). Because of the overlapping growth pattern of glial processes, it was not possible to determine with accuracy the number of GFAP+ cells in the growth factor-treated cultures. Glial cell proliferation continued in the presence of growth factors, even after confluency, which made it difficult to maintain cultures beyond 18 DIV.

Since cultured astrocytes have been demonstrated to have a high affinity DA uptake system (Pelton et al., 1981) and this parameter is used as a measure of dopaminergic neuronal function in our studies, it was important to determine the proportion of DA uptake contributed by astrocytes in growth factor-treated cultures. We compared the level of DA uptake in pure neuronal cultures with that of pure astrocytic cultures which contain predominantly GFAP+ cells, and found that [^{3}H]DA uptake into glial cells is approximately 12% of the level obtained in neuronal cultures. This amount falls within the range of values typically observed as mazindol-insensitive nonspecific uptake in our cultures. Thus, it appears that the increased levels of specific DA uptake produced by treatment with growth factors do not include uptake into nonneuronal cells.

C. Development of Dopaminergic Neurons in Neuronal-Glial Coculture

Astrocytic cultures were derived from either the mesencephalon or the striatum of newborn pups. Although oligodendrocytes and microglia could be detected by visual inspection, very few neurons were found to be present when we immunostained for NSE and cells positively stained with GFAP antibodies were the predominant cell type in the glial cultures (not shown). We co-cultured mesencephalic cells on mesencephalic or striatal glial monolayers to examine whether region-specific interactions between

Figure 9. GFAP-immunoreactive astrocytes respond to EGF and bFGF. Mesencephalic cultures were processed for GFAP immunocytochemistry at 4 and 12 DIV. A small increase in the number of GFAP+ cells with time was observed in control cultures maintained in chemically defined medium (A, B). The presence of EGF (10 ng/ml; C, D) or bFGF (10 ng/ml; E, F) in the cultures stimulated astrocytic proliferation and increased the density of GFAP+ cells.


4 DIV
12 DIV
CONTROL
EGF
FGF
A
B
C
D

dopaminergic neurons and glial cells, as described in previous studies (Denis-Donini et al., 1984; O'Malley et al., 1991), are present in our cultures. Cultures were grown for 7 days *in vitro* and the effects of the two types of glial monolayers on dopaminergic parameters were analyzed at this time.

The results clearly demonstrate stimulation of growth of dopaminergic neurons plated on glial substrate in cocultures compared to neuronal cultures plated on nonglial substrate (polyornithine). The [^{3}H]DA uptake in both mesencephalic and striatal glial-neuronal cocultures was almost 2.5-fold higher than that of controls after one week (Fig. 10). As expected from this increased DA uptake activity, dopaminergic neurons visualized with TH immunocytochemistry showed much more complex morphologies when grown on glial monolayers compared to controls grown on nonglia (Fig. 11). It can be seen that the TH immunoreactive neurons in the mixed neuron-glia cultures are much more developed than the neurons grown in the absence of glial cells, in terms of cell body size, neuritic arborization and length. The appearance of TH+ cells is similar whether they are grown on mesencephalic or striatal glia. These cells display highly branched and long, extended processes. After one week, cultures grown on glial monolayer derived from the striatum had nearly twice the number of TH+ neurons than cultures grown on nonglial substrate, indicating that the survival of dopaminergic neurons is also increased by the presence of astrocytes (Table 2). Mesencephalic glia similarly increased the dopaminergic cell survival.

Our results demonstrate that the biochemical and morphological development of dopaminergic neurons were enhanced by both mesencephalic and striatal glia. However, analyses of neuronal uptake activity and cell survival, together with morphological observations indicate that there are no apparent differences between homotypical and heterotypical glial cells in their support of dopaminergic neurons under normal condition.

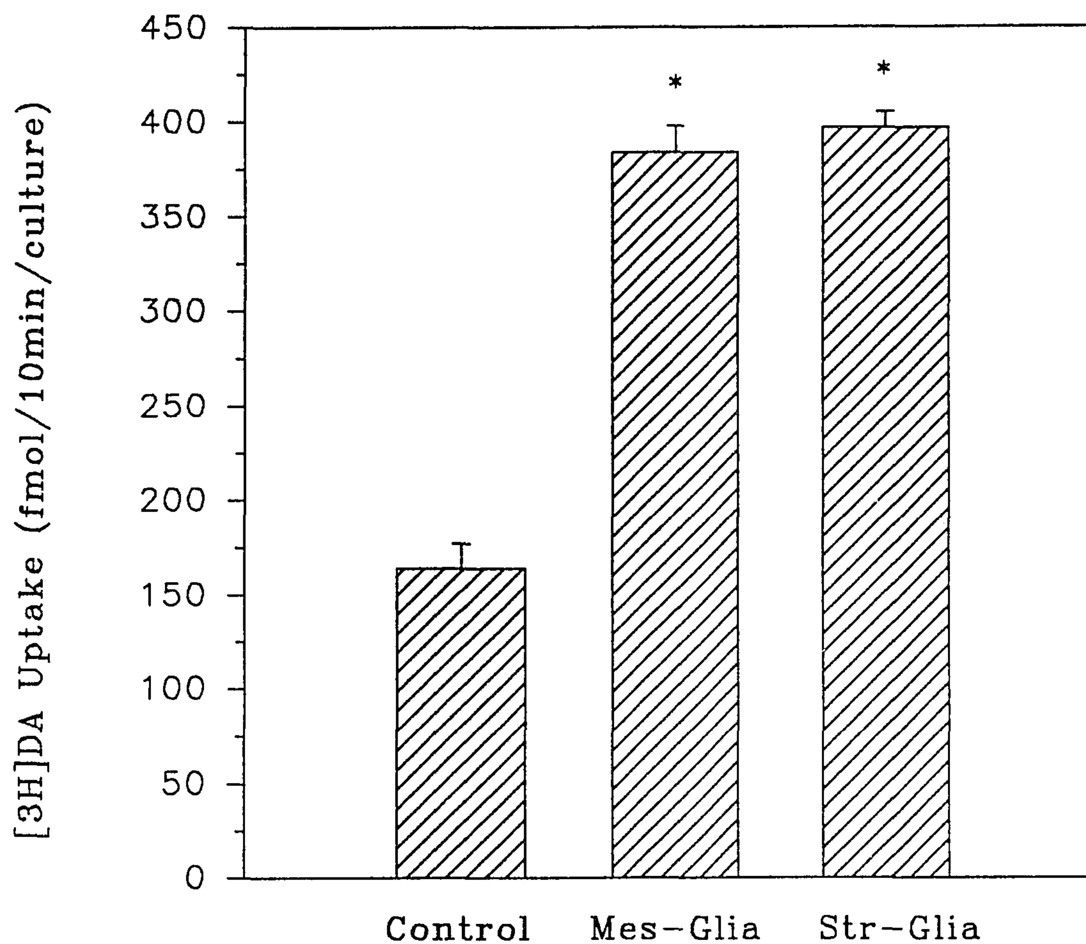
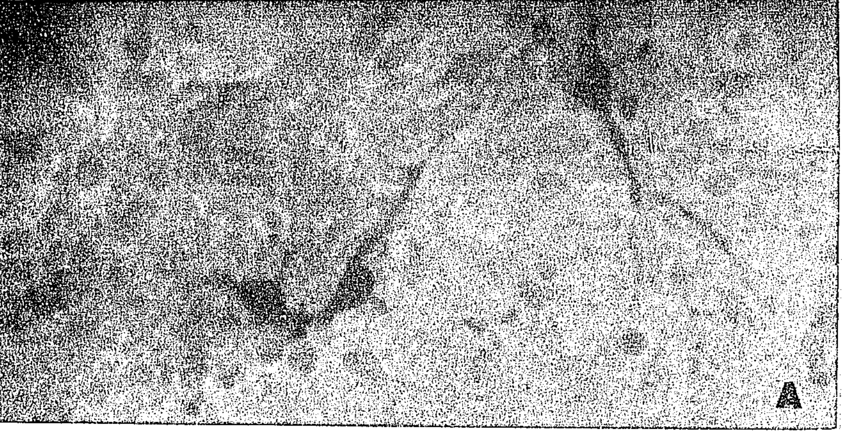
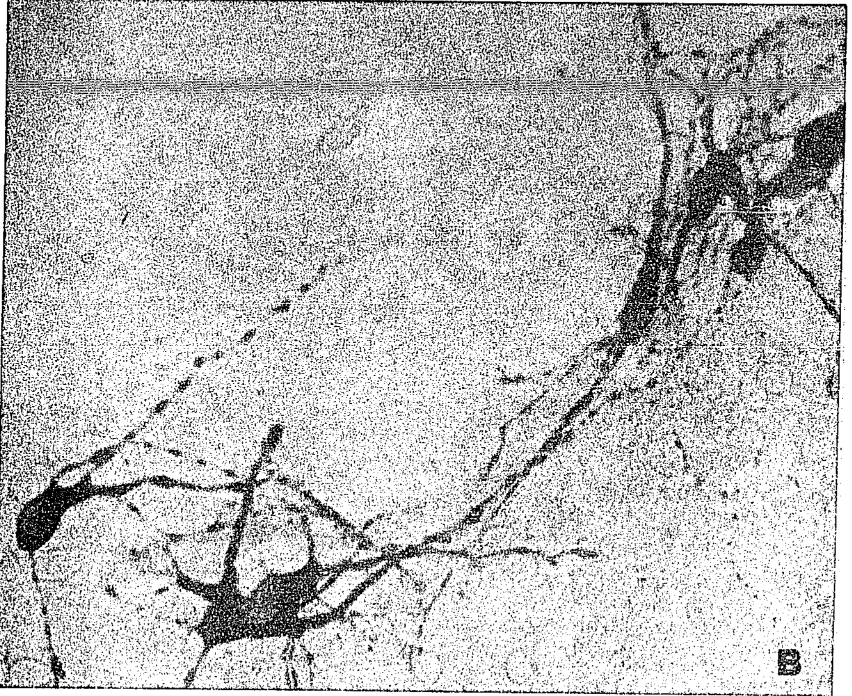


Figure 10. Effects of glial substrates on the [^{3}H]DA uptake. E14 embryonic mesencephalic cultures were plated on polyornithine or on preformed monolayer of mesencephalic or striatal glia. DA uptake levels measured at 7 DIV demonstrate significantly higher values in cultures plated on glial cells compared to controls plated on nonglial substrate (* $P < 0.001$; Independent t-test). Values represent the mean of 4 samples ± SEM.

Figure 11. Glial stimulation of dopaminergic neuronal development visualized with TH immunocytochemistry. Immunocytochemistry performed at 7 DIV reveals the enhanced morphological development of dopaminergic neurons in cultures plated on mesencephalic glial substrate (B) and on striatal glial substrate (C). Cells with much more complex morphologies compared to controls (A) are present in these cultures.


A


B

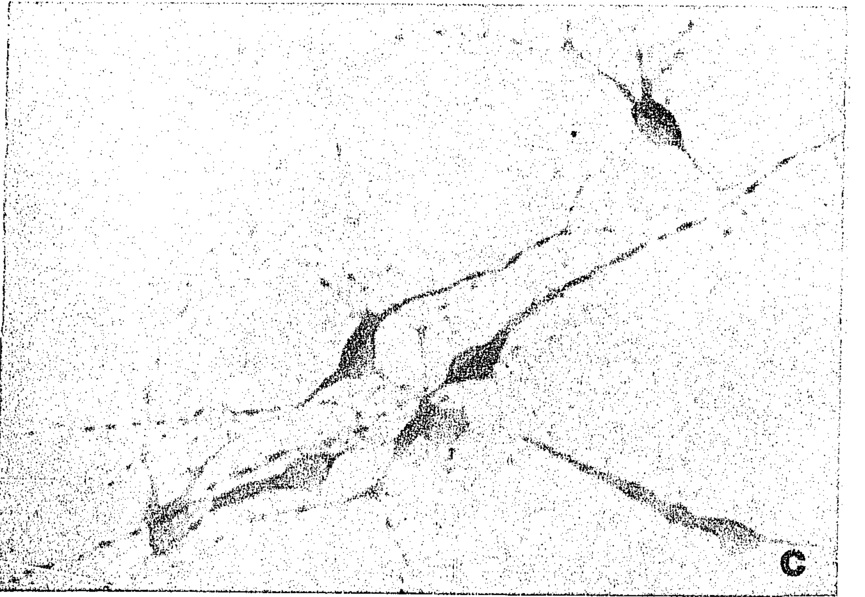

C

Table 2. Effect of glial cell substrate on the dopaminergic cell survival. The number of TH immunopositive neurons was counted at 7 DIV in cultures plated on polyornithine or on striatal glial substrate. The survival of dopaminergic neurons was significantly increased in the presence of glial cells ([a] $P < 0.05$; Independent t-test). Values represent the mean of 7 samples ± SEM.

Substratum	TH+ cells/ cm^2
Polyornithine	386±70
Striatal Glia	672±92 [a]

D. Effects of Glial Conditioned Media on Mesencephalic Culture

1. Survival and Neurite Outgrowth of Dopaminergic Neurons

In order to determine whether the trophic effects of glia on dopaminergic neurons require contact between neurons and glial cells, or whether the effects are mediated by factors released by glia into the extracellular medium, conditioned medium obtained from enriched astrocytic cultures were tested for their ability to mimic the glial effects produced in neuronal-glial cocultures. Again, glial CM derived from either mesencephalic or striatal astrocytes were used to identify possible region-specific differences in the dopaminergic neuronal response.

Some effects of CM on neuritic outgrowth and cell survival appeared almost immediately in the mesencephalic cultures. When cultures were observed only 3 h after initial plating, most cells in control cultures with chemically defined medium had no processes, whereas those with CM had already extended short neurites (not shown). There was also a noticeable difference in the number of attached cells between the two conditions so that cultures plated with CM seemed more dense than controls after 3 h *in vitro* . The effects of CM on the survival of dopaminergic neurons after 7 days in culture are shown in Table 3. Both mesencephalic and striatal CM promoted the survival of TH+ cells; however, mesencephalic CM produced a more potent effect compared to the striatal CM (4.2-fold and 2.8-fold higher than controls, respectively), suggesting a possible preference by mesencephalic dopaminergic neurons for factors derived from homotypical glial cells.

Consistent with the results of dopaminergic neuronal survival, [^{3}H]DA uptake activity was also stimulated by both types of CM, but to a greater extent by

Table 3. Effects of glial conditioned media on the dopaminergic cell survival. The number of TH+ cells was determined at 7 DIV in the cultures maintained with chemically defined medium, mesencephalic CM and striatal CM. Both types of glial CM significantly enhanced the survival of dopaminergic neurons compared to controls ([a] $P < 0.001$; [b] $P < 0.005$); but this effect was even greater after treatment of cultures with mesencephalic CM than with striatal CM ([c] $P < 0.005$). Independent t-test. Values are the mean of 3 samples ± SEM.

Condition	TH+ cells/ cm^2
Control	243±29
Mesencephalic CM	1031±86 [a, c]
Striatal CM	675±14 [b]

mesencephalic CM compared to striatal CM (3.9-fold and 2.8-fold higher than controls, respectively; Fig. 12). An increase in DA uptake was not observed after a 24 h treatment with glial CM from Day 5 to Day 6. Fig. 13 shows the enhanced morphological development of TH-immunoreactive neurons in CM-treated cultures. Neuritic outgrowth is much greater in the cultures maintained with either mesencephalic or striatal CM compared to controls.

These results show that similar trophic effects upon dopaminergic neurons are produced by both glial monolayers in neuronal-glial cocultures and by glial conditioned medium. This provides evidence that soluble trophic substances which can act upon dopaminergic neurons are secreted by cultured astrocytes derived from either the mesencephalon or the striatum.

2. Glial Cell Proliferation

Glial CM induced proliferation of glial cells, as reflected by the number of GFAP+ cells in mesencephalic cultures (Table 4). Mesencephalic and striatal CM led to a 7.5- and a 9.3-fold increase in GFAP+ cells, respectively, compared to controls. This is not unexpected since various growth factors, several of which have been found to be secreted by astrocytes, are known to have mitogenic effects on glia. Thus, it is possible that in addition to a direct trophic effect of glial CM, an increase in glial cell number could also affect the neuronal growth and survival.

E. Growth Factor Effects on the Neurotoxicity of MPP^+ and 6-OHDA on Dopaminergic Neurons

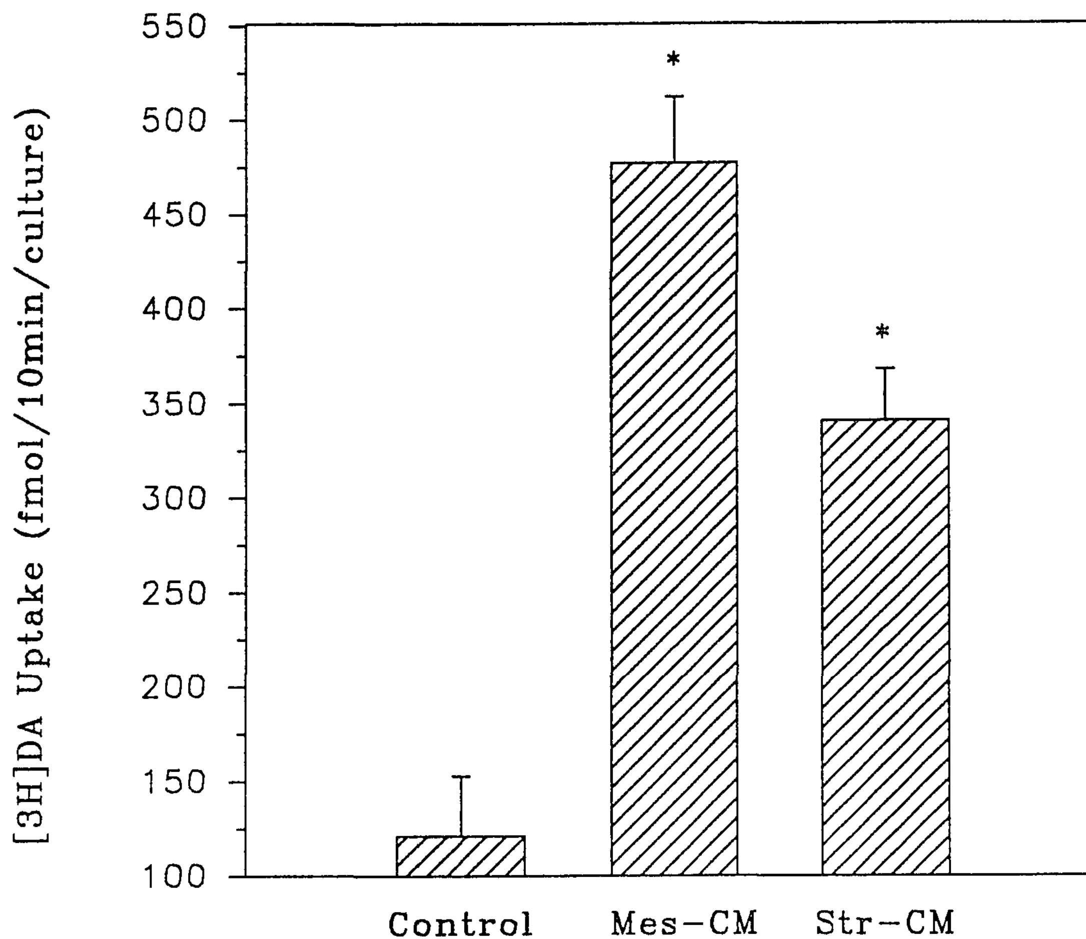
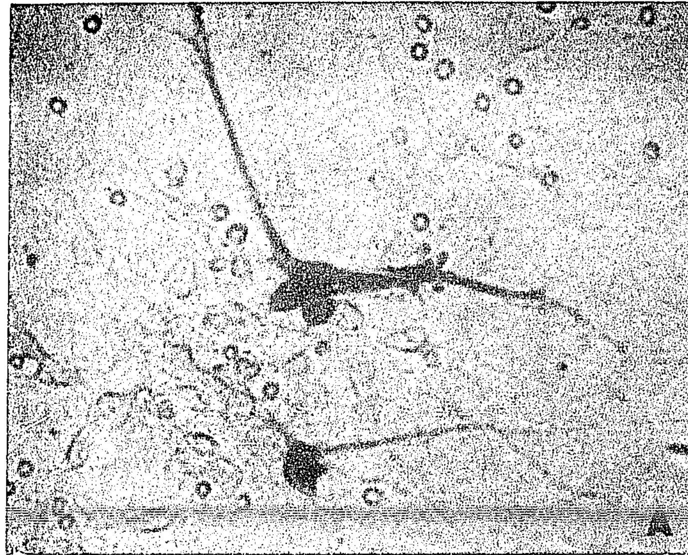
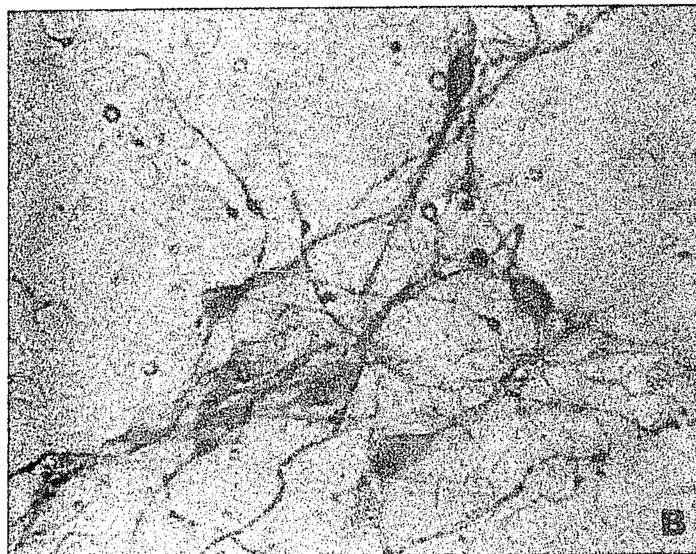


Figure 12. Effects of glial conditioned media on the [^{3}H] DA uptake. E14 embryonic mesencephalic cultures were maintained with chemically defined medium, mesencephalic CM or striatal CM, and the DA uptake levels were measured at 7 DIV. Both types of CM stimulated the DA uptake to yield significantly higher values over controls (* $P < 0.001$). The stimulation of increase in DA uptake was greater by mesencephalic CM compared to striatal CM ($P < 0.05$). Independent t-test. Values represent the mean of 4 samples ± SEM.

Figure 13. Stimulation of dopaminergic neuronal development by glial conditioned media visualized with TH immunocytochemistry. The different morphological appearance of dopaminergic neurons in mesencephalic cultures under different treatment conditions is revealed by TH immunostaining at 7 DIV. Stimulation of dopaminergic neuronal growth by mesencephalic CM (B) and striatal CM (C) is evident when compared to controls (A).


A


B

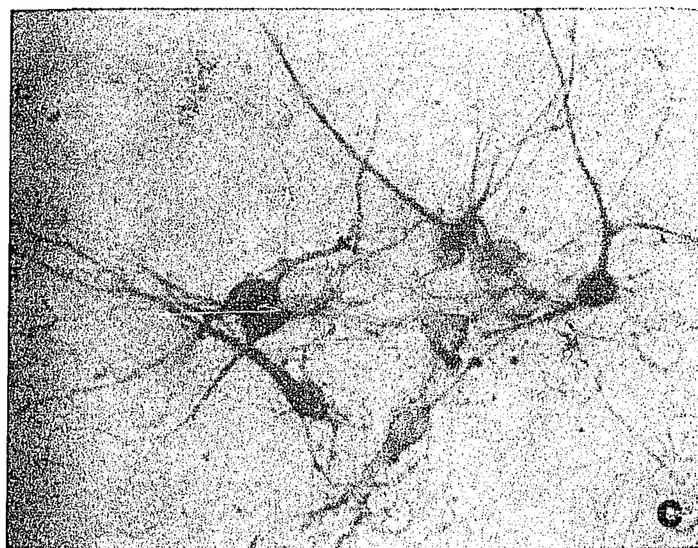

C

Table 4. Effects of glial conditioned media on the stimulation of astrocytic cell proliferation. Treatment of mesencephalic cultures with mesencephalic CM or striatal CM for 7 days resulted in an increased number of GFAP immunoreactive cells. Values are the mean of 2-4 samples ± SEM.

Condition	GFAP+ cells/ cm^2
Control	320± 72
Mesencephalic CM	2392±166
Striatal CM	2968± 36

1. Short-term Pretreatment with Growth Factors

To examine whether EGF and/or bFGF can protect dopaminergic neurons from degeneration, we treated the cultures with the neurotoxin MPP^+, at a concentration shown to selectively affect only the dopaminergic neurons (Sanchez-Ramos et al., 1986; Michel et al., 1990). Upon observation of cultures by phase contrast microscopy, we also did not find evidence of generalized toxicity after MPP^+ treatment. Cultures grown for 4 days were incubated with 10 μM MPP^+ for 24 h and the [^{3}H]DA uptake levels were measured after a 24 h incubation with toxin-free medium. In all cultures, whether or not treated with EGF or bFGF, addition of MPP^+ reduced the DA uptake to approximately 35% of their respective controls (Fig. 14), thereby suggesting that growth factor pretreatment for 4 days and their presence during MPP^+ exposure is not able to protect dopaminergic neurons against MPP^+ neurotoxicity.

Cell counts of TH immunostained cultures indicated that there was no significant loss of TH+ neurons following exposure to MPP^+ (Table 5). However, treatment of cultures with MPP^+ produced clear indications of structural damage to dopaminergic fibers. The appearance of damaged neurons was similar in all cultures, with or without EGF- and bFGF-pretreatment (Fig. 15B, E, H). Many TH+ neurons consisted of a cell soma with very short processes, while others appeared completely truncated. This suggests that while these dopaminergic neurons survived, they lost most or all of their processes, as was implied by the large decrease in DA uptake. The change in neuritic length after MPP^+ treatment was quantitated by morphometric evaluation of dopaminergic neurons stained for TH immunoreactivity. Fig. 16 shows histograms depicting the distribution of neuritic length in 60 randomly selected dopaminergic neurons under different treatment conditions. MPP^+ treatment produced similar decreases in neuritic length in control and growth factor-treated cultures. The reduction in the mean neuritic length in response to MPP^+ treatment was 58% for controls and 59%

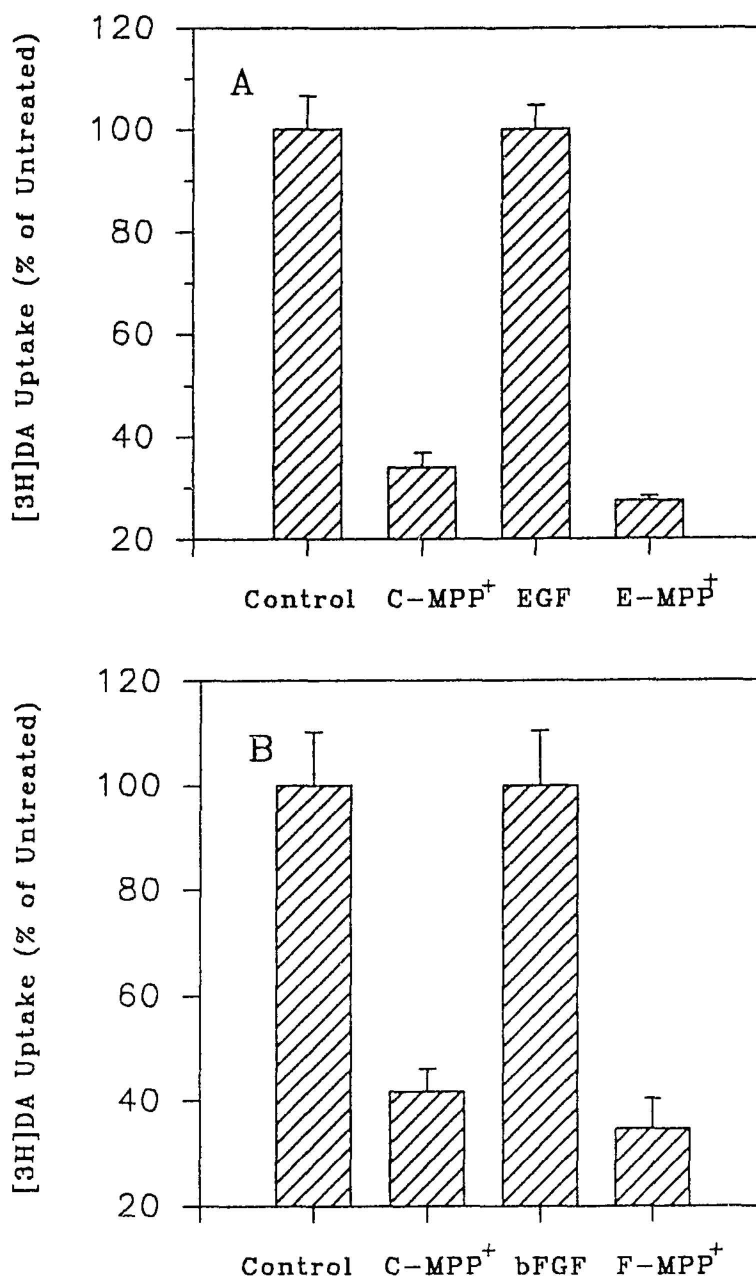
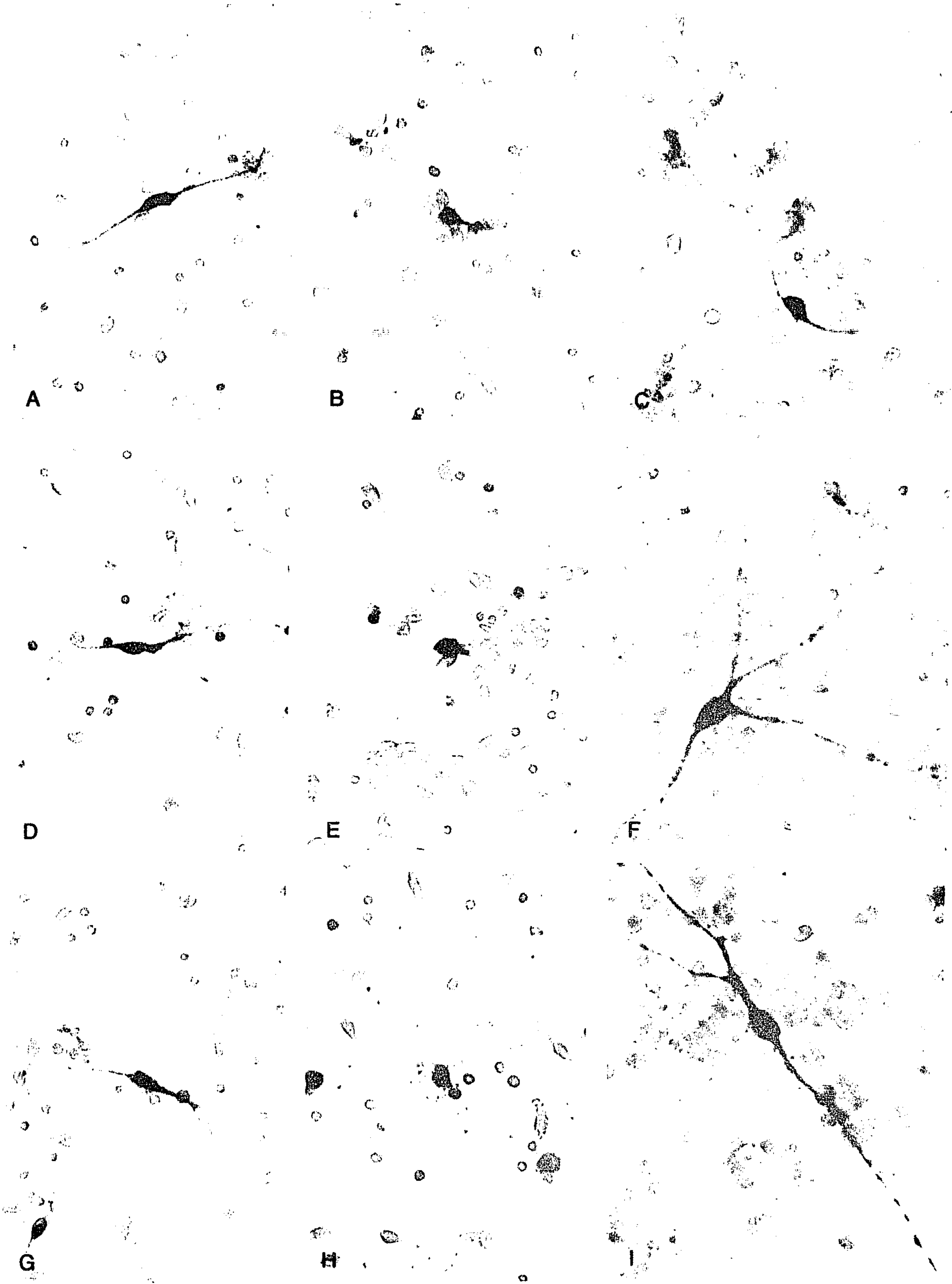


Figure 14. Effect of MPP^+ treatment on $[^3H]DA$ uptake. Mesencephalic cultures maintained in the absence or presence of EGF (10 ng/ml; A) or bFGF (10 ng/ml; B) were exposed to 10 μM MPP^+ for 24 h at 4 DIV. Cultures were incubated with toxin-free medium for 24 h and DA uptake was measured at 6 DIV and presented here as % of untreated own control. MPP^+ treatment produced approximately 60-70% reduction of DA uptake from their respective controls in all cultures. All values are the mean of 5 samples ± SEM.

Table 5. Effect of MPP^+ treatment on TH+ cell survival. Mesencephalic cultures maintained under different growth conditions were exposed to MPP^+ for 24 h at 4 DIV. The number of TH+ neurons was determined at 5 DIV. The number of TH+ cells was not affected by MPP^+ treatment in any of the groups. All values are the mean of 4 samples ± SEM.

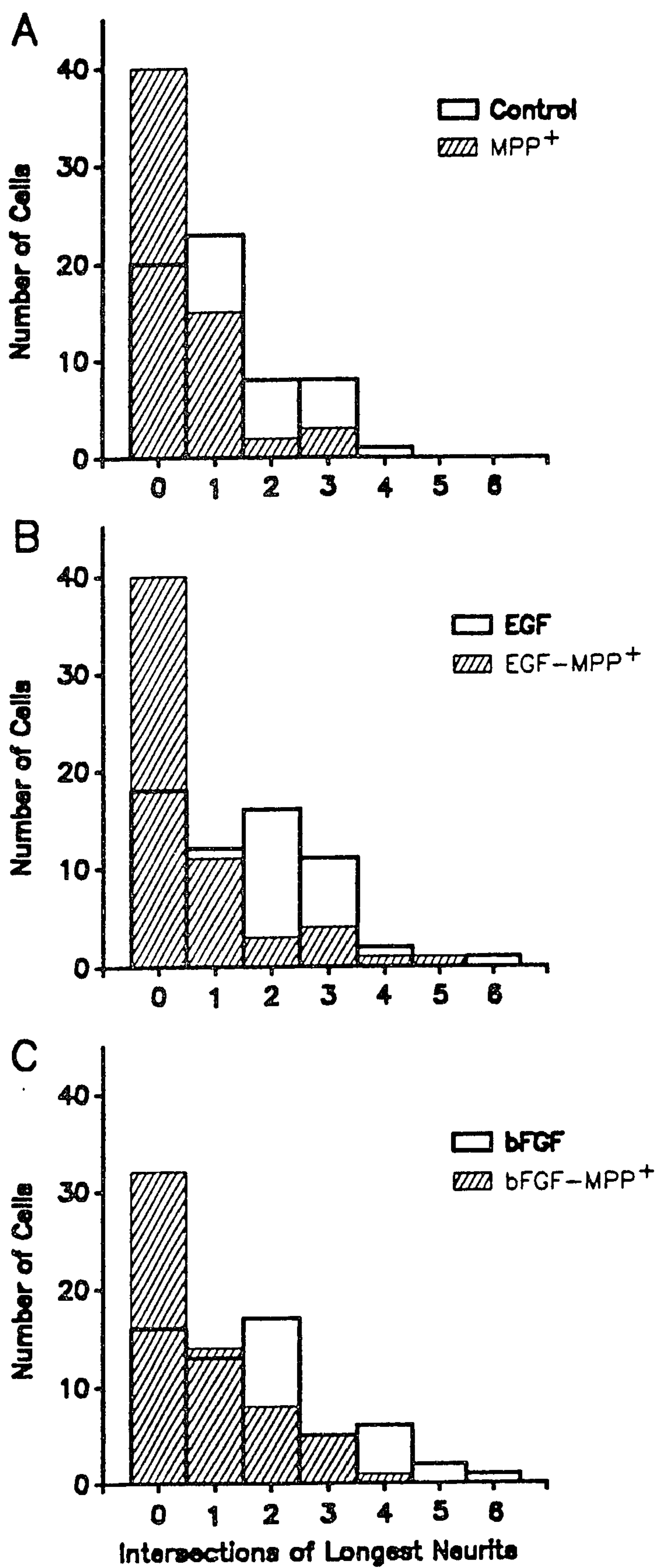
Treatment	TH+ cells/ cm^2		
	Control	EGF (10 ng/ ml)	bFGF (10 ng/ ml)
No MPP^+	117.0±13.8	115.9±16.8	130.0±8.4
MPP^+ (10 μM)	98.0±15.8	114.6±16.6	117.0±9.0

Figure 15. Effect of MPP^+ treatment on dopaminergic neurons visualized with TH immunocytochemistry, and EGF and bFGF stimulation of the regrowth of dopaminergic processes following MPP^+ treatment. Mesencephalic cultures maintained in the absence (A, B, C) or presence of EGF (10 ng/ml; D, E, F) or bFGF (10 ng/ml; G, H, I) were treated with 10 μM MPP^+ for 24 h at 4 DIV and immunostained with TH antibodies. A, D and G show TH+ neurons at 5 DIV from cultures not exposed to MPP^+, while B, E and H are from corresponding MPP^+-treated cultures. MPP^+ treatment produced truncation of nearly all dopaminergic processes in cultures with or without growth factors, while leaving the cell soma intact. The appearance of TH+ cells at 14 DIV shows some spontaneous regrowth of fibers in MPP^+-exposed dopaminergic neurons (C), but continuous presence of EGF and bFGF in the cultures after MPP^+ treatment stimulated extensive outgrowth of dopaminergic neurites (F and I, respectively).



NO MPP+ – 5 DIV
MPP+ – 5 DIV
MPP+ – 14 DIV
CONTROL
EGF
FGF
A
B
C
D
E
F
G
H
I

Figure 16. Effect of MPP^+ treatment on dopaminergic neuritic length. Mesencephalic cultures maintained in chemically defined medium (A), plus EGF (10 ng/ml; B) or plus bFGF (10 ng/ml; C) for 4 days were exposed to 10 μM MPP^+ for 24 h. Cultures were processed for TH immunocytochemistry at 5 DIV. The length of the longest neurite was measured in 60 randomly selected TH+ neurons from each treatment condition by counting the number of intersections of a reticule grid crossed by the neurite. In all treatment groups, MPP^+ exposure produced a similar shift in the distribution of number of cells toward shorter neuritic length (less number of intersections crossed). The mean neuritic length ± SEM in different treatment conditions are: (A) Control 1.12±0.14, MPP^+ 0.47±0.10; (B) EGF 1.52±0.17, E-MPP^+ 0.63±0.15; (C) bFGF 1.70±0.19, F-MPP^+ 0.82±0.14.



A
Control
MPP+
Number of Cells
B
EGF
EGF-MPP+
Number of Cells
C
bFGF
bFGF-MPP+
Number of Cells
Intersections of Longest Neurite

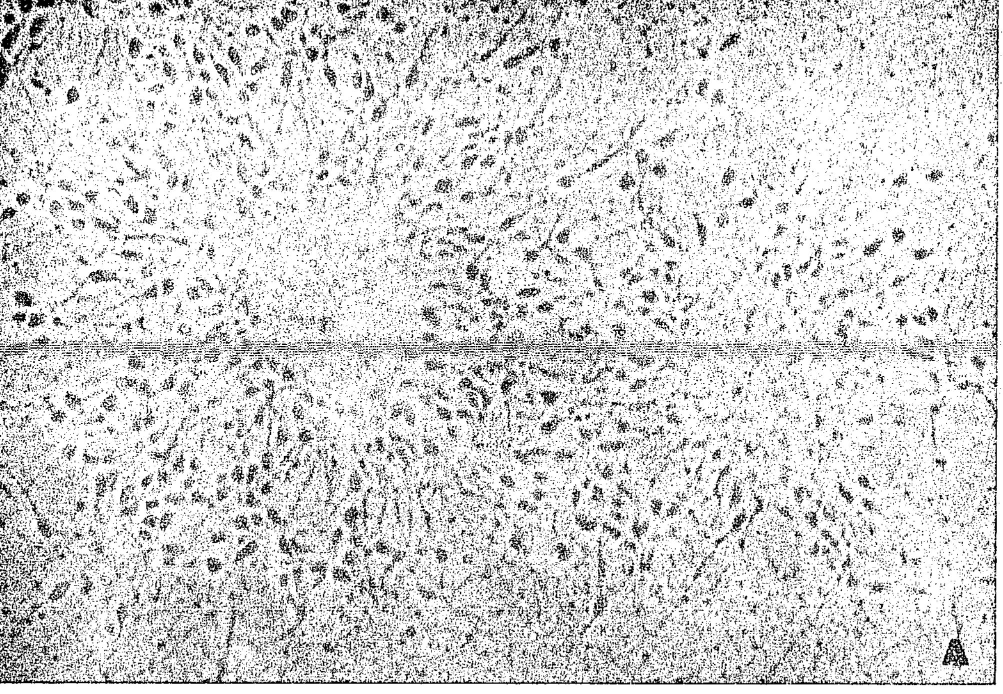
and 52% for EGF and bFGF cultures, respectively. These results present further evidence for the lack of protection from MPP^+ toxicity by a short-term treatment with EGF or bFGF.

In contrast to the selective toxicity of MPP^+ upon dopaminergic neurons, 6-OHDA at a concentration of 100 μM for 45 min at 6 DIV produced generalized toxicity in mesencephalic cultures. Fig. 17 shows the widespread cell death caused by 6-OHDA treatment, as observed by phase contrast microscopy. Most cells have lost their processes and appear to be degenerating. As a result of the nonspecific toxic action of 6-OHDA, glial cells visualized with GFAP immunocytochemistry also displayed signs of damage (not shown).

When we tested the growth factor treatments for protective effects against 6-OHDA neurotoxicity on dopaminergic neurons, we found that in contrast to the lack of protection against MPP^+ after 4 days of treatment, bFGF effectively counteracted the toxicity of 6-OHDA. All of the dopaminergic parameters measured after 6-OHDA treatment, i.e. [^{3}H]DA uptake activity (95% reduction in controls compared to 79% in bFGF cultures), number of surviving TH+ neurons (72% reduction versus 36%), and neuritic length (83% reduction versus 59%), indicated that 6-OHDA was indeed toxic to the cultures but that bFGF treatment reduced the damage produced by 6-OHDA (Table 6). Results from EGF-treated cultures were inconsistent between experiments. Fig. 18 clearly demonstrates the protective effect of bFGF treatment against 6-OHDA toxicity in dopaminergic neurons visualized with TH immunocytochemistry. In fact, the appearance of these cells is similar to dopaminergic neurons in cultures that were not treated with 6-OHDA.

2. Long-term Pretreatment with Growth Factors

Figure 17. Generalized toxicity produced by 6-OHDA visualized with phase contrast microscopy. Compared with control cultures that were not treated with a toxin (A), incubation of mesencephalic cultures with 100 μM 6-OHDA for 45 min at 6 DIV produced nonspecific damage to all cell populations (B), leading to the loss of neuritic processes and cell death.


A

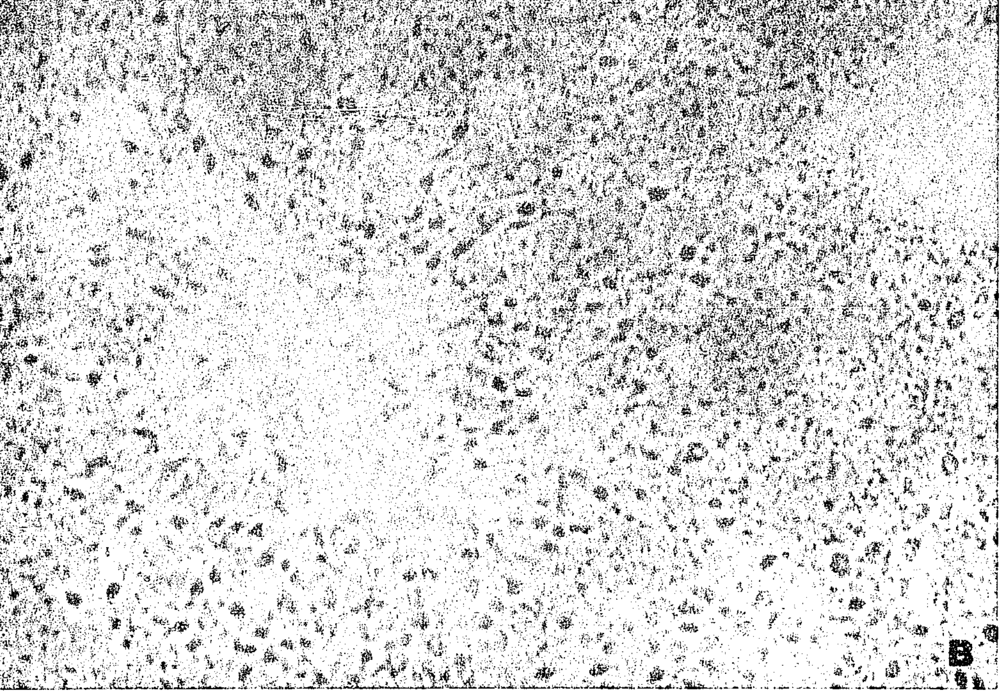
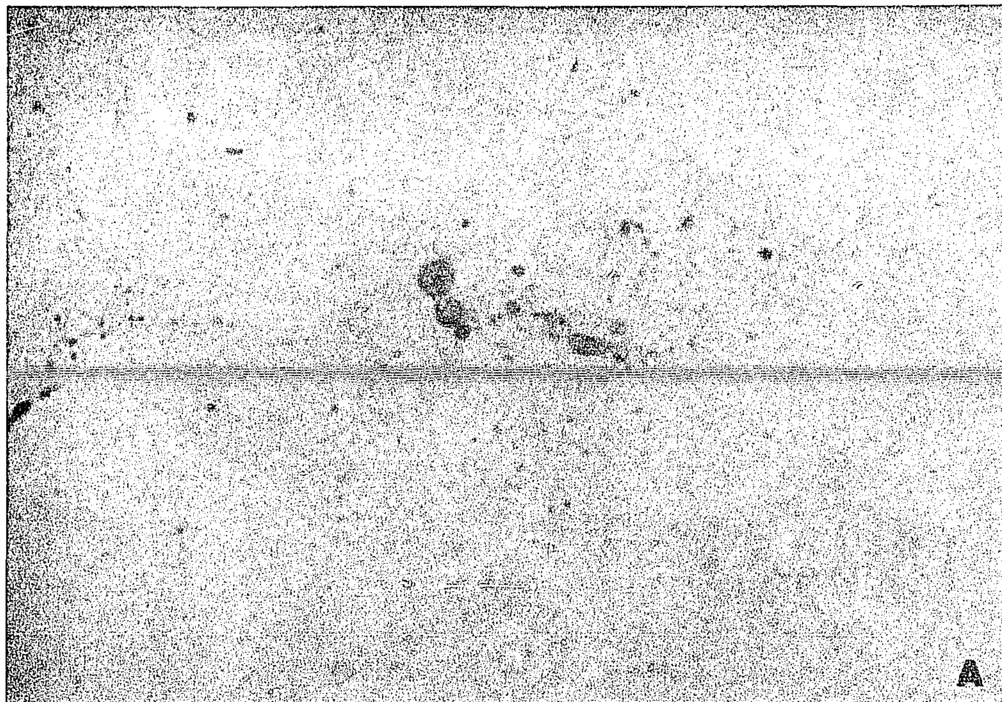
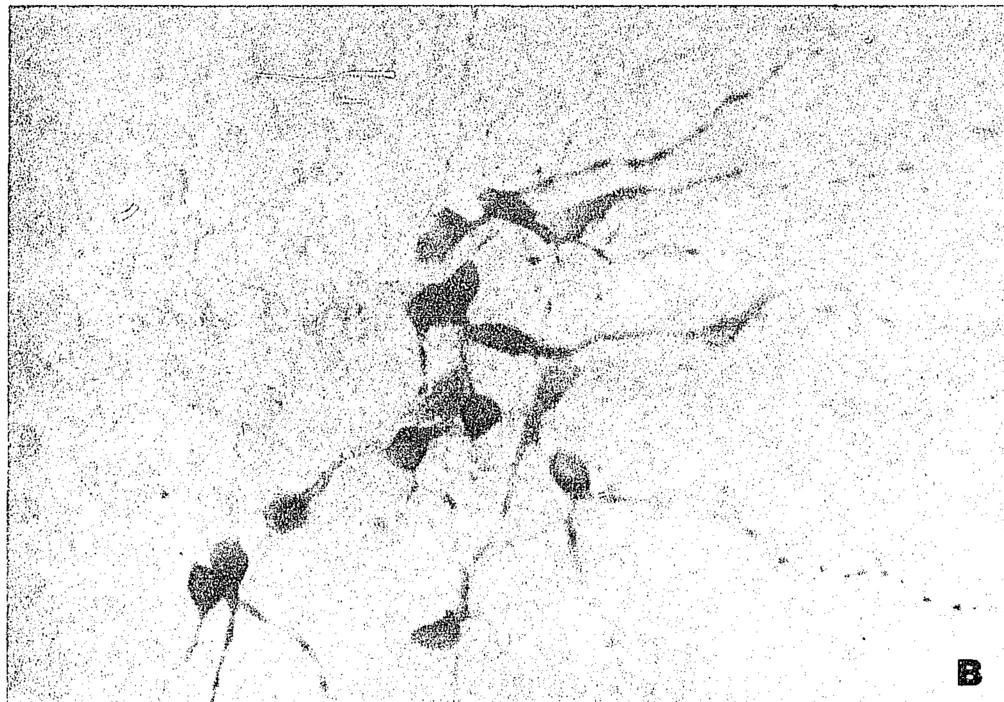

B

Table 6. Effects of bFGF treatment against 6-OHDA neurotoxicity. Exposure to 100 μM 6-OHDA for 45 min at 6 DIV in cultures grown in the continuous presence of bFGF resulted in a reduction of 6-OHDA-induced loss of dopaminergic parameters. The toxin treatment significantly decreased DA uptake levels, TH+ cell number and neurite length in all cultures, whether or not they were treated with bFGF ($P < 0.001$). However, bFGF treatment effectively reduced the damage produced by 6-OHDA ($P < 0.05$). Independent t-test.

	Control			bFGF		
	Untreated	Treated	% Untreated	Untreated	Treated	% Untreated
[^{3}H]DA Uptake (fmol/10 min/culture)	163.0±20.9	8.6±4.8	5.3±3.0	199.6±16.2	36.8±6.9	21.2±4.1
TH+ cells/cm^2	464±48	128±20	27.5±4.2	476±60	304±24	63.9±5.4
Neurite Length (Number of Intersections)	2.2±0.3	0.5±0.2	16.7±4.7	3.4±0.3	1.4±0.2	41.2±5.4

Figure 18. Protective effects of bFGF against 6-OHDA neurotoxicity on dopaminergic neurons visualized with TH immunocytochemistry. Exposure to 100 μM 6-OHDA for 45 min at 6 DIV caused cell death and extensive damage to the dopaminergic neuronal processes in control cultures (A), but bFGF treatment protected these cells so that many relatively undamaged neurons were observed in these cultures (B).


A


B

As described previously, treatment with EGF or bFGF for an extended period results in glial cell proliferation as well as increases in DA uptake and survival of dopaminergic neurons. Since a long-term pretreatment with the growth factors creates in these cultures an environment supportive of dopaminergic neuron survival and growth, we wanted to determine whether or not protection from MPP^+ toxicity could be achieved under such conditions. Cultures grown in the absence of growth factors or in the presence of EGF or bFGF for 10 days were incubated with various concentrations of MPP^+ for 48 h. The [^{3}H]DA uptake levels in control cultures exposed to MPP^+ were reduced in a dose-dependent manner, while pretreatment with EGF or bFGF attenuated the MPP^+-induced decrease in DA uptake (Fig. 19). At a concentration of 5 μM MPP^+, control cultures had an 86% reduction in uptake, while after EGF and bFGF treatments the same concentration of MPP^+ caused a 56% and a 48% reduction, respectively. These results indicate that pretreatment with growth factors for 10 days can protect the dopaminergic neurons from loss of DA uptake. However, this protective effect of growth factors was decreased at the highest MPP^+ concentration used (10 μM), indicating that the protective effects afforded by growth factor treatments can be overcome by increasing the concentration of the neurotoxin. We tested the possibility that the presence of growth factors during MPP^+ exposure was somehow responsible for counteracting the neurotoxic effects of MPP^+. When EGF or bFGF was added simultaneously with MPP^+ for 48 h, in cultures previously grown for 10 days without any growth factor, the reductions in DA uptake levels were similar to those of control cultures which were never treated with growth factors. For example, after treatment with 5 μM MPP^+ the DA uptake in control cultures was reduced by 86%, compared to 85% and 71% in the presence of EGF and bFGF, respectively. Thus, it appears that only a long-term pretreatment with EGF or bFGF is effective in raising the threshold for MPP^+ neurotoxicity in these cultures.

Growth factor pretreatment was also found to protect dopaminergic neurons from

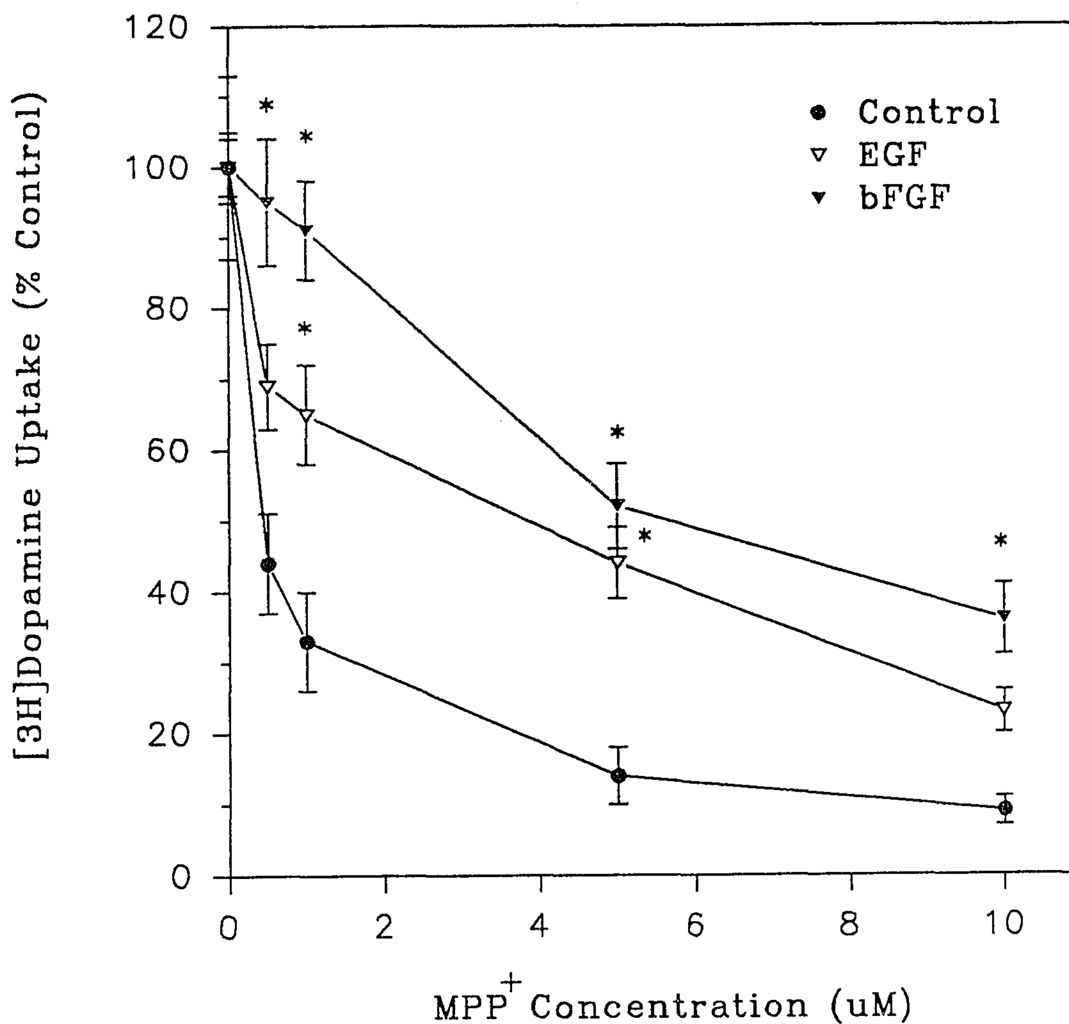


Figure 19. Protective effects of EGF and bFGF against MPP+-induced reduction in [^{3}H]DA uptake. Mesencephalic cultures maintained for 10 days in the absence or presence of EGF (10 ng/ml) or bFGF (10 ng/ml) were exposed to various concentrations of MPP+ for 48 h. DA uptake was measured at 13 DIV and presented as % of respective untreated controls. MPP+ produced a dose-dependent reduction in DA uptake in all cultures, but the presence of EGF attenuated this reduction at 1 μM and 5 μM MPP+ concentrations (* $P < 0.01$), while the presence of bFGF attenuated the reduction in DA uptake at all MPP+ concentrations used (* $P < 0.001$). ANOVA followed by Tukey's HSD post hoc test. Each point represents the mean of 5 samples ± SEM.

MPP^+-caused cell death. Although a 48 h exposure to various concentrations of MPP^+ did not result in a significant loss of TH+ cells in any of the cultures, a 72 h incubation with 10 μM MPP^+ led to a significant reduction in the number of TH+ cells in controls, but not in cultures pretreated with EGF or bFGF for 10 days (Fig. 20).

3.Inhibition of Growth Factor-Stimulated Glial Cell Proliferation

Earlier studies in our laboratory indicated that the effects of EGF on dopaminergic neurons are mediated by proliferating glial cells (Casper et al., 1991). Similarly, Engele and Bohn (1991) reported that the trophic effects of bFGF on dopaminergic neurons are also mediated by glia. In order to determine whether the protection from MPP^+ neurotoxicity after a long-term treatment with EGF or bFGF could be due to proliferating glial cells, we treated the cultures with growth factors in the presence or absence of the mitotic inhibitor FUDR. Phase contrast microscopy reveals the opposing action of FUDR on growth factor-stimulated cell proliferation and the different appearance of the cultures between these conditions (Fig. 21). As expected, treatment with FUDR greatly reduced the number of GFAP+ astrocytes in all cultures, particularly in those treated with the growth factors (77%, 91% and 98% reductions in control, EGF and bFGF cultures, respectively). When these cultures were treated at 10 DIV with 5 μM MPP^+ for 48 h, the presence of FUDR completely eliminated the protective effect of growth factors against MPP^+-induced reduction in DA uptake (Fig. 22), suggesting that proliferating glia probably mediate the growth factor effects.

F. Effects of Glial Monolayers on the Neurotoxicity of MPP^+ and 6-OHDA on Dopaminergic Neurons

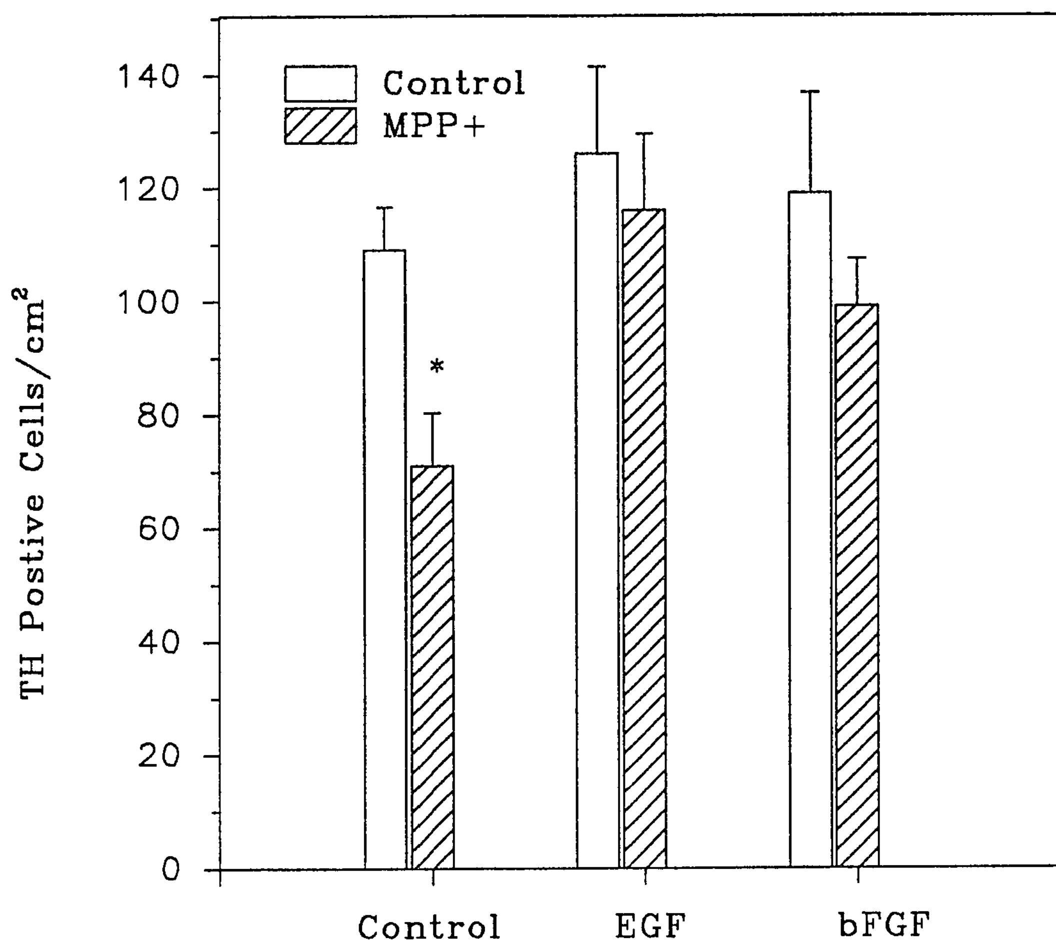
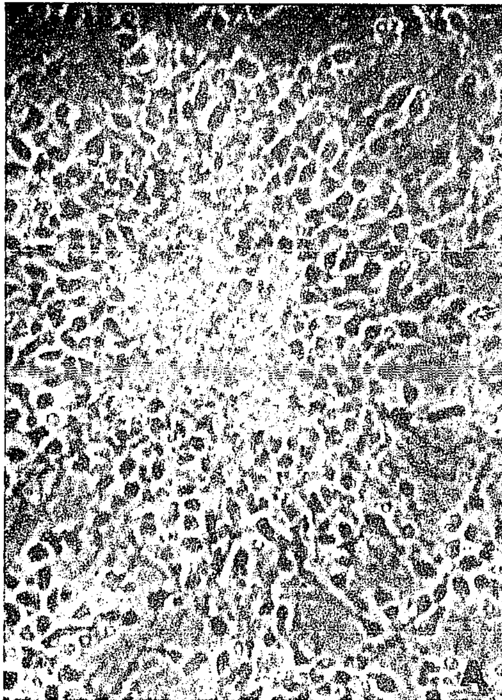
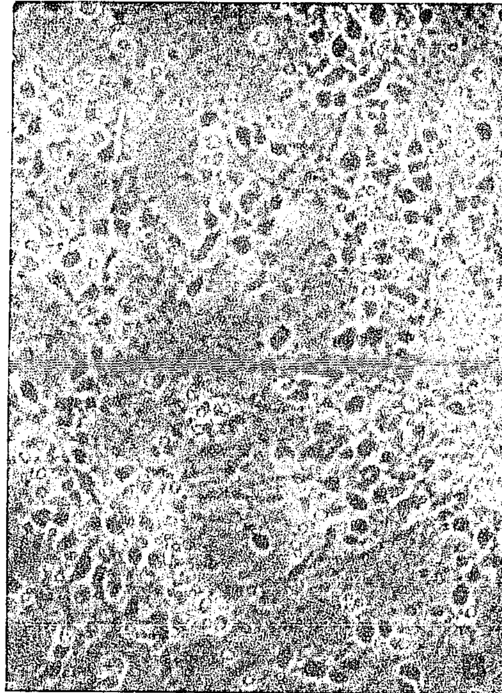
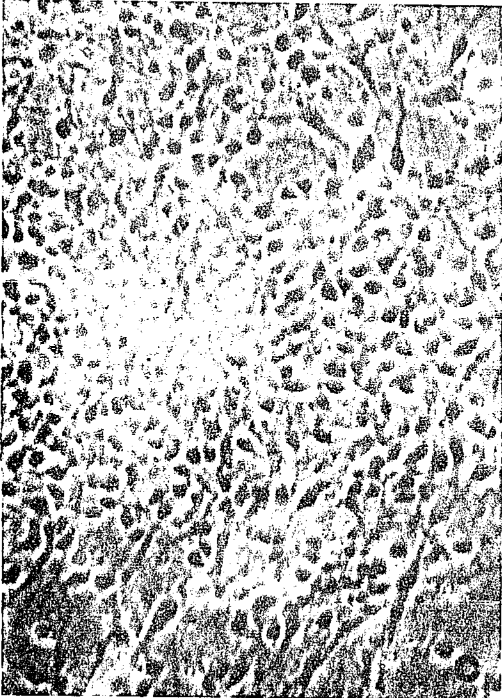


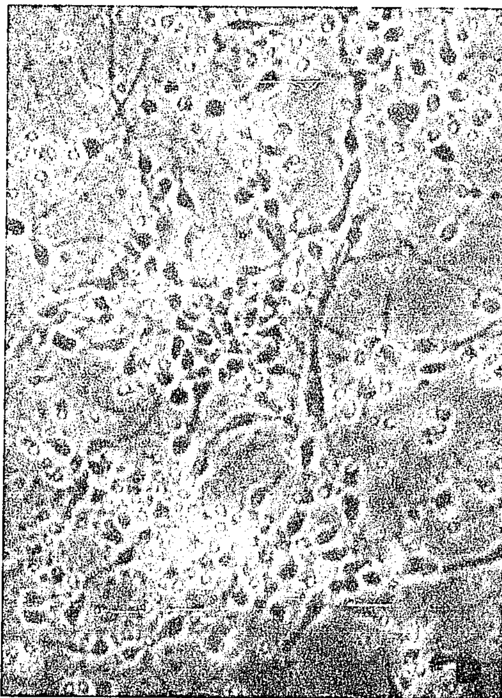
Figure 20. Protective effects of EGF and bFGF against MPP^+-induced TH+ cell loss. Mesencephalic cultures maintained for 10 days in the absence or presence of EGF (10 ng/ml) or bFGF (10 ng/ml) were exposed to 10 μM MPP^+ for 72 h. The number of surviving TH+ neurons was counted in cultures processed for TH immunocytochemistry at 13 DIV. MPP^+ produced a significant reduction in TH+ cell number only in cultures which were not treated with EGF or bFGF (* $P < 0.05$; Independent t-test). All values are the mean of 4 samples ± SEM.

Figure 21. Inhibition of glial cell proliferation by FUDR treatment visualized with phase contrast microscopy. Treatment of mesencephalic cultures with EGF (10 ng/ml) or bFGF (10 ng/ml) causes glial cell proliferation, as shown by an increased cell density in the cultures (A and C, respectively). This growth factor-stimulated increase in glial cell proliferation is inhibited with the addition of FUDR (B and D). These cultures resemble controls that were not treated with the growth factors.









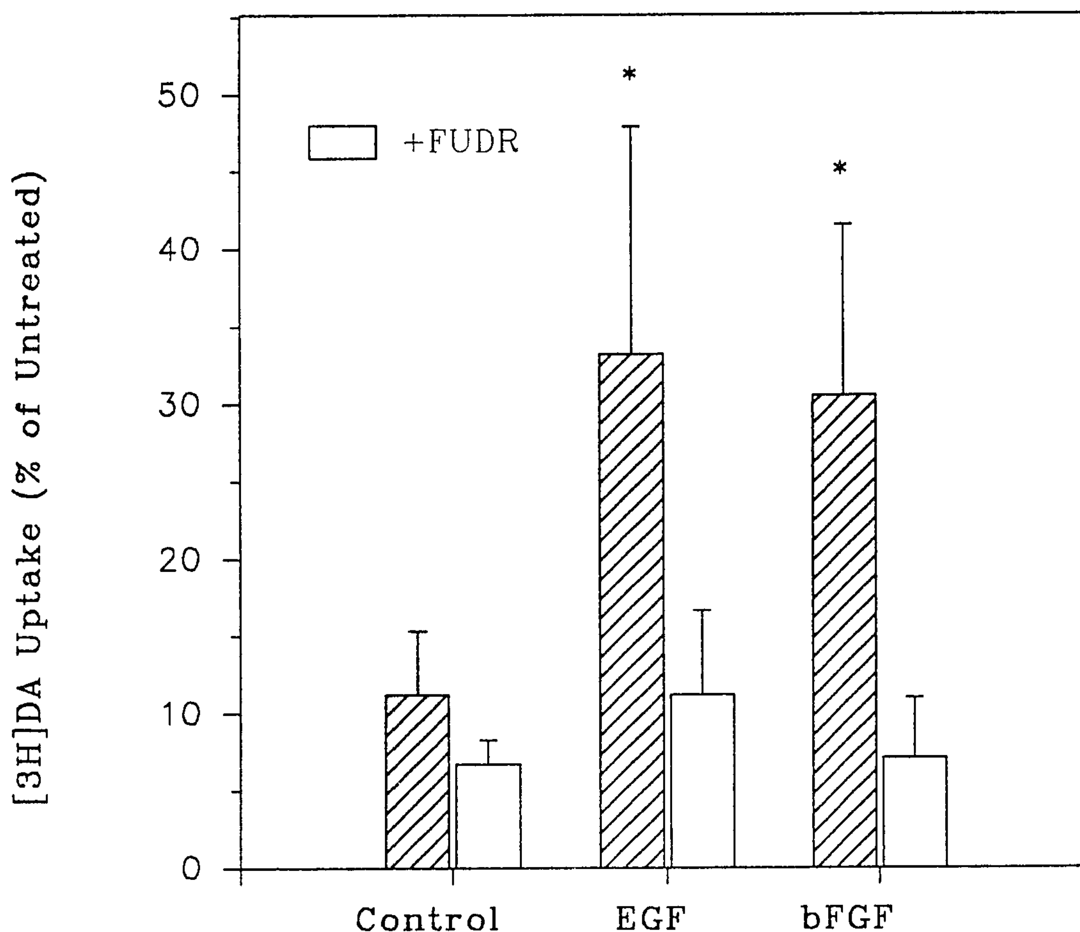


Figure 22. Inhibition of glial cell proliferation abolishes the protective effects of EGF and bFGF against MPP^+-induced reduction in [^{3}H]DA uptake. Mesencephalic cultures were maintained in the absence or presence of FUDR under three different treatment conditions: (1) chemically defined medium; (2) plus EGF (10 ng.ml); (3) plus bFGF (10 ng/ml). Cultures were exposed to 5 μM MPP^+ for 48 h and DA uptake, presented as % of respective controls not exposed to MPP^+, was measured at 13 DIV. While MPP^+ produced significantly smaller reductions in DA uptake in cultures treated with EGF or bFGF (* $P < 0.005$; ANOVA followed by Tukey's HSD post hoc test), the presence of FUDR in these cultures eliminated their protective effects and yielded similar reductions as in controls. All values are the mean of 5 samples ±SEM.

Since initial studies implicate glial cells as playing a critical role in the protective effects of growth factors against MPP^+ and 6-OHDA, we tested the ability of purified glial cells to protect dopaminergic neurons against these toxins. As described earlier, glial cell monolayers exert trophic effects on dopaminergic neurons grown in neuronal-glial cocultures.

Cultures were exposed to 100 μM MPP^+ for 60 min or 100 μM 6-OHDA for 45 min at 6 DIV and analyzed 24 h later for toxicity and glial-mediated protection. In the presence of either mesencephalic glia or striatal glia, $[^3H]$DA uptake reductions caused by MPP^+ and 6-OHDA treatments were far less than the reductions in controls (Fig. 23). Both toxins led to a significant reduction of DA uptake in all culture conditions ($p < 0.001$); however, they produced a nearly complete loss of DA uptake activity in control cultures (96%), while MPP^+ in the presence of mesencephalic or striatal glial cells caused 86.8% and 87.5% reductions, respectively, and 6-OHDA caused only 36.9% and 40.3% reductions, respectively. Interestingly, the glial protective effect against 6-OHDA toxicity was greater than that against MPP^+. This finding appears to be due to a much smaller damage produced at the neurites by 6-OHDA in the presence of glial cells compared to the damage produced by MPP^+, as demonstrated by TH immunocytochemistry (Fig. 24). Dramatic differences in the appearance of dopaminergic neurons are evident following exposure to the toxins in control cultures compared to those grown on glial monolayers, and also between the effects of MPP^+ compared to the effects of 6-OHDA, particularly in the toxicity produced at the neurites.

In confirmation of the results obtained from DA uptake assays and TH immunocytochemistry, the neuritic length was indeed damaged to a greater extent after treatment with MPP^+ than with 6-OHDA in the presence of glia (Table 7). In fact, glia was not at all effective in preventing the MPP^+-induced reduction of neuritic length, whereas 6-OHDA-induced reduction was decreased from 76.4% in control cultures to

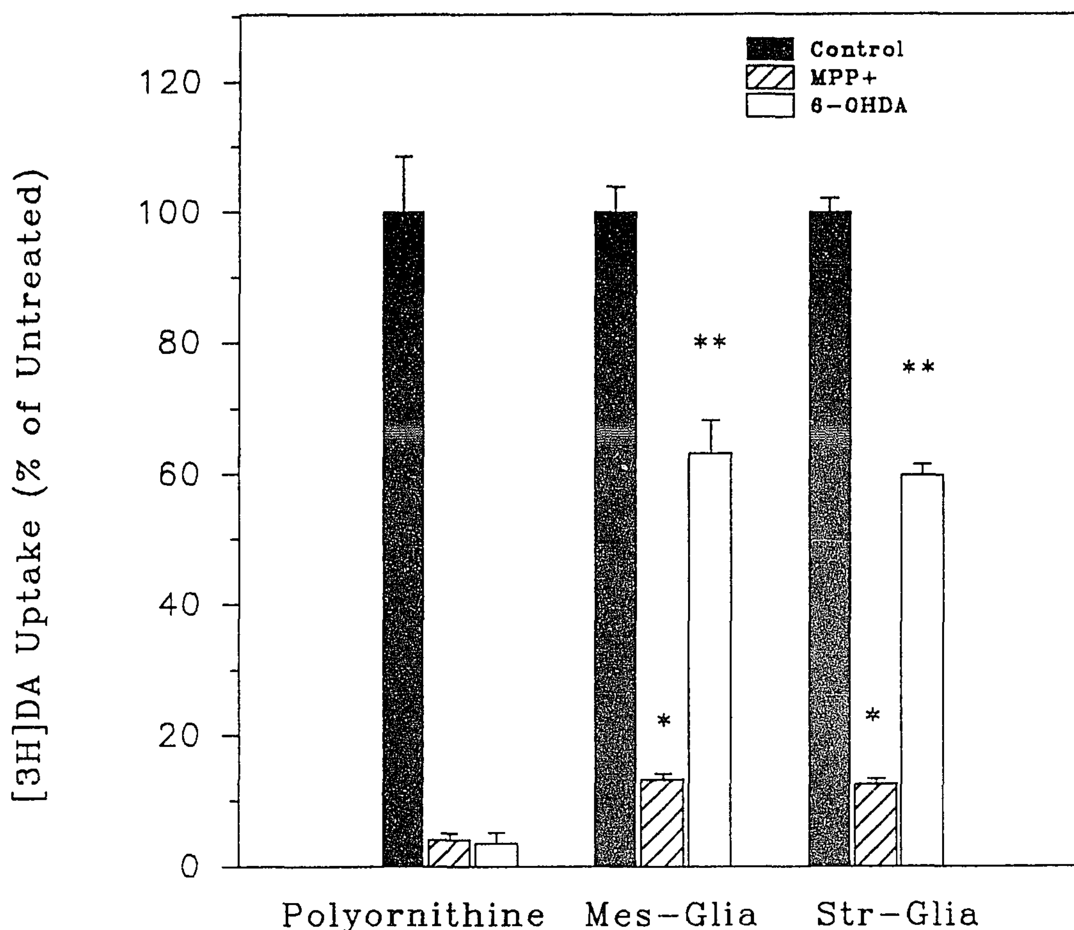


Figure 23. Protective effects of glial substrates against MPP^+- and 6-OHDA-induced reduction of $[^3H]$DA uptake. Mesencephalic cultures were plated on polyornithine, on mesencephalic glial monolayer or on striatal glial monolayer. Treatment with 100 µM MPP^+ for 60 min or 100 µM 6-OHDA for 45 min at 6 DIV produced a significant reduction of uptake in all culture conditions ($P < 0.001$). However, the presence of glial cells in the cultures afforded protection against toxin treatments, with a greater protection against 6-OHDA (** $P < 0.001$) compared to the protection against MPP^+ (* $P < 0.001$). Independent t-test. No significant difference was observed between the effects produced by mesencephalic glia and by striatal glia. Values represent the mean of 4 samples ± SEM.

Figure 24. Protective effects of glial substrate against MPP^+ and 6-OHDA toxicity on dopaminergic neurons visualized with TH immunocytochemistry. Treatment of mesencephalic cultures with 100 µM MPP^+ for 60 min or with 100 µM 6-OHDA for 45 min both produced extensive degeneration of neurons and their processes in control cultures (A, D). In cultures with glial substrate, the toxins produced a much smaller damage to dopaminergic neurons compared to controls (mesencephalic glia B, E; striatal glia C, F); this protective effect was particularly striking against 6-OHDA, where most cells had some neurites left intact (E, F).

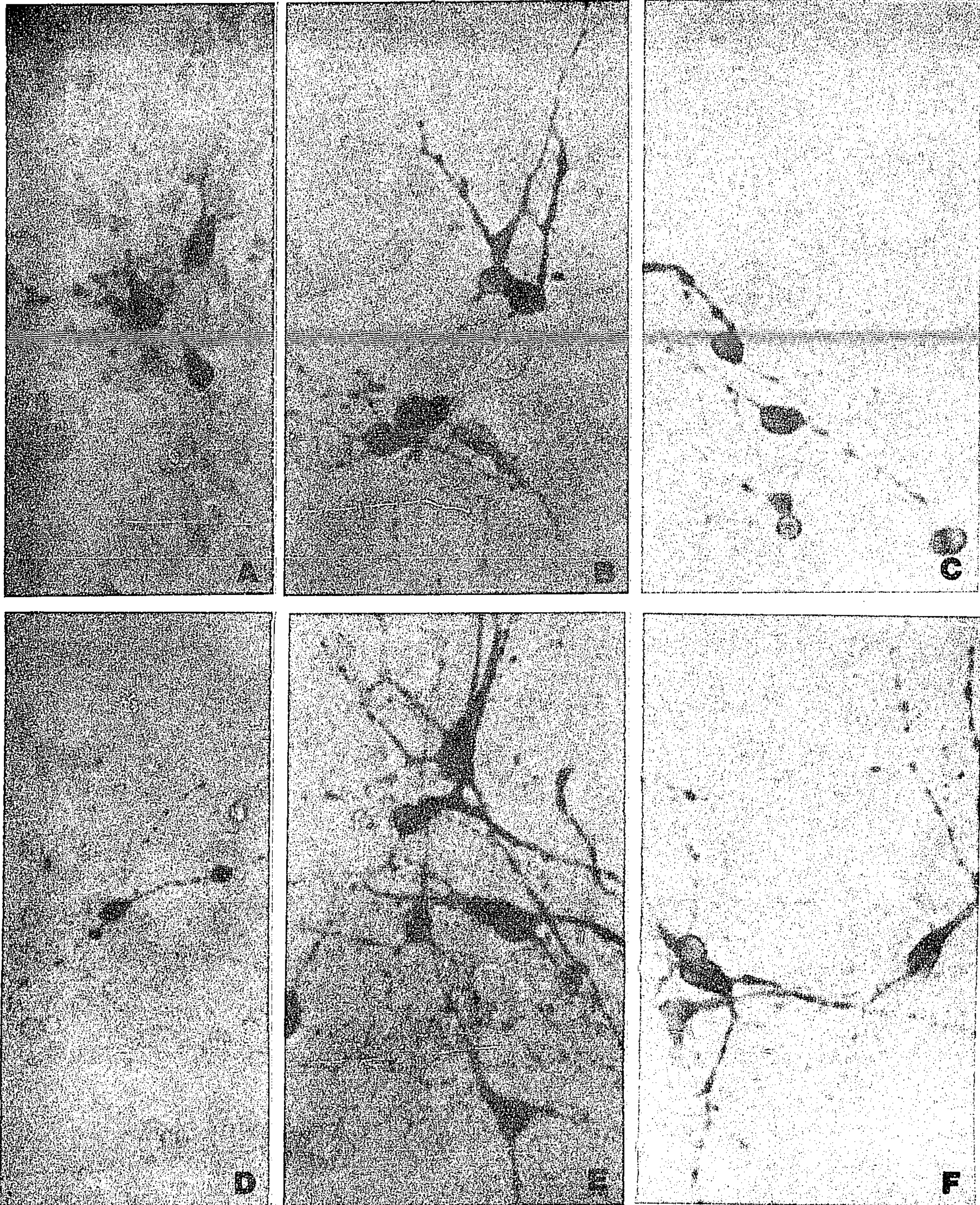

A
B
C
D
E
F

Table 7. Effects of glial substrate on MPP^+ and 6-OHDA neurotoxicity. Mesencephalic cultures were plated on polyornithine, mesencephalic glia, or striatal glia and exposed to 100 μM MPP^+ for 60 min or 100 μM 6-OHDA for 45 min at 6 DIV. The presence of glial cells in the cultures afforded some protection against 6-OHDA-induced damage to the neurites, but not against MPP^+. The glial cells effectively counteracted against TH+ cell loss caused by either of the toxins.

	MPP^+			6-OHDA		
	Control	Mes-Glia	Str-Glia	Control	Mes-Glia	Str-Glia
TH+ cells/ cm^2 (% of untreated)	55.7±16.1	111.2±11.2	102.8±0.4	30.7±5.7	90.4±0.3	92.0±10.7
Neurite Length (% of untreated)	24.1±5.9	19.3±4.2	17.1±3.6	23.6±8.6	80.3±5.8	65.7±4.4

19.7% and 34.3% by mesencephalic and striatal glia, respectively. Morphometric evaluation of these cultures demonstrated that the dopaminergic neuronal survival was increased in the presence of glial cells after treatment with the toxins, from 44.3% reduction in control cultures to a complete protection following exposure to MPP^+, and from 69.3% reduction in controls to only 8 to 10% loss following 6-OHDA treatment (Table 7). All of these results taken together indicate that there is no region-specific differences in the glial protective effects against dopaminergic neurotoxins.

It may be hypothesized that the protective effects of glia against MPP^+ and 6-OHDA are due to the uptake of these toxins into glial cells or the rapid degradation of toxins in the extracellular medium in the presence of glia, both of which would lead to decreased amounts of toxins available to the dopaminergic neurons. In order to test these hypotheses, concentration of the toxins in the extracellular medium was determined at various time intervals in control cultures and neuronal-glial cocultures. Table 8 presents the data obtained from an analysis of MPP^+ concentration in the medium at 0 time and at 60 min after addition of 100 μM. It is evident that all of the values are similar and that the presence of glial cells in the cultures did not reduce the MPP^+ concentration in the medium. The same conclusion was reached after analysis of 6-OHDA concentration in the extracellular medium by HPLC at several time points (Table 9). Therefore, it is clear that same concentration of the toxins are available for uptake into dopaminergic neurons whether or not glial cells are present in the cultures.

Since dopaminergic neurons have been demonstrated to be resistant to the toxic effects of MPP^+ and 6-OHDA in either the presence of growth factors or purified astrocytic glia, we tested the ability of EGF and bFGF to further potentiate the glial protective effects against the two toxins. It was found by measurements of DA uptake activity that EGF and bFGF did not potentiate the effects produced by glial cells alone (Table 10). This lack of additive effects of growth factors and glia supports the view that

Table 8. Concentration of MPP^+ in the culture medium. 100 μM MPP^+ was added to dishes without cells, mesencephalic neuronal cultures, and to mixed neuron-glia cocultures at 6DIV for a 60 min incubation. The concentration of MPP^+ in the extracellular medium was analyzed at the start of treatment and at the end of the the exposure period. In all conditions at both time points, the concentration of MPP^+ was found to remain at approximately 100 μM. Values are the mean of 3 samples ± SEM, expressed as μM MPP^+.

	Culture Condition		
Time (min)	No cells	Neuronal	Mixed
0	101.5±1.7	94.6±2.1	98.3±1.0
60	101.5±1.0	99.5±2.0	101.9±0.9

Table 9. Concentration of 6-OHDA in the culture medium. 100 μM 6-OHDA was added to mesencephalic neuronal cultures and to mixed neuron-glia cocultures at 6DIV for a 45 min incubation. The concentration of 6-OHDA in the extracellular medium was analyzed at the start of treatment, after 15 min, and at the end of the the exposure period. In all time points, the concentration of 6-OHDA was found to be equivalent in the two culture condition groups and also the loss of this toxin during the incubation period occurred at the same rate. Values are the mean of 3 samples ± SEM, expressed as μM 6-OHDA.

	Culture Condition	
Time (min)	Neuronal	Mixed
0	117±0.2	129±3.5
15	109± 5.7	118±5.2
45	90±1.2	102±5.5

Table 10. Lack of potentiation of glial protective effects against MPP^+ and 6-OHDA by growth factors. Mesencephalic cultures plated on striatal glial monolayer were maintained with chemically defined medium alone or in the presence of EGF (10 ng/ml) or bFGF (10 ng/ml). The growth factors, added together with glial cells, did not potentiate the reduction in the toxin-induced loss of [^{3}H]DA uptake obtained by glial cells alone. 100 μM MPP^+ was treated for 60 min and 100 μM 6-OHDA for 45 min at 6 DIV. DA uptake was then measured 24 h after removal of toxins and incubation with fresh medium without growth factors. Values represent the mean of 3-4 samples ± SEM, expressed as % of untreated.

	Striatal Glia	+ EGF	+ bFGF
MPP^+	15.3±1.4	19.2±1.4	19.2±1.9
6-OHDA	43.9±2.0	38.0±1.9	43.3±3.0

it is the increase in the number of glial cells caused by the growth factor treatments which is responsible for their protective effects against dopaminergic neurotoxicity.

G. Effects of Glial Conditioned Media on the Neurotoxicity of MPP^+ and 6-OHDA on Dopaminergic Neurons

In order to test the hypothesis that glial cells release trophic factors into the extracellular medium which in turn protect dopaminergic neurons from toxin-induced damage, we studied the protective effects of mesencephalic and striatal glial conditioned medium against MPP^+ and 6-OHDA toxicity. 100 μM MPP^+ for 60 min or 50 μM 6-OHDA for 45 min was applied to the cultures at Day 6 *in vitro* and the extent of neurotoxicity was assessed 24 h later. Mesencephalic and striatal CM treatments mimicked the glial protective effects against MPP^+- and 6-OHDA-induced reduction in DA uptake (from 88.7% to 59.2% and 63.5% , respectively after MPP^+ treatment; from 60.3% to 23.8% and 22.5%, respectively after 6-OHDA treatment; Fig. 25). Like glial cells derived from the two brain regions, there was no difference between mesencephalic CM and striatal CM in their potency of protective effects.

Visualization of dopaminergic neurons with TH immunocytochemistry revealed that the appearance of surviving cells following toxin treatment fully supports the results obtained from DA uptake assays (Fig. 26). However, photomicrographs show that the toxins were able to produce some destruction of cell structure even in the presence of CM. Toxin-induced cell loss normally varies from experiment to experiment and in this particular set of experiments, we did not observe a significant loss of TH+ cells with 100 μM MPP^+ or 50 μM 6-OHDA treatment in any of the groups.

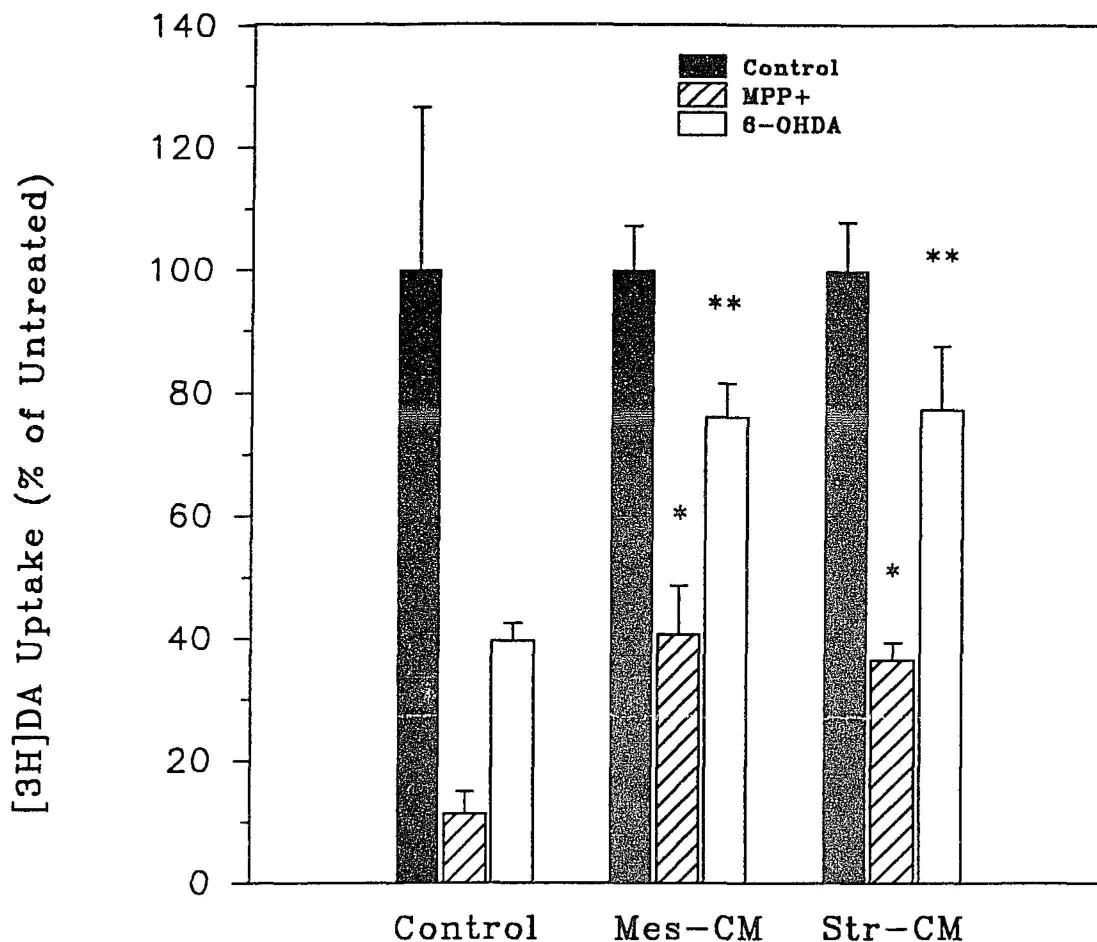
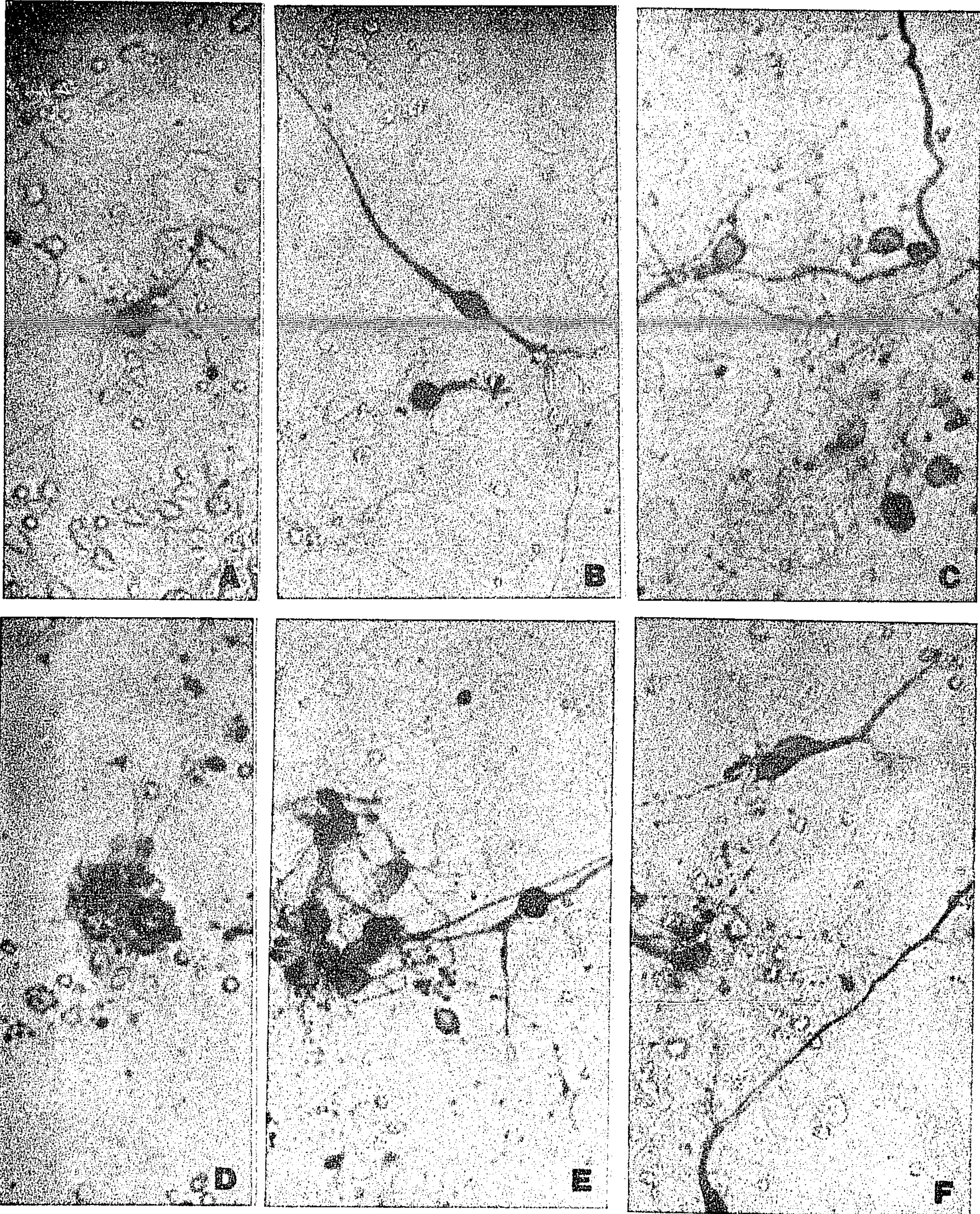


Figure 25. Protective effects of glial conditioned media against MPP$^+$- and 6-OHDA-induced reduction of [^{3}H]DA uptake. Mesencephalic cultures were maintained with chemically defined medium, mesencephalic CM or with striatal CM. Treatment with 100 μM MPP$^+$ for 60 min or 50 μM 6-OHDA for 45 min at 6 DIV produced a significant reduction of uptake in all culture conditions ($P < 0.05$), except in cultures with striatal CM after 6-OHDA treatment. Glial CM significantly reduced the toxicity of MPP$^+$ (* $P < 0.005$) and 6-OHDA (** $P < 0.01$) on DA uptake. No significant difference was observed between the effects produced by mesencephalic glia and by striatal glia. Values represent the mean of 4 samples ± SEM.

Figure 26. Protective effects of glial conditioned media against MPP^+ and 6-OHDA toxicity on dopaminergic neurons visualized with TH immunocytochemistry. Treatment of mesencephalic cultures with 100 μM MPP^+ for 60 min or with 50 μM 6-OHDA for 45 min both produced extensive degeneration of neurons and their processes in control cultures (A, D). Although the neurons in cultures with glial CM were also visibly damaged, the toxic effects on these cultures were somewhat antagonized by both mesencephalic CM (B, E) and striatal CM (C, F).


A
B
C
D
E
F

H. Growth Factor Effects on the Regrowth of Damaged Dopaminergic Neurons

We studied the ability of the growth factors to promote regrowth of damaged dopaminergic neurons by using a concentration of MPP^+ and exposure time which produced damage to the dopaminergic fibers without causing extensive cell loss. Control, EGF, and bFGF cultures were exposed to 10 μM MPP^+ for 24 h at 4 DIV and the development of the dopaminergic neurons was followed for the next two weeks (Fig. 27). Experiments described in the previous sections demonstrated that the toxicity of MPP^+ under these conditions was similar in control and growth factor-treated cultures. The cultures which were pretreated with EGF or bFGF prior to introduction of MPP^+ received the growth factors continuously until the end of the experiment. A gradual increase in $[^3H]$DA uptake over time occurred in all MPP^+-treated cultures, but they always remained lower than their respective controls. However, treatment with growth factors resulted in a pronounced increase in DA uptake in MPP^+-exposed cultures, providing evidence that regrowth of damaged dopaminergic processes was promoted by both EGF and bFGF. In fact, at 18 DIV the MPP^+-treated cultures maintained in the presence of growth factors reached levels of DA uptake that were not only higher than those of the MPP^+-treated controls, but also were several fold greater than untreated controls. For example, $[^3H]$DA uptake in EGF-MPP^+ cultures was 156.3 ± 9.4 fmol/10 min/ culture compared to 82.5 ± 4.9 in the untreated controls; similarly, bFGF-MPP^+ cultures had a DA uptake of 121.5 ± 7.6 fmol/ 10 min/ culture compared to 24.7 ± 3.3 in the untreated controls.

Consistent with the gradual increase in DA uptake in all the MPP^+-treated cultures, TH immunocytochemistry showed regrowth of dopaminergic processes in these cultures over time (Fig. 28). At 10 days after exposure to MPP^+, there was a much more extensive neurite outgrowth in growth factor-treated cultures than in controls. In order to

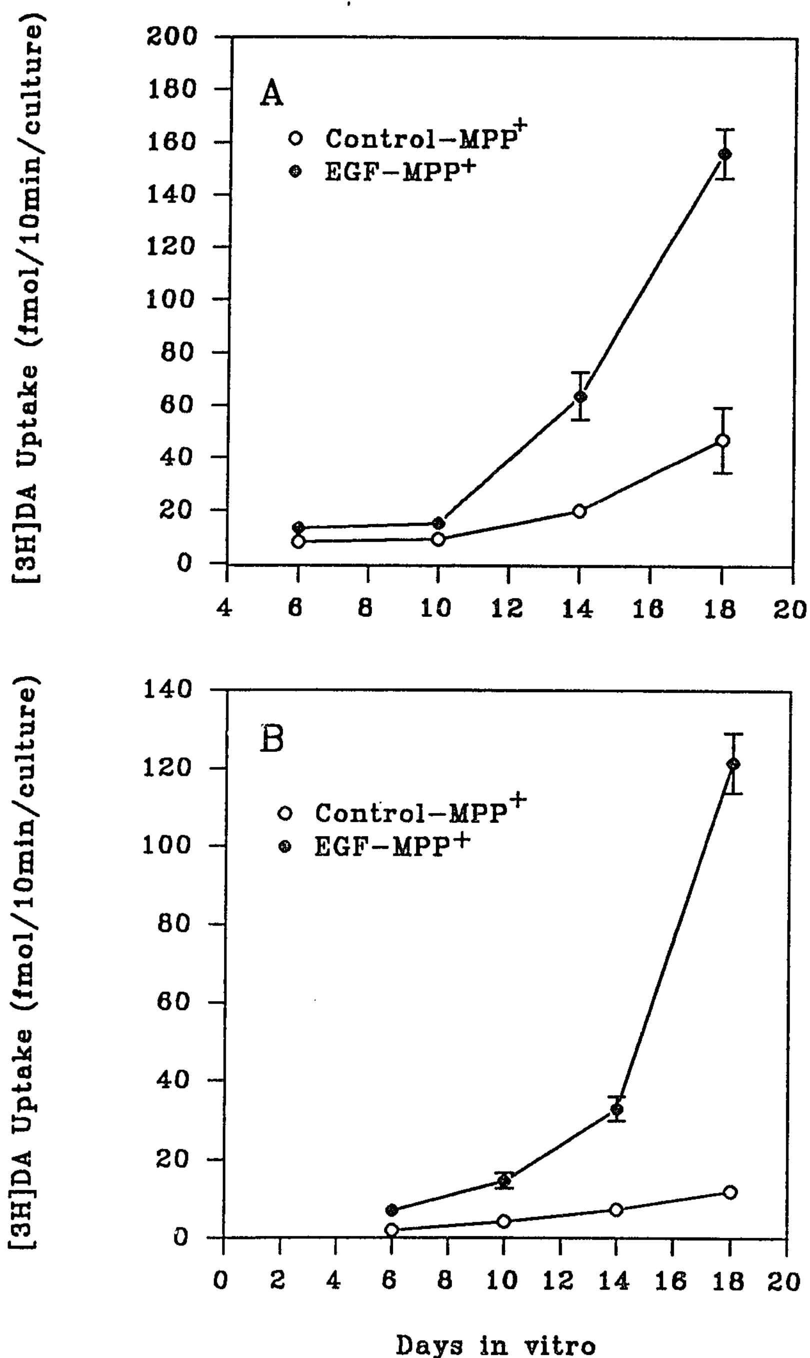
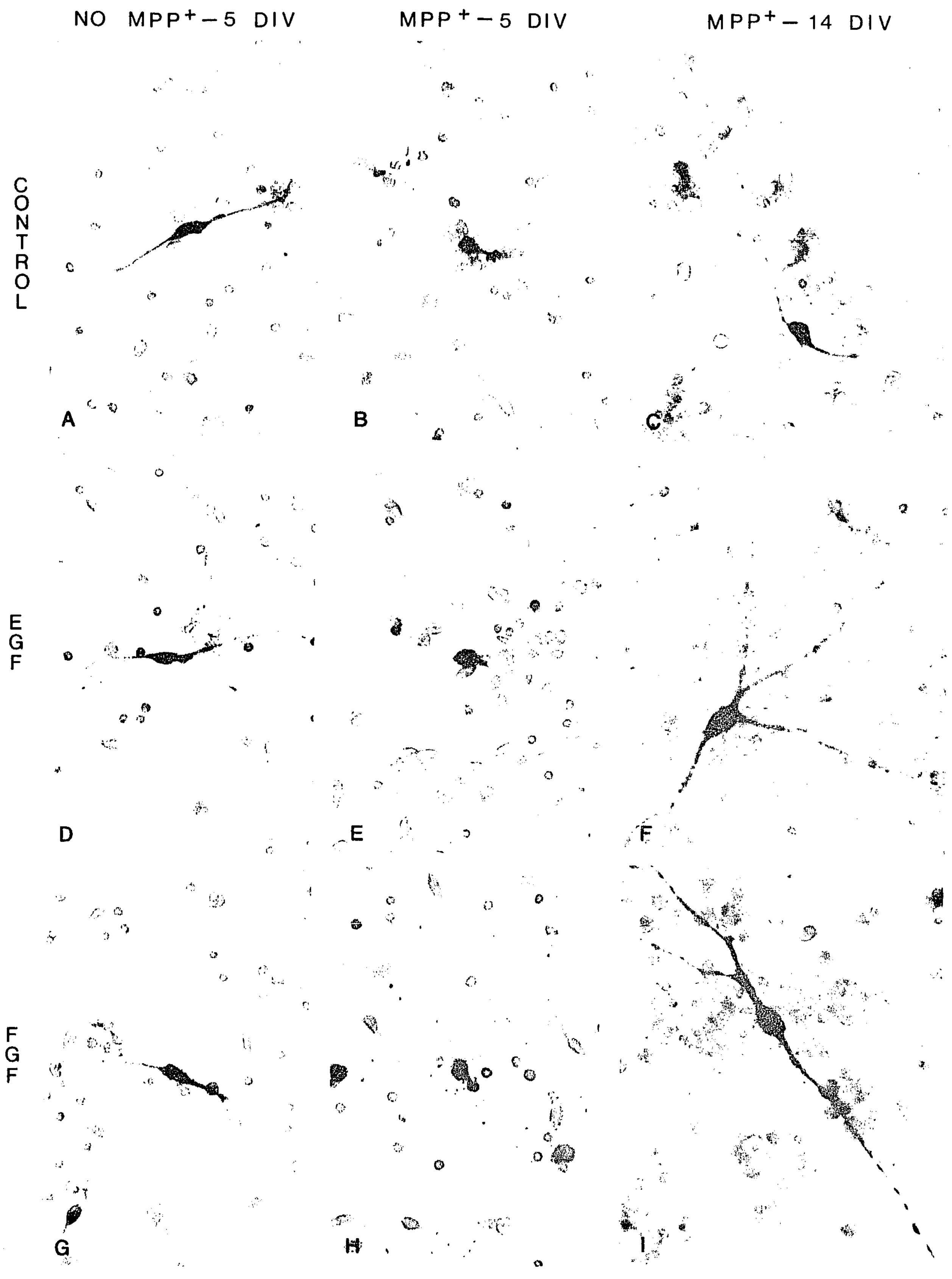


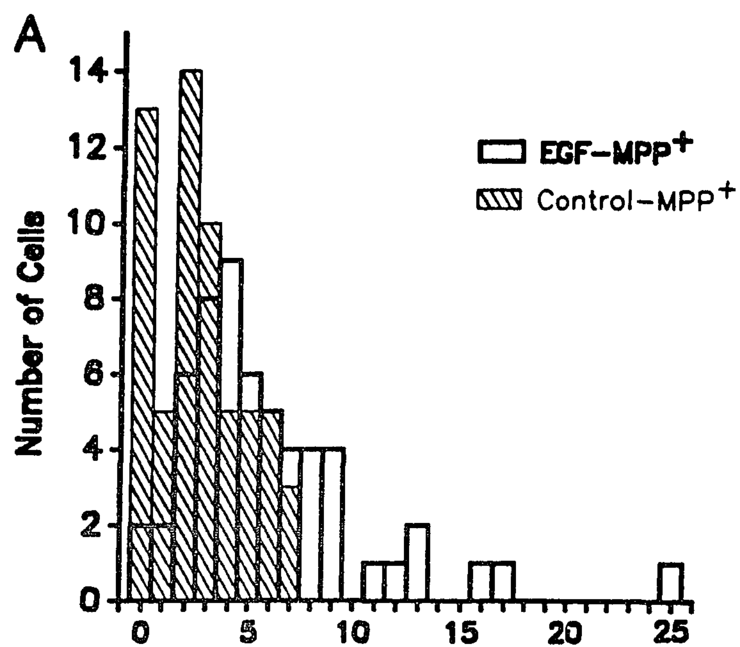
Figure 27. EGF and bFGF stimulation of the recovery of [^{3}H]DA uptake following MPP^+ treatment. Mesencephalic cultures grown in the absence or presence of EGF (10 ng/ml; A) or bFGF (10 ng/ml; B) were treated with 10 μM MPP^+ for 24 h at 4 DIV. The levels of DA uptake were measured at various times following MPP^+ treatment. EGF and bFGF were continuously present in E-MPP^+ and F-MPP^+ cultures, respectively, throughout the experiment. After an initial MPP^+-induced reduction, DA uptake increased slightly with time in C-MPP^+ cultures, while both EGF and bFGF stimulated a much larger increase in DA uptake. All E-MPP^+ values ($P < 0.05$) and all F-MPP^+ values ($P < 0.01$) are significantly greater than the respective C-MPP^+ values (Independent t-test). Each point represents the mean of 5 samples ± SEM.

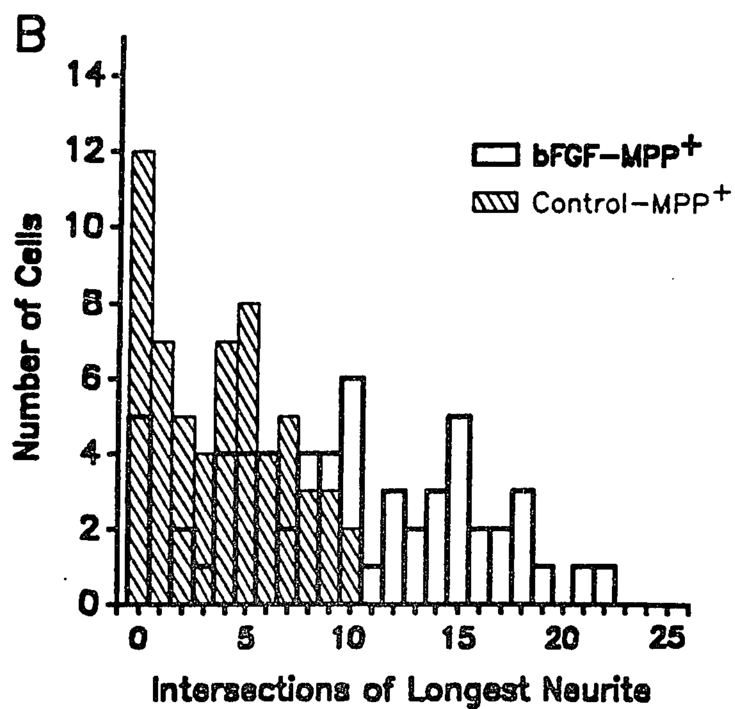
Figure 28. (Same as Figure 15) Effect of MPP^+ treatment on dopaminergic neurons visualized with TH immunocytochemistry, and EGF and bFGF stimulation of the regrowth of dopaminergic processes following MPP^+ treatment. The appearance of TH+ cells at 14 DIV shows some spontaneous regrowth of fibers in MPP^+-exposed dopaminergic neurons (C), but continuous presence of EGF and bFGF in the cultures after MPP^+ treatment stimulated extensive outgrowth of dopaminergic neurites (F and I, respectively).


NO MPP+-5 DIV
MPP+-5 DIV
MPP+-14 DIV
CONTROL
EGF
FGF
A
B
C
D
E
F
G
H
I

quantify process regrowth after MPP^+-induced damage, the length of the longest neurite was measured in 60 randomly selected TH+ neurons from each treatment group. As shown in Fig. 29, following MPP^+ treatment, there were many more dopaminergic neurons with longer neuritic length in both EGF and bFGF cultures than in controls, which further supports the finding that growth factor treatments resulted in a greater increase in DA uptake over that of controls. The mean length of the longest neurite was 2.4-fold greater than controls after EGF treatment and 2.6-fold greater after bFGF treatment.

Figure 29. EGF and bFGF increase dopaminergic neuritic length following MPP^+ treatment. Mesencephalic cultures maintained in the absence or presence of EGF (10 ng/ml) or bFGF (10 ng/ml) were exposed to 10 µM MPP^+ for 24 h at 4 DIV and allowed to recover for 10 days with or without EGF (A) and bFGF (B). The length of the longest neurite was measured as previously described. Both E-MPP^+ and F-MPP^+ cultures have many more TH+ neurons with longer neuritic length than their respective C-MPP^+ cultures at 14 DIV. The mean neuritic length ± SEM in different culture conditions are: (A) E-MPP^+ 6.33±0.62, C-MPP^+ 2.65±0.27; (B) F-MPP^+ 9.68±0.74, C-MPP^+ 3.78±0.39.


A
Number of Cells
14
12
10
8
6
4
2
0
0
5
10
15
20
25
EGF−MPP+
Control−MPP+


B
Number of Cells
14
12
10
8
6
4
2
0
0
5
10
15
20
25
bFGF−MPP+
Control−MPP+
Intersections of Longest Neurite

IV. DISCUSSION

A. Enhanced Development of Dopaminergic Neurons

1. Effects of Growth Factors

Treatment of cultures with the growth factors resulted in an increased survival and neurite outgrowth of dopaminergic neurons. Following their initial interaction with a cell surface receptor, the mechanism of action of these growth factors is not known. The stimulation of growth of dopaminergic neurons by either EGF or bFGF followed a remarkably similar course, suggesting a common mechanism of action. Furthermore, the time course of these growth factor effects closely paralleled the rate of increase in the number of glial cells, which became apparent after several days in culture. Since the localization of the growth factor receptors on dopaminergic neurons remains a question, the possibility exists that the growth factor-induced proliferation of glia led to the production of one or more dopaminergic trophic factors by these cells. Mitogenic activities of both EGF (Leutz and Schachner, 1981; Simpson et al., 1982) and bFGF (Pruss et al., 1982; Pettmann et al., 1985) on astrocytic glial cells are well documented. Toward the end of the second week *in vitro*, when significant growth factor effects on dopaminergic neurons were being observed, GFAP+ astrocytes were by far the most abundant cell population in the growth factor-treated cultures, suggesting that they were probably the cells providing trophic stimulation. Moreover, the trophic effects of EGF and bFGF on dopaminergic neurons were abolished if the mitotic inhibitor FUDR was added together with the growth factors (results not shown). This is in agreement with previous reports showing that when various agents were used to inhibit glial cell proliferation in mesencephalic cultures (Knusel et al., 1990; Casper et al., 1991; Engele

and Bohn, 1991), the neurotrophic effects of EGF and bFGF on dopaminergic neurons were eliminated. Although a direct trophic effect on CNS neurons has been ascribed to both EGF (Morrison et al., 1987) and bFGF (Walicke and Baird, 1988; Unsicker et al., 1992), results from our study support an indirect, glial-mediated trophic role of these growth factors.

2. Effects of Glial Monolayers

The effects of glial cells, in the absence of growth factors, on all dopaminergic parameters resembled those produced by a long-term, continuous treatment with EGF or bFGF. However, the glial trophic effects became apparent at a much earlier time point *in vitro* compared to the growth factor effects. Our hypothesis that the growth factor effects are glial-mediated would have predicted this early action of glia, since the putative trophic factors are probably present in these mixed cultures from the beginning. Previous studies using cocultures of neurons and astroglia have demonstrated the trophic support by glial cells for dopaminergic neuronal growth (Denis-Donini et al., 1984). Dopaminergic neurons in our cocultures of neurons and monolayers of either mesencephalic or striatal astrocytes displayed increased DA uptake activity and survival, but particularly striking was their enhanced morphological complexity. When compared to the dopaminergic neurons grown on polyornithine or with growth factor treatments at any time point, even as late as Day 18, those grown on astrocytic monolayers for one week were far more developed in terms of their cell body size, neuritic branching and neuritic length.

3. Effects of Glial Conditioned Media

Similar trophic effects on dopaminergic neurons were produced by glial CM obtained from mesencephalic or striatal astroglia to those produced by glial cell

monolayers from these brain regions. It was not evident from our experiments whether the extent of the neurotrophic effects produced by glial cells and by the CM differed. It seems likely that the same substance(s) which stimulated the development of dopaminergic neurons in the neuron-glia cocultures are contained in the CM as soluble trophic factors, thereby increasing the DA uptake activity and promoting neuronal survival and growth. It should be pointed out, however, that these effects on dopaminergic neurons by the CM were coincident with the mitogenic effect on astrocytes in the same cultures. Therefore, the enhanced dopaminergic development resulting from treatment with CM appears to be due to a combination of a direct effect by the glial-secreted products and an indirect effect by the stimulation of glial cell proliferation.

4. Summary of Results

Our initial experiments with EGF and bFGF yielded results which suggested that the growth factor trophic effects on dopaminergic neurons are mediated by glial cells. To support this hypothesis, it was important to test the ability of glial cells alone to mimic the growth factor effects, and then to follow up with experiments using glial CM to determine the possibility of production of soluble trophic factors by astrocytes in the cultures. All three culture conditions, i.e. with growth factors, glial monolayers, or glial CM, resulted in the significant enhancement of dopaminergic biochemical and morphological development. All of the results taken together suggest that the astroglia synthesize and secrete factor(s) which is(are) trophic for mesencephalic dopaminergic neurons, and that it is via this mechanism that EGF and bFGF exert their growth-promoting effects. It cannot be concluded from these experiments whether any known growth factors are among the trophic agents contained in our glial conditioned media.

B. Mechanisms of Glial Neurotrophic Effects

1. Extracellular Matrix Molecules

It has been reported that astrocytes provide a supportive substratum for neuronal attachment, migration, survival, and neurite extension *in vitro* (Noble et al., 1984). The astrocytic cell-derived substances responsible for these neuron-glia interactions may include diffusible proteins in the culture medium and also nondiffusible, membrane-associated molecules. The extracellular matrix is the substratum for cell adhesion *in vivo* , and consists of various glycoproteins, proteoglycans and glycosaminoglycans forming an insoluble network in the spaces between cells. Laminin, a glycoprotein molecule of the extracellular matrix synthesized by astrocytes, is well known for its enhancement of neurite outgrowth (Rogers et al., 1983; Matthiessen et al., 1989). It is possible that any of the extracellular matrix molecules partly mediated the glial stimulation of dopaminergic neuronal growth in our neuronal-glial cocultures. In addition, the presence of glial monolayers increased the density of the cultures so that cell-cell contacts between neurons and glia may also have potentiated the dopaminergic neuronal survival and development.

2. Secreted Factors

Trophic activities on the survival and differentiation of central neurons *in vitro* have been found in CM obtained from astrocytic cultures (Banker, 1980; Muller and Seifert, 1982; Barbin et al., 1984; Rudge et al., 1985; Patel and Hunt, 1989), suggesting that neurotrophic substances synthesized by glial cells are released into the culture medium. Similar to the results in our studies, Giulian et al. (1993) examined glial influences upon neuronal growth and survival and found that astroglial cells in cocultures and also their secretion products contained in the CM supported neuronal survival and

neuritic growth. The fact that the trophic effects produced by astrocytic monolayers in our studies were closely mimicked by glial CM provide evidence for the secretion of soluble trophic factors by astroglia as the mechanism of glial neurotrophic activity. Since the glial and CM trophic effects were analyzed for only dopaminergic neurons in our studies, we are not able to address the issue of trophic factor specificity for a particular neuronal population. It is possible that the same agent(s) which exerted the trophic effects upon dopaminergic neurons may have similar effects for other target neurons, so that many trophic factors are secreted by astrocytes to produce effects upon several different neuronal populations.

C. Neurotrophic Factors in Glial Conditioned Medium

Although the nature of the neurotrophic factors contained in glial CM is not known, several studies suggest certain identified growth factors as possible candidates. For example, it has been reported that astrocytes in culture can synthesize nerve growth factor (NGF; Yoshida and Gage, 1991); however, NGF has not been demonstrated to be trophic for dopaminergic neurons. Another growth factor which has been reported to be synthesized and secreted by astroglial cells in culture is bFGF (Ferrara et al., 1988; Hatten et al., 1988). Interestingly, bFGF is reported to function as an autocrine factor to stimulate the growth of astrocytes themselves (Araujo and Cotman, 1992; Gomez-Pinilla et al., 1992), which then synthesize and release more bFGF. Since bFGF added to our cultures with inhibited glial cell proliferation did not produce dopaminergic neurotrophic effects, it appears that some other trophic factor(s) must be secreted by astrocytes which can act upon the dopaminergic neurons. However, it cannot be ruled out that bFGF acts synergistically with some unidentified glial-derived factor to exert the trophic effects

observed in our cultures. If bFGF is in fact one of the active participants, Araujo and Cotman's (1992) finding that EGF treatment of astroglial cultures increased the release of bFGF from these cells becomes significant for our studies where the neurotrophic effects of EGF are believed to have resulted from an increase in the number of astrocytes. On the other hand, it is possible that the only action of bFGF in our cultures is to induce the production of other trophic agents by astroglial cells.

D. Region-Specific Differences in Neuron-Glia Interactions

The main projection targets for dopaminergic neurons from the substantia nigra are in the striatum. In order to examine region-specific neuron-glia interactions for dopaminergic neurons, tissues from the mesencephalon and from the striatum, where dopaminergic somata and axon terminals, respectively, are located, were chosen to prepare glial cell monolayers and glial CM. Our aim was to find indications that different dopaminergic trophic substances, both membrane-associated and diffusible, may be synthesized by astrocytes derived from the two brain regions.

Several studies have reported that astrocytes from different brain regions display distinct biochemical and physiological properties (Perraud et al., 1990), and that regional heterogeneity exists in glial-released proteins (Zahs et al., 1993). More significantly, astrocytes derived from different brain regions have been demonstrated to differ in their support of dopaminergic neuronal survival (O'Malley et al., 1991) and their morphological maturation in culture (Denis-Donini et al., 1984; Chamak et al., 1987a, 1987b). In all of these studies, it was found that astrocytes derived from the mesencephalon had greater effects on the promotion of survival and growth of mesencephalic dopaminergic neurons compared to astrocytes derived from the striatum.

For example, Chamak et al. (1987b) demonstrated that the dopaminergic neurons grown on mesencephalic astrocytes have more complex morphological traits than those grown on striatal astrocytes. Similarly, Rousselet et al. (1988) found that the mesencephalic glial CM enhances the number of primary neurites in mesencephalic neurons.

In our studies, we did not observe these different region-specific effects for dopaminergic neurons grown on mesencephalic astrocytes or on striatal astrocytes. Dopaminergic neuronal development in the presence of either type of glial cells was enhanced to the same extent. However, we did find a larger effect of mesencephalic CM compared to striatal CM in supporting neuronal survival and also in increasing the DA uptake activity. One explanation for this discrepancy between astrocytes and astrocytic CM may be that the additional contributing factors present in the neuronal-glial cocultures (i.e., extracellular matrix molecules and cell-cell contacts) produce neurotrophic effects which overshadow the effects produced by secreted glial products alone. Thus, the total effect produced by a combination of all these factors in the cocultures may not allow for the identification of region-specific interactions. Our results suggest that different trophic factors are present in the two types of glial CM and that the growth and survival of mesencephalic dopaminergic neurons are preferentially influenced by factors produced by astrocytes derived from the same brain region.

E. Protection Against MPP^+ and 6-OHDA Neurotoxicity

1. Effects of Growth Factors

In our culture system, prolonged incubation with EGF or bFGF was necessary to obtain positive protective effects against MPP^+ toxicity, suggesting that their trophic effects were indirectly mediated, probably as a result of stimulation of glial cell

proliferation by these growth factors. This was supported by observations of a lack of protection from toxicity when cultures were exposed to MPP^+ at 4 DIV, before growth factors had caused extensive glial cell proliferation, and a lack of protection from toxicity when cultures were treated with growth factors just for the duration of MPP^+ exposure. In contrast, if MPP^+ was applied to the cultures after they were treated with EGF or bFGF for 10 days, when the glial cell number had greatly increased, the reductions in DA uptake and TH+ neuronal survival were significantly smaller than in non-growth factor-treated controls. Moreover, when FUDR was included in the medium together with growth factors, the protective effect against MPP^+-induced reduction in DA uptake was completely eliminated, suggesting that it was mediated by the dividing cell population.

In contrast to the lack of protective effects against MPP^+ neurotoxicity after a 4-day pretreatment with the growth factors, bFGF was able to protect against the toxicity of 6-OHDA after 6 days of treatment, which is still before significant stimulation of dopaminergic neuronal development or glial proliferation has occurred. This is suggestive of different mechanisms of bFGF-induced resistance to dopaminergic neurotoxins: an indirect, glial-mediated effect against MPP^+ neurotoxicity and a direct effect against 6-OHDA. It cannot be completely ruled out, however, that the small number of contaminating glial cells in the bFGF-treated cultures were stimulated to synthesize factors which then produced the observed protective effects.

2. *Effects of Glial Monolayers and Glial Conditioned Media*

Both astrocytic glial cells and glial CM in the same cultures with neurons significantly reduced the MPP^+ and 6-OHDA dopaminergic neurotoxicity. The extent of protection against these toxins appeared to be the same with either glial monolayers or with the CM, suggesting that the glial-derived molecules responsible for these effects are contained in the CM as soluble trophic factors. We did not find any region-specific

differences in the protective effects between those produced by mesencephalic glial cells or CM and those produced by striatal glia or CM.

Our results demonstrate much greater protective effects against 6-OHDA toxicity compared to those against MPP^+ by both glial cells and by CM, which indicates different mechanisms of action of the two neurotoxins. It is known that although both of these toxins are actively accumulated within the dopaminergic neurons via the high-affinity DA uptake system (Kostrzewa and Jacobowitz, 1974; Chiba et al., 1985; Javitch et al., 1985), the mechanisms by which they induce toxicity within the cell are different. MPP^+ is believed to induce cell death by inhibiting mitochondrial respiration, leading to ATP depletion (Nicklas et al., 1985). On the other hand, 6-OHDA toxicity is produced by the formation of cytotoxic oxygen radical species (Heikkila and Cohen, 1972; Sachs and Jonsson, 1975). The observed differences between the protective effects against the two toxins was primarily due to the degree of resistance against neuritic degeneration following toxic treatment. The presence of glial cells or treatment with glial CM greatly reduced the damage produced by 6-OHDA at the neurites, whereas MPP^+ damage to neuritic length was only slightly reduced. It may be possible that different glial-derived molecules are targeted to act against these two dopaminergic toxins.

3. Summary of Results

To support the hypothesis that the protective effects of growth factors against MPP^+ are mediated by glial cells, it was important to demonstrate that glial cells alone could also protect the dopaminergic neurons against toxin-induced damage. In further support of the role of glial cells in the neuroprotective effects obtained with growth factor treatments, the addition of EGF or bFGF to the neuronal-glial coculture did not potentiate the glial protective effects against MPP^+. Furthermore, bFGF did not potentiate the glial protective effects against 6-OHDA, although it was suggested that bFGF may exert a

direct action on the dopaminergic neurons treated with this toxin. This lack of an additive effect between bFGF and glial cells suggests that bFGF may be one of the factors secreted by astrocytes. The finding that the glial CM was able to closely mimic the effects produced by glial cells leads to the conclusion that it is by the action of secreted glial products that all treatment conditions in our studies resulted in the protection of dopaminergic neurons against MPP^+ and 6-OHDA toxicity.

F. Neuroprotective Mechanisms

1. Glial Cells

Potential mechanisms by which glial cells could mediate the protection of dopaminergic neurons from the toxic effects of MPP^+ and 6-OHDA include (1) the prevention of accumulation of the toxins within dopaminergic neurons by interfering with the DA uptake carrier, or (2) decreased concentration of the toxins in the extracellular medium as a result of uptake or degradation of the toxins by glial cells, as well as (3) the stimulation of protective mechanisms for dopaminergic neurons through the secretion of other putative trophic molecules.

The first possibility seems to be unlikely, since both MPP^+ and 6-OHDA accumulate within dopaminergic neurons through the high affinity DA reuptake system and, as our studies have demonstrated, DA uptake levels are greatly increased after EGF or bFGF treatment and in the presence of glial monolayers or glial CM. Secondly, it also appears unlikely that glial uptake of MPP^+ may have produced significant lowering of the concentration of the toxin available to dopaminergic neurons, since studies have shown that even in the presence of astrocytes MPP^+ is not stored within the cells but is accumulated mostly in the medium (Mytilineou and Friedman, 1988; Schinelli et al.,

1988). In fact, DiMonte et al. (1992) has recently shown that one possible mechanism for the resistance of astrocytes to MPP^+ toxicity is that MPP^+, which is generated within the astrocytes, can cross the cell membranes and escape to the extracellular compartment. To rule out the possibility of either glial uptake or degradation of the toxins, we analyzed the concentrations of MPP^+ and 6-OHDA in the extracellular medium during their incubation period and found that the same concentrations of the toxins were available to dopaminergic neurons whether or not glial cells were present in the cultures. Therefore, it appears that the third possibility is the most likely mechanism of glial-mediated protection against these toxins -- through the secretion of neurotrophic substances by astrocytes.

2. Glial Secretion of Trophic Factors

The results of our studies indicate that the glial cells condition the culture medium with one or more substances which can protect the dopaminergic neurons against toxicity produced by MPP^+ or 6-OHDA. Astrocytes have been shown to produce identified peptide growth factors such as NGF (Ono et al., 1991; Yoshida and Gage, 1991) and bFGF (Ferrara et al., 1988; Hatten et al., 1988). Although it does not appear to be the mechanism operating in our cultures, a direct effect of growth factors against neurotoxin-induced cell death has been shown through the protection of hippocampal neurons from glutamate neurotoxicity by FGF (Mattson et al., 1989; Mattson and Rychlik, 1990); by the reduction of neuroblastoma cell death caused by 6-OHDA after treatment with NGF (Tiffany-Castiglioni and Perez-Polo, 1981); and by the protection of mesencephalic dopaminergic neurons from MPP^+-induced cell death by BDNF (Hyman et al., 1991; Beck et al., 1992). Although BDNF has been demonstrated to be neurotrophic for mesencephalic dopaminergic neurons, it appears unlikely that BDNF is the trophic factor

secreted by glial cells in our cultures since BDNF mRNA has been found in neurons, but not in glial cells (Ernfors et al., 1990; Hofer et al., 1990).

3. Defense System Against Oxygen Radicals

The highly reactive products of 6-OHDA autooxidation, including superoxide radicals, hydrogen peroxide and hydroxyl radicals, have been shown to cause neuronal cell destruction (Sachs and Jonsson, 1975). The normal cellular defense system against these cytotoxic oxygen species consists of antioxidative enzymes such as superoxide dismutase (SOD), glutathione peroxidase, glutathione reductase and catalase. An augmentation in the level of these antioxidative enzymes which can remove the toxic products may thus protect the neurons against 6-OHDA toxicity. It has been reported that glial cells are better equipped with antioxidant defense mechanisms than neurons (Raps et al., 1989; Geremia et al., 1990). In these studies, an enhanced SOD activity, leading to reduced lipid peroxidative injury, has been found in glial cells (Geremia et al., 1990), and the concentration of glutathione was found to be high in cultured astrocytes compared to neurons (Raps et al., 1989).

Growth factors have been implicated as protective agents against oxidative damage. Tiffany-Castiglioni and Perez-Polo (1981) studied the effects of NGF treatment on the toxicity of 6-OHDA in neuroblastoma cells. They found that NGF significantly increased the cell survival after 6-OHDA treatment and that the mechanism of protection was by an enhanced resistance to free radical products, particularly to hydrogen peroxide. A study by Jackson et al. (1990) also found a protective effect of NGF against 6-OHDA toxicity. These investigators observed an enhanced catalase activity in PC12 cells, which led to the resistance to peroxidative insult. Another growth factor that has been demonstrated to be effective against the toxicity of 6-OHDA is BDNF. Spina et al. (1992) found an involvement of the glutathione system against oxidative stress in the

protective effect of BDNF against 6-OHDA-induced dopaminergic cell loss in mesencephalic cultures.

Recent studies have addressed the question of free radical involvement in the mechanism of MPTP neurotoxicity. It has been observed that the interaction of MPP^+ with mitochondrial complex I generates free radicals, such as superoxide, and that transgenic mice with increased SOD activity are resistant to the toxic effects of MPTP (Przedborski et al., 1992). Others found that MPTP caused a reduction in the cellular glutathione levels in mice and that this effect could be reversed by antioxidants (Yong et al., 1986). These results provide supporting evidence for an oxygen radical mediation in MPTP neurotoxicity.

If the neurotoxic actions of both MPP^+ and 6-OHDA are indeed caused by the formation of oxygen radicals, it is possible that an induction by glial cells and glial-derived factors of free radical detoxifying mechanisms mentioned above could account for the protective effects against these neurotoxins observed in our studies.

G. MPP^+ Neurotoxicity

In the present study we have used the neurotoxin MPP^+ to investigate the process of nerve fiber degeneration in dopaminergic neurons and the ensuing recovery following damage. The mechanisms by which MPTP causes dopaminergic neuronal degeneration are not yet fully understood and the site of neurotoxic action of MPTP has been debated. Some reports provide evidence that the initial damage occurs at the nerve terminals (Burns et al., 1983; Kitt et al., 1986; Ricaurte et al., 1986), while others suggest that the insult begins at the cell soma (D'Amato et al., 1986; Sundstrom et al., 1988). If the primary damage of MPTP is terminal degeneration, the toxin may not cause cell death

and thus allow the possibility of recovery. Our results demonstrate that dopaminergic neuronal processes are most sensitive to MPP^+ damage. By selecting the appropriate MPP^+ concentration and exposure time, we were able to produce severe reductions in DA uptake (65%) and in the length of dopaminergic fibers (56%) without loss of neuronal somata. These results are in agreement with findings from a recent study which examined the MPTP-induced degenerative process in the primate brain (Herkenham et al., 1991). By utilizing autoradiography to localize $[^{14}C]MPP^+$ accumulation sites following MPTP injections, the authors found that MPP^+ was taken up by dopaminergic terminals and subsequently transported to the cell body in a retrograde fashion. They postulate that dopaminergic terminals die first, followed by a slower degeneration of the neurons at a dose-dependent rate. Our morphological observations on TH immunostained neurons after MPP^+ treatment are consistent with their findings.

H. Neuronal Recovery from Injury

Regrowth of dopaminergic fibers after lesion of the nigrostriatal pathway have not been demonstrated. However, spontaneous recovery following MPTP treatment has been observed in primates (Eidelberg et al., 1986; Waters et al., 1987), cats (Schneider and Rothblat, 1991) and mice (Hallman et al., 1985; Ricaurte et al., 1986). In culture, it was previously suggested that mesencephalic dopaminergic neurons did not regenerate processes after MPP^+ damage (Michel et al., 1990). In contrast, our studies demonstrate that regrowth of dopaminergic fibers can occur spontaneously following MPP^+-induced damage, but it often occurs with a delay of several days (see Fig. 27). This delayed recovery is possibly one of the reasons for the apparent discrepancy between our results

and those of Michel et al. (1990), who followed recovery for only 6 days after MPP^+ treatment. The regrowth of dopaminergic fibers after damage observed in our experiments, is not an unexpected finding. Many studies indicate that the neurons of the central nervous system (CNS) have an inherent ability to regenerate processes, after they have been severed by either mechanical or chemical injury (Bjorklund and Stenevi, 1979; Kalil and Reh, 1979; Fawcett, 1992). The environment surrounding the damaged neurons appears to be the critical factor which prevents or restricts significant neuronal regeneration in the CNS. For example, the formation of reactive astrocytes at the site of injury (McKeon et al., 1991) or the presence of oligodendrocytes (Schwab and Caroni, 1988) may act as inhibitory environmental influences. Experiments have shown that substitution of the CNS environment with a more permissive one, such as the peripheral nervous system neuroglia, can induce injured central neurons to regenerate processes (Aguayo et al., 1981; Benfey and Aguayo, 1982). In the peripheral nervous system, regeneration of damaged neurons can be enhanced by selective trophic factors. NGF and BDNF have been demonstrated to promote the regrowth of axotomized peripheral neurons (Lindsay, 1988).

Although dopaminergic neurons, under our culture conditions, were able to grow fibers after MPP^+ damage, the extent of growth did not approach that of undamaged fibers even after 2 weeks following lesion. To determine whether the regrowth of MPP^+-damaged dopaminergic fibers in culture can be stimulated by the action of growth factors, we examined the effect of EGF and bFGF on the recovery of growth after exposure to MPP^+. Initially, we determined the effects of treatment with EGF or bFGF on the development of dopaminergic neurons in our culture system. In accord with findings from previous studies (Ferrari et al., 1989; Casper et al., 1991; Engele and Bohn, 1991), both EGF and bFGF stimulated the growth of dopaminergic fibers and promoted the survival of dopaminergic neurons in culture. Treatment with these growth factors

resulted in an increase in [^{3}H]DA uptake, which was more pronounced during the second and third week in vitro. Also during this time, a striking increase in the neuritic length of TH+ neurons was observed in cultures treated with growth factors. In addition to promoting growth during normal development in culture, treatment with EGF or bFGF also enhanced the regrowth of MPP$^+$-damaged dopaminergic fibers. This was confirmed both by measurements of [^{3}H]DA uptake and by morphological evaluation of cultures immunostained with antibodies to TH. At 10 days after MPP$^+$ exposure, the DA uptake levels and dopaminergic neuritic lengths of EGF and bFGF cultures were 2.4- to 3.0-fold greater than their controls. In fact, growth factor treatment of MPP$^+$-damaged dopaminergic neurons resulted in growth which was greater than that of undamaged controls not treated with EGF or bFGF. In a recent publication, Hadjiconstantinou et al. (1991) also described stimulation of DA uptake by EGF in mesencephalic cultures treated with MPP$^+$. The authors also found that partial recovery of dopaminergic parameters could be achieved by treating MPTP-lesioned mice with EGF. Similarly, Otto and Unsicker (1990) reported that treatment of MPTP-lesioned mice with bFGF resulted in a recovery of DA and TH levels and reappearance of TH-immunoreactive fibers.

I. Neurotrophic Factors and Degenerative Disease

An interesting idea has been proposed that the lack of neurotrophic factors in the brain may contribute to the development of degenerative diseases, such as Alzheimer's, Parkinson's and amyotrophic lateral sclerosis (ALS; Appel, 1981). If so, then the exogenous administration of trophic factors should be able to interrupt the degenerative disease process. It has been hypothesized that a deficit in NGF may be involved in the degeneration of forebrain cholinergic neurons observed in Alzheimer's disease and that

administration of NGF or NGF-like compounds may be able to treat the disease (Hefti and Weiner, 1986). Reports of protective effects of some trophic agents against neuronal cell death in the mammalian CNS and their promotion of recovery after experimental lesions, provide supporting evidence for these hypotheses and hope for the eventual therapeutic use of neurotrophic factors (see review by Hefti et al., 1989).

J. Conclusion

Many investigations are currently undergoing to search for factors which can promote neuronal growth and survival in the CNS. The aim of this thesis was to study factors which are trophic for the mesencephalic dopaminergic neurons, under normal and toxic conditions. We found that the astrocytic glial cells play an essential role in the trophic effects observed with EGF and bFGF, most likely through the production and secretion of molecules into the culture medium. Further research is necessary to determine the exact mechanisms by which glial cells can offer protection to the neurons against neurotoxin-induced damage and stimulate recovery of surviving neurons, and to identify the molecules responsible for these trophic effects. Identification of specific dopaminergic growth factors *in vitro* can eventually become useful in clinical applications to increase transplant survival and to interfere with the progression of Parkinson's disease. By increasing the availability of specific growth factors to the aging CNS, it may become possible to enhance the growth and long-term survival of neurons and to prevent their degeneration following injury, while promoting recovery processes.

V. REFERENCES

Abraham J.A., Mergia A., Whang J.L., Tumolo A., Friedman J., Hjerrild K.A., Gospodarowicz D., and Fiddes J.C. (1986) Nucleotide sequence of a bovine clone encoding the angiogenic protein, basic fibroblast growth factor. Science 233, 545-548.

Adamson E.D. and Meek J. (1984) The ontogeny of epidermal growth factor receptors during mouse development. Dev. Biol. 103, 62-70.

Agid Y. (1991) Parkinson's disease: pathophysiology. Lancet 337, 1321-1324.

Aguayo A.J., David S., and Bray G.M. (1981) Influences of the glial environment on the elongation of axons after injury. J. Exp. Biol. 95, 231-240.

Anderson K.J., Dam D., Lee S., and Cotman C.W. (1988) Basic fibroblast growth factor prevents death of lesioned cholinergic neurons in vivo. Nature 332, 360-361.

Appel S.H. (1981) A unifying hypothesis for the cause of amyotrophic lateral sclerosis, parkinsonism, and alzheimer disease. Ann. Neurol. 10, 499-505.

Araujo D.M. and Cotman C.W. (1992) Basic FGF in astroglial, microglial, and neuronal cultures: characterization of binding sites and modulation of release by lymphokines and trophic factors. J. Neurosci. 12, 1668-1678.

Banker G.A. (1980) Trophic interactions between astroglial cells and hippocampal neurons in culture. Science 209, 809-810.

Barbin G., Selak I., Manthorpe M., and Varon S. (1984) Use of central neuronal cultures for the detection of neuronotrophic agents. Neurosci. 12, 33-43.

Beck K.D., Knusel B., Winslow J.W., Rosenthal A., Burton L.E., Nikolics K., and Hefti F. (1992) Pretreatment of dopaminergic neurons in culture with brain-derived neurotrophic factor attenuates toxicity of 1-methyl-4-phenylpyridinium. Neurodegeneration 1, 27-36.

Benfey M. and Aguayo A. J. (1982) Extensive elongation of axons from rat brain into peripheral nerve grafts. Nature 296, 150-152.

Bjorklund A. and Stenevi U. (1979) Regeneration of monoaminergic and cholinergic neurons in the mammalian central nervous system. Physiol. Rev. 59, 62-100.

Bohlen P., Esch F., Baird A., Jones K.L., and Gospodarowicz D. (1985) Human brain fibroblast growth factor. Isolation and partial chemical characterization. FEBS Lett. 185, 177-181.

Bohn M.C., Cupit L., Marciano F., and Gash D.M. (1987) Adrenal medulla grafts enhance recovery of striatal dopaminergic fibers. Science 237, 913-916.

Bottenstein J.E. and Sato G.H. (1979) Growth of a rat neuroblastoma cell line in serum-free supplemented medium. Proc. Natl. Acad. Sci. USA 76, 514-517.

Bradbury A.J., Costall B., Domeney A.M., Jenner P., Kelly M.E., Marsden C.D., and Naylor R.J. (1986) 1-Methyl-4-phenylpyridine is neurotoxic to the nigrostriatal dopamine pathway. Nature 319, 56-57.

Breese G.R. and Traylor T.D. (1970) Effects of 6-hydroxydopamine on brain norepinephrine and dopamine: Evidence of selective degeneration of catecholamine neurons. J. Pharmacol. Exp. Ther. 174, 413-420.

Burns R.S., Chiueh C.C., Markey S.P., Ebert M.H., Jacobowitz D.M., and Kopin I.J. (1983) A primate model of parkinsonism: selective destruction of dopaminergic neurons in the pars compacta of the substantia nigra by N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. Proc. Natl. Acad. Sci. USA 80, 4546-4550.

Caday C.G., Klagsbrun M., Fanning P.J., Mirzabegian A., and Finklestein S.P. (1990) Fibroblast growth factor (FGF) levels in the developing rat brain. Dev. Brain Res. 52, 241-246.

Carpenter G. (1979) Epidermal growth factor. Ann. Rev. Biochem. 48, 193-216.

Casper D., Mytilineou C., and Blum M. (1991) EGF enhances the survival of dopamine neurons in rat embryonic mesencephalon primary cell culture. J. Neurosci. Res. 30, 372-381.

Chamak B., Fellous A., Autillo-Touati A., Barbin G., and Prochiantz A. (1987a) Are neuro-astroglial neuronotrophic interactions regionally specified? Ann. N.Y. Acad. Sci. 495, 528-536.

Chamak B., Fellous A., Glowinski J., and Prochiantz A. (1987b) MAP2 expression and neuritic outgrowth and branching are coregulated through region-specific neuro-astroglial interactions. J. Neurosci. 7, 3163-3170.

Chiba K., Trevor A., and Castagnoli, Jr., N. (1984) Metabolism of the neurotoxic tertiary amine, MPTP, by brain monoamine oxidase. Biochem. Biophys. Res. Commun. 120, 574-578.

Chiba K., Trevor A.J., and Castagnoli, Jr., N. (1985) Active uptake of MPP^+, a metabolite of MPTP, by brain synaptosomes. Biochem. Biophys. Res. Commun. 128, 1228-1232.

Chiueh C.C., Markey S.P., Burns R.S., Johannessen J.N., Pert A., and Kopin I.J. (1984) Neurochemical and behavioral effects of systemic and intranigral administration of N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine in the rat. Eur. J. Pharmacol. 100, 189-194.

Cleeter M.W.J., Cooper J.M., and Schapira A.H.V. (1992) Irreversible inhibition of mitochondrial complex I by 1-methyl-4-phenylpyridinium: evidence for free radical involvement. J. Neurochem. 58, 786-789.

Cohen G., Pasik P., Cohen B., Leist A., Mytilineou C., and Yahr M. (1984) Pargyline and deprenyl prevent the neurotoxicity of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in monkeys. Eur. J. Pharmacol. 106, 209-210.

Cohen S. (1962) Isolation of a mouse submaxillary protein accelerating incisor eruption and eyelid opening in the newborn animal. J. Biol. Chem. 237, 1535-1562.

D'Amato R.J., Lipman Z.P., and Snyder S.H. (1986) Selectivity of the parkinsonian neurotoxin MPTP: toxic metabolite MPP^+ binds to neuromelanin. Science 231, 987-989.

Davis G.C., Williams A.C., Markey S.P., Ebert M.H., Calne E.D., Reichert C.M., and Kopin I.J. (1979) Chronic parkinsonism secondary to intravenous injection of meperidine analogues. Psychiatry Res. 1, 249-254.

Denis-Donini S., Glowinski J., and Prochiantz A. (1984) Glial heterogeneity may define the three-dimensional shape of mouse mesencephalic dopaminergic neurones. Nature 307, 641-643.

Di Monte D.A., Wu E.Y., Irwin I., Delanney L.E., and Langston J.W. (1992) Production and disposition of 1-methyl-4-phenylpyridinium in paimary cultures of mouse astrocytes. Glia 5, 48-55.

DiPorzio U., daguet M.-C., Glowinski J., and Prochiantz A. (1980) Effect of striatal cells on in vitro maturation of mesencephalic dopaminergic neurones grown in serum-free conditions. Nature 288, 370-373.

Eccleston P.A. and Silberberg D.H. (1985) Fibroblast growth factor is a mitogen for oligodendrocytes in vitro. Dev. Brain Res. 21, 315-318.

Eidelberg E., Brooks B.A., Morgan W.W., Walden J.G., and Kokemoor R.H. (1986) Variability and functional recovery in the N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine model of parkinsonism in monkeys. Neuroscience 18, 817-822.

Engele J. and Bohn M.C. (1991) The neurotrophic effects of fibroblast growth factors on dopaminergic neurons *in vitro* are mediated by mesencephalic glia. J. Neurosci. 11, 3070-3078.

Ernfors P., Lonnerberg P., Ayer-LeLievre C., and Persson H. (1990) Developmental and regional expression of basic fibroblast growth factor mRNA in the rat central nervous system. J. Neurosci. Res. 27, 10-15.

Esch F., Baird A., Ling N., Ueno N., Hill F., Denoroy L., Klepper R., Gospodarowicz D., Bohlen P., and Guillemin R. (1985) 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 82, 6507-6511.

Fallon J.H., Seroogy K.B., Loughlin S.E., Morrison R.S., Bradshaw R.A., Knauer D.J., and Cunningham D.D. (1984) Epidermal growth factor immunoreactive material in the central nervous system: location and development. Science 224, 1107-1109.

Fawcett J.W. (1992) Intrinsic neuronal determinants of regeneration. Trends Neurosci. 15, 5-8.

Ferguson I.A., Schweitzer J.B. and Johnson Jr., E.M. (1990) Basic fibroblast growth factor: receptor-mediated internalization, metabolism, and anterograde axonal transport in retinal ganglion cells. J. Neurosci. 10, 2176-2189.

Ferrara N., Ousley F., and Gospodarowicz D. (1988) Bovine brain astrocytes express basic fibroblast growth factor, a neurotropic and angiogenic mitogen. Brain Res. 462, 223-232.

Ferrari G., Minozzi M.-C., Toffano G., Leon A., and Skaper S.D. (1989) Basic fibroblast growth factor promotes the survival and development of mesencephalic neurons in culture. Dev. Biol. 133, 140-147.

Finkelstein S.P., Apostolides P.J., Caday C.G., Prosser J., Philips M.F., and Klagsbrun M. (1988) Increased basic fibroblast growth factor (bFGF) immunoreactivity at the site of local brain wounds. Brain Res. 460, 253-259.

Frautschy S.A., Walicke P.A., and Baird A. (1991) Localization of basic fibroblast growth factor and its mRNA after CNS injury. Brain Res. 553, 291-299.

Freese A., Finkelstein S. P., and DiFiglia M. (1992) Basic fibroblast growth factor protects striatal neurons *in vitro* from NMDA-receptor mediated excitotoxicity. Brain Res. 575, 351-355.

Gensburger C., Labourdette G., and Sensenbrenner M. (1987) Brain basic fibroblast growth factor stimulates the proliferation of rat neuronal precursor cells in vitro. FEBS 217, 1-5.

Geremia E., Baratta D., Zafarana S., Giordano R., Pinizzoto M.R., La Rosa M.G., and Garozzo A. (1990) Antioxidant enzymatic systems in neuronal and glial cell enriched fractions of rat brain during aging. Neurochem. Res. 15, 719-723.

Gerlach M., Riederer P., Przuntek H., and Youdim M.B.H. (1991) MPTP mechanisms of neurotoxicity and their implications for Parkinson's disease. Eur. J. Pharmacol. 208, 273-286.

Giulian D., Vaca K., and Corpuz M. (1993) Brain glial release factors with opposing actions upon neuronal survival. J. Neurosci. 13, 29-37.

Gomez-Pinilla F., Knauer D.J., and Nieto-Sampedro M. (1988) Epidermal growth factor receptor immunoreactivity in rat brain. Development and cellular localization. Brain Res. 438, 385-390.

Gomez-Pinilla F., Lee J.W.-K., and Cotman C.W. (1992) Basic FGF in adult rat brain: cellular distribution and response to entorhinal lesion and fimbria-fornix transection. J. Neurosci. 12, 345-355.

Gospodarowicz D., Cheng J., Lui G.M., Baird A., and Bohlen P. (1984) Isolation by heparin-Sepharose affinity chromatography of brain fibroblast growth factor: Identity with pituitary fibroblast growth factor. Proc. Natl. Acad. Sci. USA 81, 6963-6967.

Gospodarowicz D., Ferrara N., Schweigerer L., and Neufeld G. (1987) Structural characterization and biological functions of fibroblast growth factor. Endocrinol. Rev. 8, 95-114.

Gray C.W. and Patel A.J. (1992) Characterization of a neurotrophic factor produced by cultured astrocytes involved in the regulation of subcortical cholinergic neurons. Brain Res. 574, 257-265.

Grothe C., Otto D., and Unsicker K. (1989) Basic fibroblast growth factor promotes *in vitro* survival and cholinergic development of rat septal neurons: comparison with the effects of nerve growth factor. Neuroscience 31, 649-661.

Gupta M., Gupta B.K., Thomas R., Bruemmer V., Sladek, Jr., J.R., and Felten D.L. (1986) Aged mice are more sensitive to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine treatment than young adults. Neurosci. Lett. 70, 326-331.

Hadjiconstantinou M., Fitkin J.G., Dalia A., and Neff N.H. (1991) Epidermal growth factor enhances striatal dopaminergic parameters in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated mouse. J. Neurochem. 57, 479-482.

Hallman H., Lange J., Olson L., Stromberg I., and Jonsson G. (1985) Neurochemical and histochemical characterization of neurotoxic effects of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine on brain catecholamine neurones in the mouse. J. Neurochem. 44, 117-127.

Hasegawa E., Takeshige K., Oishi T., Murai Y. and Minakami S. (1990) 1-Methyl-4-phenylpyridinium (MPP^+) induces NADH-dependent superoxide formation and enhances NADH-dependent lipid peroxidation in bovine heart submitochondrial particles. Biochem. Biophys. Res. Commun. 170, 1049-1055.

Hatten M.E., Lynch M., Rydel R.E., Sanchez J., Joseph-Silverstein J., Moscatelli D., and Rifkin D.B. (1988) In vitro neurite extension by granule neurons is dependent upon astroglial-derived fibroblast growth factor. Dev. Biol. 125, 280-289.

Hefti F. (1986) Nerve growth factor promotes survival of septal cholinergic neurons after fimbrial transections. J. Neurosci. 6, 2155-2162.

Hefti F., Hartikka J., and Knusel B. (1989) Function of neurotrophic factors in the adult and aging brain and their possible use in the treatment of neurodegenerative diseases. Neurobiology of Aging 10, 515-533.

Hefti F. and Weiner W.J. (1986) Nerve growth factor and Alzheimer's disease. Ann. Neurol. 20, 275-281.

Heikkila R. and Cohen G. (1972) Further studies on the generation of hydrogen peroxide by 6-hydroxydopamine: Potentiation by ascorbic acid. Mol. Pharmacol. 8, 241-248.

Heikkila R.E., Hess A., and Duvoisin R.C. (1984) Dopaminergic neurotoxicity of 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine in mice. Science 224, 1451-1453.

Heikkila R.E., Nicklas W.J., and Duvoisin R.C. (1985) Dopaminergic toxicity after stereotaxic administration of the 1-methyl-4-phenylpyridinium ion (MPP^+) to rats. Neurosci. Lett. 59, 135-140.

Herkenham M., Little M.C., Bankiewicz K., Yang S.-C., Markey S.P., and Johannessen J.N. (1991) Selective retention of MPP^+ within the monoaminergic systems of the primate brain following MPTP administration: an in vivo autoradiographic study. Neuroscience 40, 133-158.

Hiramatsu M., Kashimata M., Sato A., Murayama M., and Minami N. (1988) Influence of age on epidermal growth factor receptor level in the rat brain. Experientia 44, 23-25.

Hofer M., Pagliusi S.R., Hohn A., Leibrock J., and Barde Y.-A. (1990) Regional distribution of brain-derived neurotrophic factor mRNA in the adult mouse brain. EMBO J. 9, 2459-2464.

Houlgatte R., Mallat M., Brachet P., and Prochiantz A. (1989) Secretion of nerve growth factor in culture of glial cells and neurons derived from different regions of the mouse brain. J. Neurosci. Res. 24, 143-152.

Hyman C., Hofer M., Barde Y.-A., Juhasz M., Yancopoulos G.D., Squinto S.P., and Lindsay R.M. (1991) BDNF is a neurotrophic factor for dopaminergic neurons of the substantia nigra. Nature 350, 230-232.

Jackson G.R., Apffel L., Werrbach-Perez K., and Perez-Polo J.R. (1990) Role of nerve growth factor in oxidant-antioxidant balance and neuronal injury. I.Stimulation of hydrogen peroxide resistance. J. Neurosci. Res. 25, 360-368.

Janet T., Grothe C., Pettmann B., Unsicker K., and Sensenbrenner M. (1988) Immunocytochemical demonstration of fibroblast growth factor in cultured chick and rat neurons. J. Neurosci. Res. 19, 195-201.

Javitch J.A., D'Amato R.J., Strittmatter S.M., and Snyder S.H. (1985) 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 82, 2173-2177.

Johannessen J.N., Adams J.D., Schuller H.M., Bacon J.P., and Markey S.P. (1986) 1-Methyl-4-phenylpyridine induces oxidative stress in the rodent. Life Sci. 38, 743-749.

Johannessen J.N., Chiueh C.C., Bacon J.P., Garrick N.A., Burns R.S., Weise V.K., Kopin I.J., Parisi J.E., and Markey S.P. (1989) Effects of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine in the dog: Effect of pargyline pretreatment. J. Neurochem. 53, 582-589.

Johannessen J.N., Chiueh C.C., Burns R.S., and Markey S.P. (1985) Differences in the metabolism of MPTP in the rodent and primate parallel differences in sensitivity to its neurotoxic effects. Life Sci. 36, 219-224.

Jonsson G. (1976) Studies on the mechanisms of 6-hydroxydopamine cytotoxicity. Med. Biol. 54, 406-420.

Kalil K. and Reh T. (1979) Regrowth of severed axons in the neonatal central nervous system: establishment of normal connections. Science 205, 1158-1160.

Kinoshita A., Yamada K., Hayakawa T., Kataoka K., Mushiroi T., Kohmura E., and Mogami H. (1990) Modification of anoxic neuronal injury by human recombinant epidermal growth factor and its possible mechanism. J. Neurosci. Res. 25, 324-330.

Kitt C.A., Cork L.C., Eidelberg F., Joh T.H., and Price D.L. (1986) Injury of nigral neurons exposed to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine: a tyrosine hydroxylase immunocytochemical study in monkey. Neuroscience 17, 1089-1103.

Knusel B., Michel P.P., Schwaber J.S., and Hefti F. (1990) Selective and nonselective stimulation of central cholinergic and dopaminergic development in vitro by nerve growth factor, basic fibroblast growth factor, epidermal growth factor, insulin and the insulin-like growth factors I and II. J. Neurosci. 10, 558-570.

Kostrzewa R.M. and Jacobowitz D.M. (1974) Pharmacological action of 6-hydroxydopamine. Pharmacol. Rev. 26, 199-288.

Krueger M.J., Singer T.P., Casida J.E., and Ramsay R.R. (1990) Evidence that the blockade of mitochondrial respiration by the neurotoxin 1-methyl-4-phenylpyridinium (MPP+) involves binding at the same site as the respiratory inhibitor, rotenone. Biochem. Biophys. Res. Commun. 169, 123-128.

Lakshmanan J., Weichsel M.E., and Fisher D.A. (1986) Epidermal growth factor in synaptosomal fractions of mouse cerebral cortex. J. Neurochem. 46, 1081-1085.

Langston J.W., Ballard P.A., Tetrud J.W., and Irwin I. (1983) Chronic parkinsonism in human due to a product of meperidine-analog synthesis. Science 219, 979-980.

Langston J.W., Irwin I., and DeLanney L.E. (1987) The biotransformation of MPTP and disposition of MPP^+: The effects of aging. Life Sci. 40, 749-754.

Langston J. W., Irwin I., Langston E.B., and Forno L.S. (1984) Pargyline prevents MPTP-induced parkinsonism in primates. Science 225, 1480-1482.

Lazar L.M. and Blum M. (1992) Regional distribution and developmental expression of epidermal growth factor and transforming growth factor-mRNA in mouse brain by a quantitative nuclease protection assay. J. Neurosci. 12, 1688-1697.

Leutz A. and Schachner M. (1981) Epidermal growth factor stimulates DNA-synthesis of astrocytes in primary cerebellar cultures. Cell Tiss. Res. 220, 393-404.

Lindsay R.M. (1979) Adult rat brain astrocytes support survival of both NGF-dependent and NGF-insensitive neurons. Nature 282, 80-82.

Lindsay R.M. (1988) Nerve growth factors (NGF, BDNF) enhance axonal regeneration but are not required for survival of adult sensory neurons. J. Neurosci. 8, 2394-2405.

Liuzzi F.J. and Lasek R.J. (1987) Astrocytes block axonal regeneration in mammals by activating the physiological stop pathway. Science 237, 642-645.

Lowry O., Rosebrough N.J., Farr A.L., and Randall R.J. (1951) Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265-275.

Lundborg G., Longo F.M., and Varon S. (1982) Nerve regeneration model and trophic factors in vivo. Brain Res. 232, 157-161.

Manthorpe M., Longo F.M., and Varon S. (1982) Comparative features of spinal neuronotrophic factors in fluids collected in vitro and in vivo. J. Neurosci. Res. 8, 241-250.

Manthorpe M., Nieto-Sampedro M., Skaper S.D., Lewis E.R., Barbin G., Longo F.M., Cotman C.W., and Varon S. (1983) Neuronotrophic activity in brain wounds of the developing rat. Correlation with implant survival in the wound cavity. Brain Res. 267, 47-56.

Manthorpe M., Rudge J.S., and Varon S. (1986) Astroglial cell contributions to neuronal survival and neuritic growth, in Astrocytes (Federoff S. and Vernadakis A., eds), Vol. 2, pp.315-376. Academic Press, New York.

Marsden C.D. (1990) Parkinson's disease. Lancet 335, 948-952.

Matsuda S., Saito H., and Nishiyama N. (1990) Effect of basic fibroblast growth factor on neurons cultured from various regions of postnatal rat brain. Brain Res. 520, 310-316.

Matthiessen H.P., Schmalenbach C., and Muller H.W. (1989) Astroglia-released neurite growth-inducing activity for embryonic hippocampal neurons is associated with laminin bound in a sulfated complex and free fibronectin. Glia 22, 177-188.

Mattson M.P., Murrain M., Guthrie P.B., and Kater S.B. (1989) Fibroblast growth factor and glutamate: opposing roles in the generation and degeneration of hippocampal neuroarchitecture. J. Neurosci. 9, 3728-3740.

Mattson M.P. and Rychlik B. (1990) Glia protect hippocampal neurons against excitatory amino acid-induced degeneration: involvement of fibroblast growth factor. Int. J. Devl. Neurosci. 8, 399-415.

McGeer P.L., Itagaki S., Akiyama H., and McGeer E.G. (1988) Rate of cell death in parkinsomism indicates active neuropathological process. Ann. Neurol. 24, 574-576.

McKeon R.J., schreiber R.C., Rudge J.S., and Silver J. (1991) Reduction of neurite outgrowth in a model of glial scarring following CNS injury is correlated with the expression of inhibitory molecules on reactive astrocytes. J. Neurosci. 11, 3398-3411.

Michel P.P., Dandapani B.K., Knusel B., Sanchez-Ramos J., and Hefti F. (1990) Toxicity of 1-methyl-4-phenylpyridinium for rat dopaminergic neurons in culture: selectivity and irreversibility. J. Neurochem. 54, 1102-1109.

Morrison R.S., Kornblum H.I., Leslie F.M., and Bradshaw R.A. (1987) Trophic stimulation of cultured neurons from neonatal rat brain by epidermal growth factor. Science 238, 72-75.

Morrison R.S., Sharma A., DeVellis J., and Bradshaw R.A. (1986) Basic fibroblast growth factor supports the survival of cerebral cortical neurons in primary cultures. Proc. Natl. Acad. Sci. USA 83, 7537-7541.

Muller H.W. and Seifert W. (1982) A neurotrophic factor (NTF) released from primary glial cultures supports survival and fiber outgrowth of cultured hippocampal neurons. J. Neurosci. Res. 8, 195-204.

Murphy M., Drago J., and Bartlett P.F. (1990) Fibroblast growth factor stimulates the proliferation and differentiation of neural precursor cells in vitro. J. Neurosci. Res. 25, 463-475.

Mytilineou C. and Cohen G. (1984) 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine destroys dopamine neurons in explants of rat embryo mesencephalon. Science 225, 529-531.

Mytilineou C. and Danias P. (1989) 6-Hydroxydopamine toxicity to dopamine neurons in culture: Potentiation by the addition of superoxide dismutase and N-acetylcysteine. Biochem. Pharmacol. 38, 1872-1875.

Mytilineou C. and Friedman L. (1988) Studies on the metabolism and toxicity of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine in cultures of embryonic rat mesencephalon. J. Neurochem. 51, 750-755.

Mytilineou C., Cohen G., and Heikkila R.E. (1985) 1-Methyl-4-phenylpyridine (MPP+) is toxic to mesencephalic dopamine neurons in culture. Neurosci. Lett. 57, 19-24.

Mytilineou C., Park T.H., and Shen J. (1992) Epidermal growth factor-induced survival and proliferation of neuronal precursor cells from embryonic rat mesencephalon. Neurosci. Lett. 135, 62-66.

Nicklas W.J., Vyas I., and Heikkila R.E. (1985) Inhibition of NADH-linked oxidation in brain mitochondria by 10methyl-4-phenylpyridine, a metabolite of the neurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. Life Sci. 36, 2503-2508.

Nieto-Sampedro M., Lewis E.R., Cotman C.W., Manthorpe M., Skaper S.D., Barbin G., Longo F.M., and Varon S. (1982) Brain injury causes a time dependent increase in neuronotrophic activity at the lesion site. Science 217, 860-861.

Nieto-Sampedro M., Manthorpe M., Barbin G., Varon S., and Cotman C.W. (1983) Injury-induced neuronotrophic activity in adult rat brain: correlation with survival of delayed implants in the wound cavity. J. Neurosci. 3, 2219-2229.

Noble M., Fok-Seang J., and Cohen J. (1984) Glia are a unique substrate for the in vitro growth of central nervous system neurons. J. Neurosci. 4, 1892-1903.

O'Malley E.K., Black I.B., and Dreyfus C.F. (1991) Local support cells promote survival of substantia nigra dopaminergic neurons in culture. Exp. Neurol. 112, 40-48.

Ono T., Saito H., Kishimoto T., Okumoto T., and Miyamoto K. (1991) Stimulation of biosynthesis of nerve growth factor by acidic fibroblast growth factor in cultured mouse astrocytes. Neurosci. Lett. 126, 18-20.

Otto D. and Unsicker K. (1990) Basic FGF reverses chemical and morphological deficits in the nigrostriatal system of MPTP-treated mice. J. Neurosci. 10, 1912-1921.

Pandiella A., Beguinot L., Vicentini L.M., and Meldolesi J. (1989) Transmembrane signalling at the epidermal growth factor receptor. Trends Pharmacol. 10, 411-414.

Parker, Jr., W.D., Boyson S.J., and Parks J.K. (1989) Abnormalities of the electron transport chain in idiopathic Parkinson's disease. Ann. Neurol. 26, 719-723.

The Parkinson Study Group (1989) Effect of deprenyl on the progression of disability in early Parkinson's disease. New Engl. J. Med. 32, 1364-1371.

Patel A.J. and Hunt A. (1989) Regulation of production by primary cultures of rat forebrain astrocytes of a trophic factor important for the development of cholinergic neurons. Neurosci. Lett. 99, 223-228.

Pelton , II, E.W., Kimelberg H.K., Shipherd S.V., and Bourke R.S. (1981) Dopamine and norepinephrine uptake and metabolism by astroglial cells in culture. Life Sci. 28, 1655-1663.

Perraud F., Lagourdette G., Eclancher F., and Sensenbrenner M. (1990) Primary cultures of astrocytes from different brain areas of newborn rats and effects of basic fibroblast growth factor. Dev. Neurosci. 12, 11-21.

Pettmann B., Weibel M., Sensenbrenner M., and Labourdette G. (1985) Purification of two astroglial growth factors from bovine brain. FEBS Lett. 189, 102-108.

Pettmann B., Labourdette G., Weibel M., and Sensenbrenner M. (1986) The brain fibroblast growth factor is localized in neurons. Neurosci. Lett. 68, 175-180.

Probstmeier R. and Schachner M. (1986) Epidermal growth factor is not detectable in developing and adult rodent brain by a sensitive double-site enzyme immunoassay. Neurosci. Lett. 63, 290-294.

Pruss R.M., Bartlett P.F., Gavrilovic J., lisak R.P., and Rattray S. (1982) Mitogens for glial cells: a comparison of the response of cultured astrocytes, oligodendrocytes and Schwann cells. Dev. Brain Res. 2, 19-35.

Przedborski S., Kostic V., Jackson-Lewis V., Naini A.B., Simonetti S., Fahn S., Carlson E., Epstein C.J., and Cadet J. L. (1992) Transgenic mice with increased Cu/Zn-superoxide

dismutase activity are resistant to N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced neurotoxicity. J. Neurosci. 12, 1658-1667.

Rall L.B., Scott J., Bell G.I., Crawford R.J., Penschow J.D., Niall H.D., and Coghlan J.P. (1985) Mouse prepro-epidermal growth factor synthesis by kidney and other tissues. Nature 313, 228-231.

Ramsay R.R. and Singer T.P. (1986) Energy-dependent uptake of N-methyl-4-phenylpyridinium, the neurotoxic metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, by mitochondria. J. Biol. Chem. 261, 7585-7587.

Ransom B.R., Kunis D.M., Irwin I., and Langston J.W. (1987) Astrocytes convert the parkinsonism inducing neurotoxin, MPTP, to its active metabolite, MPP^+. Neurosci. Lett. 75, 323-328.

Raps S.P., Lai J.C.K., Hertz L., and Cooper A.J.L. (1989) Glutathione is present in high concentrations in cultured astrocytes but not in cultured neurons. Brain Res. 493, 398-401.

Ricaurte G.A., langston J.W., Delanney L.E., Irwin I., Peroutka S.J., and Forno L.S. (1986) Fate of nigrostriatal neurons in young and mature mice given 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine: a neurochemical and morphological reassessment. Brain Res. 376, 117-124.

Rogers S.L., Letourneau P.C., Palm S.L., McCarthy J., and Furcht L.T. (1983) Neurite extension by peripheral and central nervous system neurons in response to substratum-bound fibronectin and laminin. Dev. Biol. 98, 212-220.

Rousselet A., Fetler L., Chamak B., and Prochiantz A. (1988) Rat mesencephalic neurons in culture exhibit different morphological traits in the presence of media conditioned on mesencephalic or striatal astroglia. Dev. Biol. 129, 495-504.

Rudge J.S., Manthorpe M., and Varon S. (1985) The output of neuronotrophic and neurite-promoting agents from rat brain astroglial cells: a microculture method for screening potential regulatory molecules. Dev. Brain Res. 19, 161-172.

Sachs C.H. and Jonsson G. (1975) Mechanisms of action of 6-hydroxydopamine. Pharmacol. 24, 1-8.

Sanchez-Ramos J., Barrett J.N., Goldstein M., Weiner W.J., and Hefti F. (1986) 1-Methyl-4-phenylpyridinium (MPP+) but not 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) selectively destroys dopaminergic neurons in cultures of dissociated rat mesencephalic neurons. Neurosci. Lett. 72, 215-220.

Sanchez-Ramos J.R., Michel P., Weiner W.J., and Hefti F. (1988) Selective destruction of cultured dopaminergic neurons from fetal rat mesencephalon by 1-methyl-4-phenylpyridinium: cytochemical and morphological evidence. J. Neurochem. 50, 1934-1944.

Sayre L.M. (1989) Biochemical mechanism of action of the dopaminergic neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Toxicol. Lett. 48, 121-149.

Schapira A.H.V., Cooper J.M., Dexter D., Clark J.B., Jenner P., and Marsden C.D. (1990) Mitochondrial complex I deficiency in Parkinson's disease. J. Neurochem. 54, 823-827.

Schinelli S., Zuddas A., Kopin I.J., Barker J.L., and DiPorzio U. (1988) 1-Methyl-4-phenylpyridinium uptake in dissociated cell cultures from the embryonic mesencephalon. J. Neurochem. 50, 1900-1907.

Schneider J.S. and Rothblat D.S. (1991) Neurochemical evaluation of the striatum in symptomatic and recovered MPTP-treated cats, Neuroscience 44, 421-429.

Schneider J.S., Yuwiler A., and Markham C.H. (1986) Production of a Parkinson-like syndrome in the cat with N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP): behavior, histology, and biochemistry. Exp. Neurol. 91, 293-307.

Schwab M.E. and Caroni P. (1988) Oligodendrocytes and CNS myelin are nonpermissive substrates for neurite growth and fibroblast spreading in vitro. J. Neurosci. 8, 2381-2393.

Simpson D.L., Morrison R., DeVellis J., and Herschman H.R. (1982) Epidermal growth factor binding and mitogenic activity on purified populations of cells from the central nervous system. J. Neurosci. Res. 8, 453-462.

Spina M.B., Squinto S.P., Miller J., Lindsay R.M., and Hyman C. (1992) Brain-derived neurotrophic factor protects dopamine neurons against 6-hydroxydopamine and N-methyl-4-phenylpyridinium ion toxicity: involvement of the glutathione system. J. Neurochem. 59, 99-106.

Strolin-Benedetti M. and Dostert P. (1989) Monoamine oxidase, brain ageing and degenerative diseases. Biochem. Pharmacol. 38, 555-561.

Sundstrom E., Luthman J., Goldstein M., and Jonsson G. (1988) Time course of MPTP-induced degeneration of the nigrostriatal dopamine system in C57BL/6 mice. Brain Res. Bull. 21, 257-263.

Tetrud J.W. and Langston J. W. (1989) The effect of deprenyl (Selegiline) on the natural history of Parkinson's disease. Science 245, 519-522.

Tiffany-Castiglioni E. and Perez-Polo J.R. (1981) Stimulation of resistance to 6-hydroxydopamine in a human neuroblastoma cell line by nerve growth factor. Neurosci. Lett. 26, 157-161.

Torelli S., Dell'Era P., Ennas M.G., Sogos V., Gremo F., Ragnotti G., and Presta M. (1990) Basic fibroblast growth factor in neuronal cultures of human fetal brain. J. Neurosci. Res. 27, 78-83.

Torres-Aleman I., Naftolin F., and Robbins R. (1990) Trophic effects of basic fibroblast growth factor on fetal rat hypothalamic cells: interactions with insulin-like growth factor I. Dev. Brain Res. 52, 253-257.

Ungerstedt U. (1968) 6-Hydroxydopamine induced degeneration of central monoamine neurons. Eur. J. Pharmacol. 5, 107-110.

Unsicker K., Reichert-Preibsch H., Schmidt R., Pettmann B., Labourdette G., and Sensenbrenner M. (1987) Astroglial and fibroblast growth factor have neurotrophic functions for cultured peripheral and central nervous system neurons. Proc. Natl. Acad. Sci. USA 84, 5459-5463.

Unsicker K., Reichert-Preibsch H., and Wewetzer K. (1992) Stimulation of neuron survival by basic FGF and CNTF is a direct effect and not mediated by non-neuronal cells: evidence from single cell cultures. Dev. Brain Res. 65, 285-288.

Uretsky N.J. and Iversen L.L. (1970) Effects of 6-hydroxydopamine on catecholamine containing neurones in rat brain. J. Neurochem. 17, 269-278.

Varon S. and Somjen G. (1979) Neuron-glia interactions. Neurosci. Res. Prog. Bull. 17, 1-239.

Wagner J.A. (1991) The fibroblast growth factors: an emerging family of neural growth factors. Curr. Topics Microbiol. Immunol. 165, 95-118.

Walicke P., Cowan E.M., Ueno N., Baird A., and Guillemin R. (1986) Fibroblast growth factor promotes survival of dissociated hippocampal neurons and enhances neurite extension. Proc. Natl. Acad. Sci. USA 83, 3012-3016.

Walicke P.A. (1988) Basic and acidic fibroblast growth facotrs have trophic effects on neurons from multiple CNS regions. J. Neurosci. 8, 2618-2627.

Walicke P.A. and Baird A. (1988) Neurotrophic effects of basic and acidic fibroblast growth factors are not mediated through glial cells. Dev. Brain Res. 40, 71-79.

Wanaka A., Johnson Jr., E.M., Milbrandt J. (1990) Localization of FGF receptor mRNA in the adult rat central nervous system by *in situ* hybridization. Neuron 5, 267-281.

Wang S.L., Shiverick K.T., Ogilvie S., Dunn W.A., and Raizada M.K. (1989) Characterization of epidermal growth factor receptors in astrocytic glial and neuronal cells in primary culture. Endocrinology 124, 240-247.

Waters C.M., Hunt S.P., Jenner p., and Marsden C.D. (1987) An immunohistochemical study of the acute and long-term effects of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine in the marmoset. Neuroscience 23, 1025-1039.

Werner M.H., Nanney L.B., Stoscheck C.M., and King L.E. (1988) Localization of immunoreactive epidermal growth factor receptors in human nervous system. J. Histochem. Cytochem. 36, 81-86.

Westermann R., Grothe C., and Unsicker K. (1990) Basic fibroblast growth factor (bFGF), a multifunctional growth factor for neuroectodermal cells. J. Cell Sci. Suppl. 13, 97-117.

Westlund K.N., Denney R.M., Kochersperger L.M., Rose R.M., and Abell C.W. (1985) Distinct monoamine oxidase A and B populations in primate brain. Science 230, 181-183.

Williams L.R., Varon S., Peterson G.M., Victorin K., Fischer W., Bjorklund A., and Gage F.H. (1986) Continuous infusion of nerve growth factor prevents basal forebrain neuronal death after fimbria fornix transection. Proc. Natl. Acad. Sci. USA 83, 9231-9235.

Woodward W.R., Nishi R., Meshul C.K., Williams T.E., Coulombe M., and Eckenstein F.P. (1992) Nuclear and cytoplasmic localization of basic fibroblast growth factor in astrocytes and CA2 hippocampal neurons. J. Neurosci. 12, 142-152.

Yong V.W., Perry T.L., and Krisman A.A. (1986) Depletion of glutathione in brainstem of mice caused by N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine is prevented by antioxidant pretreatment. Neurosci. Lett. 63, 56-60.

Yoshida K. and Gage F.H. (1991) Fibroblast growth factors stimulate nerve growth factor synthesis and secretion by astrocytes. Brain Res. 538, 118-126.

Zahs K.R., Bigornia V., and Deschepper C.F. (1993) Characterization of "plasma proteins" secreted by cultured rat macroglial cells. Glia 7, 121-133.